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Development of PCR-based detection methods for the quarantine phytopathogen *Synchytrium endobioticum*, causal agent of potato wart disease

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Abstract

PCR-based methods were developed for the detection and quantification of the potato pathogen *Synchytrium endobioticum* in soil extracts and *in planta*. PCR primers, based on the internal transcribed spacer region of the multi-copy gene rDNA were tested for specificity, sensitivity and reproducibility in conventional and real-time PCR assays. Soil extraction procedures compared included the Hendrickx centrifugation (HC) procedure, nested wet sieving (NWS) and a method used by the Plant Protection Service (PPS). The primers amplified a 472 bp product from *S. endobioticum* DNA, but did not amplify DNA from other potato pathogens, other plant pathogens, and related species. Standard cell disruption and DNA extraction and purification methods were optimized for amplification of *S. endobioticum* DNA from resting sporangia. DNA was successfully amplified from a single sporangium and equivalent DNA preparations from soil extracts. Low levels of target DNA in water did not amplify, possibly due to DNA loss during final purification steps. A real-time PCR assay, developed for soil-based extracts using primers and probe based on the rDNA gene sequences, involved co-amplification of target DNA along with an internal DNA fragment. Both conventional and real-time PCR methods performed well with HC- and NWS-extracts having a threshold sensitivity of 10 sporangia per PCR assay. Of the three soil extraction methods, only with the HC method could 100 g soil samples be efficiently processed in one single PCR assay. Such a high capacity assay could be useful for routine soil analysis in respect to disease risk assessments and to secure de-scheduling according to EPPO guidelines.

Introduction

The chytridiomycete, *Synchytrium endobioticum* is an obligate plant pathogen that causes a serious potato disease in cool, moist climates worldwide. It infects plant tissue via zoospores and survives as thick-walled sporangia, also known as resting spores. Upon infection, it induces the host to produce wart-like outgrowths (warts) on tubers, stolons and sprouts. Warts consist of hypertrophic host cells each containing a single sporangium, which develop into infectious zoospores (Hampson, 1985; Hampson et al., 1994). In response to ill-defined environmental triggers, zoospores conjugate to form a zygote, which develop into resting sporangia. These resting sporangia survive inter-host periods for up to 20 years (Hampson, 1993). Pathotypes of *S. endobioticum* have been identified

based on a differential set of potato genotypes (Hampson, 1993; Langerfeld and Stachewicz, 1993). Some pathotypes have a considerably wider host range in terms of pathogenicity on different potato cultivars than others and these broad host range pathotypes pose a serious threat for potato production in northwest Europe (Baayen et al., 2005). The most effective way to control the disease is by use of resistant cultivars and effective containment procedures in case of field infection (Hampson, 1993).

Legislative regulations have been in force throughout Europe to prevent inter-field spread of potato wart. According to the European and Mediterranean Plant Protection Organization (EPPO), infected fields should be scheduled for 20 years (EPPO) or longer if soil tests disclose the presence of *S. endobioticum* sporangia. In the European Union (EU), potato production is not permitted until the presence of sporangia can no longer be demonstrated in an infected field. Various methods have been developed for direct enumeration of resting spores in soil (Pratt, 1976; Laidlaw, 1985). Several methods have been adapted for routine use and to meet EPPO requirements (van Leeuwen et al., 2005). Basically the methods are directed to concentrate sporangia from soil particles using sieving and centrifugation techniques. Soil extracts are subject to microscopic observation for the presence of sporangia (reviewed by van Leeuwen et al., 2005). Recently, zonal centrifugation (Hendrickx, 1995) has been used for routine extraction of nematode eggs from soil samples up to 200 g. The zonal centrifuge technique was adapted at Applied Plant Research and fine-tuned for routine extraction of wart sporangia from soil samples as an alternative to the sieve-centrifugation method presently in use at Plant Protection Service.

Recently, the ITS region of the multi-copy rDNA gene of *S. endobiotictum* has been sequenced and specific PCR primers and probes have been identified (Lévesque et al., 2001). Both the developments pertaining to soil extraction and PCR identification of *S. endobioticum* were motives for further development and validation of sensitive and specific detection methods for potato wart sporangia directly in soil samples (Lévesque et al., 2002). The aim of the present study was to develop a PCR-based method for detection of *S. endobioticum* sporangia in soil extracts.

Materials and methods

Organisms: cultivation and DNA extraction

Synchytrium endobioticum sporangia of pathotypes 1, 2, 6, 8 and 18 were produced on young susceptible potato sprouts (cv. Deodara) by inoculating them with a wart compost according to the Spieckermann method described in the EPPO Diagnostic Protocol for *S. endobioticum* (Baayen and Stachewicz, 2004). After 10 week incubation under moist and dark conditions at 16–18 °C mature (dark coloured) warts were harvested, freed from adhering soil by thorough agitation in tap water, and stored at −20 °C. Thawed warts were homogenized to fine slurry using a pestle and mortar. Compost inocula of pathotypes 1, 2, 6, 8 and 18 were obtained from the Plant Protection Service, the Netherlands (Baayen et al., 2005). Sporangia were separated from the homogenate or compost by wet-sieving and centrifugation using the method of the Dutch Plant Protection Service (PPS method) as described below.

Non-chytrid pathogens listed in Table 1 were obtained from the collection held at Plant Research International, Wageningen, the Netherlands. Cultivation was according to current laboratory practice. For fungi this involved cultivation in potato dextrose broth (PDB; Oxoid DM 139) and extraction of DNA from freeze-dried mycelium using Puregene kit 02F01 (BIOzyme, Blaenavon, UK). For bacteria the methods of van Beckhoven et al. (2002) were used and for second stage *Meloidogyne* juvenile nematodes, the methods of Curran et al. (1986) and Zijlstra et al. (1997) were used. Chytrid DNA-preparations other than *S. endobioticum* and *Synchytrium taraxaci* were kindly provided by Dr. T. Turner, Duke University, NC, USA.

Soils and preparation of soil dilution series

In this study, four different artificially infested soils were used from Dutch agricultural fields. Potatoes had not been grown in these fields for at least 10 years. Soil samples were collected from the fields G13 located near Wageningen (loamy

Table 1. Origin of micro-organisms tested for amplification with *Synchytrium endobioticum*-specific F49/R502 primers

Species	Strain No.	Host	Location	Year
S. endobioticum pathotype 1	PD1–1990	Potato	Netherlands	2002
S. endobioticum pathotype 2	PD2–1988	Potato	Netherlands	1988
S. endobioticum pathotype 6	PD6–1991	Potato	Netherlands	1991
S. endobioticum pathotype 8	PD8–1998	Potato	Germany	1998
S. endobioticum pathotype 18	PD18–2001	Potato	Netherlands	2001
S. taraxaci	PRI-Star	*Taraxicum officinalis*	Netherlands	2002
Chrytridium confervea	CBS675.73	*Tribonema bombycinum*	Canada	1973
Rhizoclosmatium sp.[a]	JEL 347			
Rhizophydium sp.[a]	JEL 136			
Rhizophlyctis sp.[a]	BK 47–07			
Phytophthora fragariae	FVF28	Strawberry	Netherlands	NK
P. infestans	Vk98014	Potato	Netherlands	1998
Rhizoctonia solani AG 2–1	PRI 21R86	Cauliflower	Netherlands	1998
R. solani AG 2–2	PRI 22R92	Sugarbeet	Netherlands	1997
Verticillium dahliae[b]	V44			
Phoma exiqua	PD1	Potato	Netherlands	NK
Ralstonia solanum	IPO1609	Potato	Netherlands	1995
Clavibacter michiganensis subsp. *sepedonicus*	IPO498	Potato	Netherlands	1978
Meloidogyne hapla	Hbf	Potato	Maryland, US	1994
M. fallax	Fa	Potato	Netherlands	1993
M. chitwoodi	Ca	Potato	Netherlands	1989

[a]DNA supplied by Dr T James, Duke University, NC, USA.
[b]Supplied by Dr RC Rowe, Ohio State University, OH, USA.
NK – not known.

sand, pH 4.5–5.5, under permanent grassland), ZO located near Veldhoven (sandy soil, pH 5.4, previous crop sugar beet), NO located near Emmen (sandy reclaimed peat soil, pH 4.4, under permanent natural flora), and KL located near Sexbierum (clay soil, pH 7.8, under permanent grassland). ZO soil amended with 5% v/v potting soil was used as reference soil throughout this study (REF soil). Following collection, the soils were air-dried, sieved through 3 mm screens and stored at 4 °C.

To determine the sensitivity of the PCR tests, soil samples were spiked by adding appropriate aliquots of wart compost with known numbers of sporangia then serially 1:5 diluted with inoculum-free soil in a cement mixer.

Extraction of sporangia from soil and compost samples

We used three different soil extraction methods for sporangia based on specific gravity (<1.4) and dimensions (25–75 μm diam; near iso-diametric in shape) of sporangia compared to soil particles. The methods included nested wet-sieving (NWS) technique according to Pratt (1976), Hendrickx centrifugation (HC) according to Hendrickx (1995), and the Plant Protection Service (PPS) method, a combination of nested wet-sieving and centrifugation (van Leeuwen et al., 2005). Briefly, the NWS soil extraction method involved wet-sieving soil samples (20 g dry weight) through nested 75 and 25 μm mesh screens using a manual sieve shaker. Washing with tap water was done until no fine material remained in the sieves. The resulting soil slurries (NWS extracts) retained on the 25 μm mesh screen were used for DNA extraction. The PPS method involved wet-sieving soil samples (20 g dry weight) followed by centrifugation of the resulting soil slurries in near saturated $CaCl_2$ (specific weight 1.4) extraction solution at 800 g for 5 min. The supernatant containing sporangia was sieved over a 25 μm mesh screen and thoroughly washed in tap water to remove excess of $CaCl_2$. The resulting PPS extract was re-suspended in tap water for microscopic counting and DNA extraction. The HC method, briefly, involved suspending soil samples (200 g dry weight) in 1 l tap water by thoroughly mixing in a blender. After allowing for

sedimentation, 500 ml of the suspension was centrifuged in the Hendrickx centrifuge (Instrumentenmakerij de Koning, Zierikzee, the Netherlands), containing $CaCl_2$ extraction solution (specific weight 1.4) in an open top rotor. After spinning at 15,000 × *g* for 5 min the HC-extract retained on top of the $CaCl_2$ layer was removed from the centrifuge rotor, concentrated on a 25 μm mesh screen and suspended in water for microscopic counting and DNA extraction. Sporangia from fresh wart tissue were extracted using $CsCl_2$ (specific weight 1.4) or non-ionic Nycodenz® (Nycomed Pharma AS, Oslo, Norway). Sporangial counts in HC-, NWS- or PPS-extracts were done on 2 ml suspensions in a 45 mm diam counting vessel with a 10 mm square grid marked at the bottom of the vessel. High-density sporangial suspensions were counted in disposable Glasstis slides with a 0.33 mm square grid (Hycor Biomedical Inc. Edinburgh, UK).

DNA-extraction

The Ultra Clean Soil DNA extraction kit (UC kit; MoBio Laboratories Carlsbad, USA) provided the highest amplifiable DNA yield from *S. endobioticum* in preliminary tests with soil extracts. The UC kit was subjected to further optimization regarding cell disruption and PCR performance. The final extraction procedure for sporangial suspensions and soil extracts was as follows: aliquots of 0.5 ml aqueous sporangial suspensions or soil extracts were transferred to 2 ml bead solution vials provided by the UC kit and amended with three stainless steel beads (3.2 mm diam). The contents of the vials were thoroughly shaken in a Biospec mini bead beater (Stratech Scientific, Luton, UK) or in a Hybaid Ribolyser multiple bead beater (Thermo Electron Corporation, Delft, the Netherlands) at 5000 rpm for 100 s. DNA was then extracted as recommended in the UC kit instructions. DNA extracted from *S. endobioticum* with the UC kit amplified more consistently with an additional purification step involving passage through 2 ml capless Axygen spin vials (MKS-100; Axygen Scientific, Union City, USA). These columns were prepared by addition of dry polyvinylpolypyrrolidone (PVPP; Sigma P-6755) and packed in 1 cm high columns. Before use, the PVPP-columns were washed twice with 150 μl double deionized water (SDW) and excess SDW was removed by centrifugation (Eppendorf Centrifuge 5417C) at 1500 × *g* for 4 min. After washing, 50 μl DNA extracts were loaded onto the columns, which were centrifuged at 1500 × *g* for 5 min. The resulting eluates were transferred to fresh tubes for PCR.

PCR amplification

Universal primers, ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990), and *S. endobioticum* specific primers F49 (5′-CAACACCATGTGAACTG-3′) and R502 (5′-ACATACACAATTCGAGTTT-3′) (Lévesque et al., 2001) were used for PCR amplification. The components for 25 μl PCR reactions were: 10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 600 nM of each F49 and R502 primers, 60 μM dNTPs, 1 unit Taq DNA polymerase (Roche, Basel, Switzerland) and 1 μl serially diluted template DNA or 5 μl of a 1:10 dilution. The cycling parameters were: denaturating step 2 min 95 °C; 35 cycles of amplification (30 s 95 °C; 30 s 57 °C; 60 s 72 °C); 10 min 72 °C. The amplification reaction was performed in a Peltier thermocycler (PTC200, MJ Research, Biozym, Germany). After amplification, the DNA products were analyzed by electrophoresis in a 1.0% (W/V) agarose gel. The gels were run at 120 V for 1 h in buffer containing 50 mM Tris, 45 mM boric acid and 0.5 mM EDTA at pH 8.4, stained with ethidium bromide (0.5 μg ml^{-1}) and photographed under UV light with a gel documentation system (Herolab, E.A.S.Y. 440k, Wiesloch, Germany).

Real-time PCR amplification

Taqman primer pairs used were *S. endobioticum* specific primers F49 (5′-CAACACCATGTGAACTG–3′) and R213 (5′-AAGTTGTTTAATAATTGTTGTA-3′) (Lévesque et al., 2001) and -TGGTGTATGTGAACGGCTTGCCCAC-3′) designed from ITS-rDNA sequence of *S. endobioticum*. The fluorogenic P1 probe was labelled at the 5′end with the fluorescent reporter dye FAM (6-carboxy fluorescein) and modified at the 3′end with a quencher dye TAMRA (6-carboxy-tetramethyl-rhodamine). The qPCR™ Core Kit (Eurogentec, Belgium) was used for real-time PCR experiments, which were carried out in

optical tubes and caps (Applied Biosystems, Foster City, USA). Each 30 μl PCR reaction consisted of 1× reaction buffer containing ROX (5, 6-carboxy-X-rhodamine) passive reference, 200 μM dNTP, 5 mM $MgCl_2$, 0.75 U HotGoldStar enzyme (Eurogentec, Belgium), 500 nM of each F49 and R213 primers, 200 nM TaqManP1 probe and 1 μl serial diluted target DNA or 5 μl 1:10 diluted DNA sample. Internal Amplification Control (IAC) was added to check reactions for inhibition and false negative results. The components were 75 nM of each IAC primer, 50 nM IAC probe and 10 fg IAC containing plasmid DNA (Klerks et al., 2004). The cycle conditions were a hot start of 10 min at 95 °C, followed by 40 cycles of 2-step amplification (15 s 95 °C; 60 s 55 °C). The real-time quantitative PCR was performed in an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, USA).

DNA cloning and sequencing of amplified ITS regions

PCR products were cloned into pGEM®-Teasy (Promega) plasmids and propagated in *Escherichia coli* strain JM109. Inserts were re-amplified from clones using the primers of the original PCR product by picking the bacterial colonies with a toothpick directly into the PCR reaction. Electrophoresis and data collection were performed on a DNA sequencer (3100 Genetic Analyser, Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands) and sequence data were analyzed using DNASTAR software (DNASTAR Inc, USA).

Results

DNA extracts from sporangia

Commercial bead disruption protocols were ineffective in cell disruption, leaving 10–50% of *S. endobioticum* sporangia visually intact. Complete cell disruption was achieved at maximum beating frequency with three extra steel beads in the UC extraction procedure, indicating high mechanical strength of sporangial cell walls. *Synchytrium endobioticum* sporangial DNA appeared as an extensive smear of low molecular DNA in electrophoresis gels, which clearly contrasted with the high molecular weight bands of similarly extracted DNA from *Rhizoctonia solani* AG 3 sclerotia (Figure 1). DNA-fragmentation was evident from both ionic ($CaCl_2$, $CsCl_2$) and non-ionic (Nycodenz) extraction liquids, irrespective of whether the source of sporangia was wart compost or fresh wart tissue. The cell content of extracted sporangia from both sources looked similar in microscopic preparations showing internal granular composition and no visible plasmolysis. The DNA samples from wart- or compost-extracted sporangia were amplifiable in PCR yielding multiple bands of 400–750 bp with the universal ITS1/ITS4 primer set. Aliquots of 10^6 sporangia extracted in $CaCl_2$ or $CsCl_2$ yielded 0.5 and 5.0 μg DNA, respectively. Assuming all extracted DNA was of *S. endobioticum* origin, a single sporangium represented 0.5 and 5.0 pg DNA equivalents.

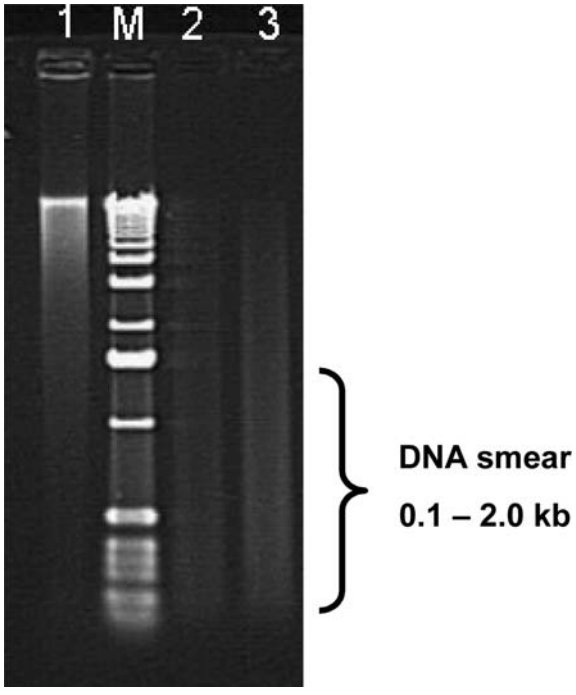

Figure 1. EtBr-stained agarose gel of genomic DNA obtained from cultured *Rhizoctonia solani* sclerotia and $CaCl_2$-extracted potato wart sporangia of *Synchytrium endobioticum* using modified UC procedure for DNA extraction. Lanes 1–3: DNA from *R. solani* sclerotia, *S. endobioticum* pathotype 1, *S. endobioticum* pathotype 2, respectively. M indicates a 1kb size marker.

Sequencing of PCR amplicons

Amplification of $CaCl_2$- or $CsCl_2$-extracted sporangia of pathotype 2 using ITS1/ITS4 yielded 7 bands in the 400–750 bp range, indicating the presence of a mixture of amplifiable DNA sources. DNA from all 7 bands was sequenced and the resulting sequences were blasted in GenBank (NCBI). The blast results showed one single band of 650 bp with unknown sequence, possibly from ITS of *S. endobioticum* rDNA. Subsequent comparison with the ITS of rDNA of *S. endobioticum* pathotype 1 (Lévesque et al., 2001) showed 100% sequence consensus. Using the *S. endobioticum* specific primers F49/R502 (Lévesque et al., 2001) a single PCR product of the predicted size (472 bp) was amplified using DNA from representative isolates of pathotypes 1, 2, 6, 8 and 18. The sequence of the 472 bp PCR products of each of the aforementioned pathotypes showed 100% nucleotide consensus with one another (data not shown).

Specificity of S. endobioticum *primers*

PCR with primer pair F49/R502 failed to amplify extracts from any of the organisms listed in Table 1, except for *S. endobioticum* strains of pathotypes 1, 2, 6, 8 and 18. No other *Chytridiomycota* or biotrophic members of *Synchytrium* yielded a PCR signal, unless universal primers ITS1/ITS4 were used. In addition, symptomless potato stems were PCR-negative, but extracts from potato stems were wart-like symptoms were positive in PCR. Our results confirmed the results of Lévesque et al. (2001) on the specificity of the F49/R502 primer set.

Sensitivity of PCR with spiked DNA or sporangia

The standardized PCR with primer set F49/R502 was used to estimate the threshold value for *S. endobioticum* detection in aqueous samples spiked with 1 ng *S. endobioticum* DNA. A 472 bp product was obtained from each dilution of a 1:10 serial dilution series of target DNA at decreasing intensity to 1.0 pg (Figure 2). The threshold value for whole sporangia was tested in 0.5 ml aqueous samples or NWS-extracts from REF soil, spiked with 1, 5 and 25 sporangia per PCR assay. Purification on PVPP spin columns was required to obtain amplifiable DNA from NWS extracts. NWS-extracts were PCR-positive at descending concentrations of sporangia (Figure 3), and even single sporangia per assay gave a 472 bp product in two out of five replicate experiments. Unlike NWS-extracts, aqueous suspensions gave no visible 472 bp product in PCR, in spite of 100% cell disruption of the sporangia. Possibly, the failure of PCR to amplify DNA from aqueous suspensions is due to the large binding capacity of the MoBio and PVPP columns, relative to the amount of DNA that was present in the aqueous sample.

PCR of soil extracts

NWS extracts from REF soil with descending concentrations of sporangia were more PCR-conducive than the dry-sieved soil extracts (Table 2). DNA extraction by NWS, HC and PPS methods was optimized by combining with UC extraction, PVPP spin column clean up and a 10-fold dilution of the DNA. The extended procedure was used to evaluate possible interaction of PCR with soil types. NWS and HC soil extracts from four test soils (REF, G13, NO and ZO) were spiked with descending concentrations of target DNA (100, 10 and 1 pg per PCR assay). PCR with the specific primer set F49/R502 resulted in a visible 472 bp product in all spiked DNA samples, irrespective of soil type and

Table 2. Amplicon intensity obtained with F49/R502 primers of serially diluted DNA from nested wet or dry sieved extracts of REF-soil spiked with *Synchytrium endobioticum* sporangia (625 per g soil and 1:5 soil dilutions with non-spiked REF soil)

Number of sporangia added (g^{-1} REF soil)	PCR signal intensity*			
	DNA from wet-sieved soil		DNA from dry-sieved soil	
	1:10 dilution	1:100 dilution	1:10 dilution	1:100 dilution
625	++	++	+	++
125	++	++	++	+
25	++	++	0	+
5	++	+	0	0
1	++	0	0	0
0.2	++	0	0	0
0.04	+	0	0	0
Non-inoculated REF soil	0	0	0	0

++, high amplicon intensity; +, low; 0, no signal.

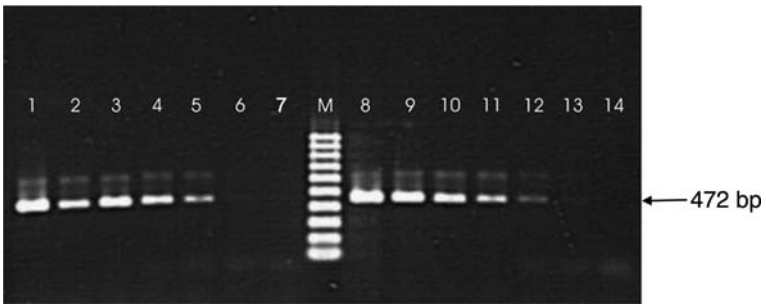

Figure 2. EtBr-stained agarose gel of amplification products obtained after PCR with F49/R502 primers of 1:10 serial dilutions of 100 ng *Synchytrium endobioticum* DNA. Lanes 1–7: 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg DNA of pathotype 1; Lanes 8–14: 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg DNA of pathotype 2. M indicates a 100 bp size marker.

extraction method (data not shown). Hence, the extended procedure was a necessary amendment for the PCR method although sensitivity may be reduced due to DNA losses on the PVPP column and a 10-fold dilution step.

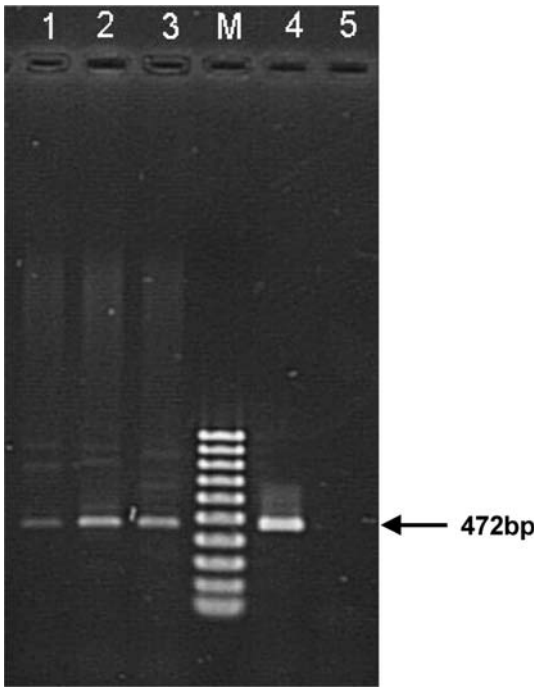

Figure 3. EtBr-stained agarose gel of amplification products obtained after PCR with F49/R502 primers of DNA from NWS (nested wet sieve) extracts from REF soil, enriched with variable numbers of *Synchytrium endobioticum* sporangia. Lanes 1–5:1,2 and 5 sporangia added to PCR assay, positive (100 pg *S*. DNA in PCR) and negative controls. M indicates a 100 bp ladder.

Detection of S. endobioticum *in soil extracts*

Validation of the PCR was assessed in PPS, NWS and HC soil extracts with known numbers of sporangia. Soil extracts from 100 g soil were analyzed for the number of sporangia and tested in this study for PCR amplification with the F49/R502 primer pair. The HC- and PPS-extractions always yielded less than 0.5 ml soil extract (slurry), irrespective of soil type. Hence, the HC and PPS techniques met the 0.5 ml format requirement for the UC kit. The NWS-extracts, however, were considerably larger than HC- and PPS-extracts from 100 g soil samples and amounted to 5 and 10 ml slurry for sandy (REF, NO, ZO) and loamy sand (G13) soils, respectively. NWS-extracts from no more than 10 g sandy soil samples or 5 g loamy sand soil could be processed for single PCR assays. Sporangia concentrated in 0.5 ml HC and PPS soil slurries were readily distinguishable from competitive soil particles in 20 ml suspension liquid (water or near saturated $CaCl_2$) for microscopic counting. Sporangial counts in NWS-extracts were, however, extremely difficult and time-consuming due to interfering soil particles. For NWS extracts, sporangial counts were estimated indirectly from HC-counts in twin samples of REF soil and corrected for an average recovery efficiency factor of 36%. Based on the sporangial counts and PCR response it was shown that HC and NWS gave positive and consistent PCR amplifications at 10 or more sporangia per assay (Figures 4a, b); the PPS approach was less sensitive with a threshold of several hundreds of

sporangia per PCR assay (Figure 4c). The data shown in Figure 4a were from REF soil only and in Figures 4b and 4c from REF, ZO, NO and KL soils together. It is clear that the PCR efficiency was dependent on the soil extraction method used.

Quantitative detection of S. endobioticum *in soil*

All the dilutions of *S. endobioticum* DNA (0.1–10,000 pg per μl) were consistently amplified in the real-time PCR assay with primer pair F49/R213 and P1 Taqman probe (Figure 5). The Ct value is the number of PCR cycles resulting in a fluorescence signal above the threshold. Inhibition of each reaction was monitored by incorporating an IAC in the assay. As expected, PVPP spin column purified DNA samples showed 2–4 higher Ct values on average compared with untreated samples, indicating substantial loss of target DNA in the extended DNA purification procedure. The real-time PCR assay could detect and quantify *S. endobioticum* in HC-extracts at sporangial densities of 5 or more per 100 g samples as shown for REF soil (Table 3).

Discussion

The classical method for the detection of *S. endobioticum* sporangia in soil is microscopic analysis of soil extracts obtained by nested wet sieving and centrifugation (Pratt, 1976; van Leeuwen et al., 2005). In this study, internal transcribed sequence information of *S. endobioticum* rDNA gene was used to validate specificity of conventional and real-time PCR primers and probe specific to all pathotypes occurring in the Netherlands and many other countries in the EU (Baayen et al., 2005). The PCR method we developed was sensitive and consistent for HC and NWS soil extracts and can therefore be used as a fast and reliable alternative for the current time-consuming and time-intensive microscope procedure. Real-time PCR was more sensitive than conventional PCR and can be used for routine quantitative soil analysis to trace new infections and to support de-scheduling procedures. Real-time PCR are more valuable when using an additional non-competitive IAC to monitor for inhibition of target amplification.

The ITS of rDNA has shown useful sequence information for species-specific diagnostics. Negative test results obtained with DNA extracted from a number of other potato pathogens and micro-organisms confirmed the specificity of the F49/R502 primer pair. The PCR assays can therefore be used to support visual detection of *S. endobioticum* in potato plant tissue and to test the zoospore production by potato cultivars in resistance bioassays, as reported by Niepold and Stachewicz (2004). The ITS-rDNA primers developed for pathotype 1 (Lévesque et al., 2001) were shown to be relevant for diagnosis at the species level. Intraspecies sequence variation in the ITS-rDNA region, however, was low and did not provide a basis for discrimination between pathotypes of *S. endobioticum*.

The ITS region of rDNA of nearly all economically important fungal and oomycete potato pathogens have been sequenced and used for disease diagnosis and pathogen detection. Examples include *Rhizoctonia solani* (Lees et al., 2002), *Spongospora subterranea* f.sp. *subterranea* (Bell et al., 1999; van de Graaf et al., 2003), *Helminthosporium solani* (Cullen et al., 2001) *Colletotrichum coccoides* (Cullen et al., 2002) and *Phytophthora infestans* (Tooley et al., 1997) in soil, water and *in planta*. Published and newly identified pathogen-specific sequences within the ITS of rDNA genes form the basis of simple and multiplex molecular assays (Bonants et al., 2001).

Liberation of DNA from sporangia was difficult and could only be achieved using intense mechanical forces. Vortexing or bead beating with commercially available beads (UC kit) did not provide sufficient disruption. Enhancement of bead beating with stainless steel balls (Lees et al., 2002) gave near 100% cell disruption of sporangia. Disrupted sporangia released low molecular weight but amplifiable DNA fragments in contrast to the predominantly high molecular weight DNA obtained from *Rhizoctonia solani* AG 3 *sclerotia*. Evidently, *S. endobioticum* DNA is particularly sensitive to breakage by mechanical forces. An alternative explanation may be that DNA in resting sporangia in compost or wart tissue may have been subjected to enzymatic degradation before being processed for extraction.

Soil constituents inhibited PCR amplification of DNA in dry soil extracts, which has also been documented by Tebbe and Vahjen (1993) and Tsai

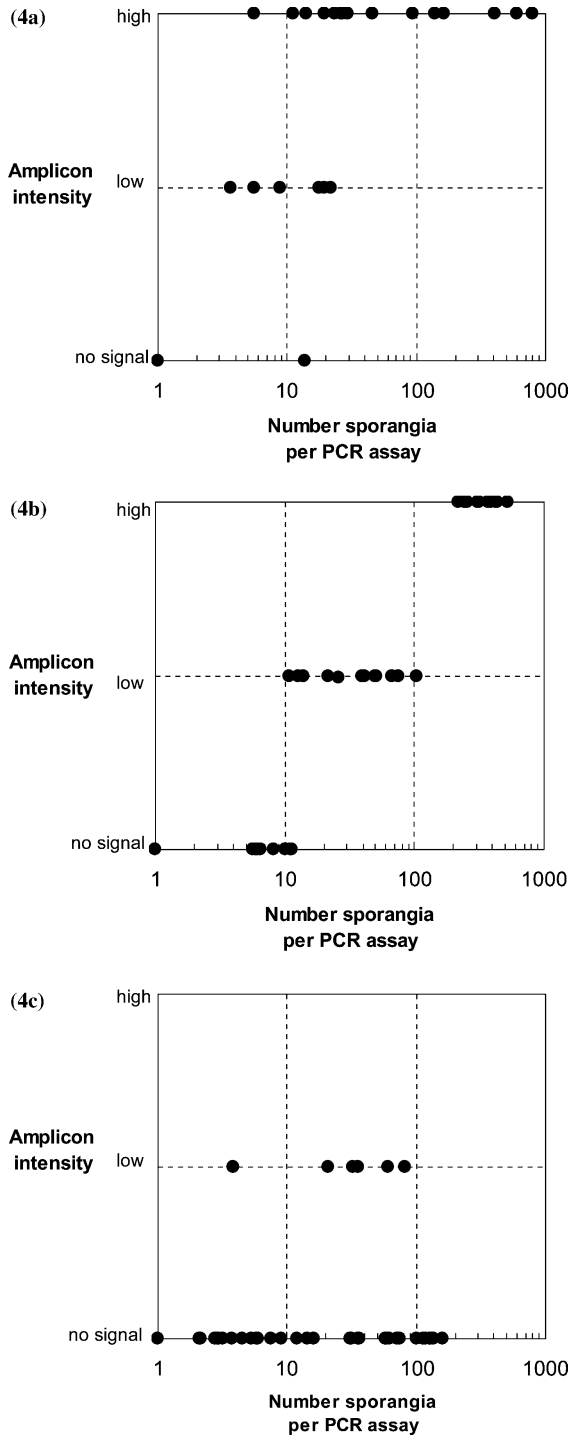

Figure 4. Sporangial counts and amplicon intensities in different types of soil extracts from unspiked and spiked soils with different levels of sporangia of *Synchytrium endobioticum*: NWS extracts from 10 g REF soil (a), HC extracts from 100 g REF, ZO, NO, KL soils (b) and PPS extracts from 20 g REF, ZO, NO, KL soils (c).

and Olsen (1992). By washing the soil extracts in water, as part of the HC and NWS extraction procedures, and with additional purification on a PVPP column and subsequent dilution, inhibition was minimized but at the sacrifice of sensitivity. The use of other commercial soil extraction kits may improve the levels of amplifiable DNA extracted from soil or water samples. Evidently, the use of the UC kit was not applicable to aqueous samples in spite of 100% disruption of sporangia (data not shown). A relatively large proportion of target DNA has been shown to irreversibly bind to UC kit components and to the PVPP column. Only relatively dense aqueous suspensions (>25 sporangia per assay) provided sufficient DNA for PCR amplification.

The overall sensitivity of the PCR method is a function of recovery efficiency of soil extraction methods, of DNA purification with the UC kit, and the PCR reaction itself. The PCR assay is sensitive, being able to detect a single sporangium or DNA-equivalent in 0.5 ml HCZ or NWS soil extract. The PCR assay is thus relevant for evaluation of field soils for *S. endobioticum* infestation or the EPPO-approved de-scheduling procedures (EPPO, 1999). Of the three sporangial extraction methods explored in this study, the HC and PPS methods are preferred for their high capacity to concentrate sporangia from 100 g soil samples to less than 0.5 ml slurry of soil extract, thereby achieving sporangial recoveries of 38% in REF soil and 60–70% in natural field soils (data not shown). The HC extract is appropriate for the capacity of the commercially available UC DNA extraction kit. HC extracts of 100 g soil samples could thus be processed in one single PCR assay. The extraction efficiency of NWS is 100%, but the final extract is concentrated on the sieve in relatively large volumes of up to 10 ml slurry in fine grain soils such as the loamy sand soil G13. NWS extracts exceed the 0.5 ml capacity of the UC kit and 10–20 DNA-extractions need to be done for a single PCR assay of a 100 g soil sample.

Not all soil extracts produced amplifiable DNA. In the final stage of this study, it became apparent that the PPS-extracts that remained unwashed, unlike the HC samples, probably contained traces of $CaCl_2$, which may interact with Mg^{++} availability and inhibit Taq polymerase.

Direct microscopic examination of soil for sporangia is currently being used in the EPPO

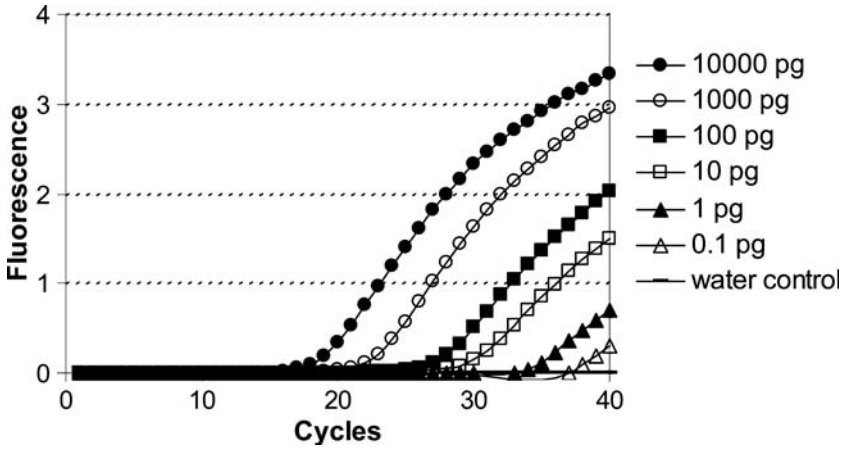

Figure 5. Real-time amplification and detection of a 10-fold dilution series of *Synchytrium endobioticum* DNA using the Taqman PCR; water without spiked DNA served as control.

protocol (EPPO, 1999) as one of the requirements for de-scheduling of infected potato fields. A negative result, however, is not a sufficient criterion for de-scheduling and a biological assay is required for confirmation (EPPO Phytosanitary Procedure PM 3/59). Our results suggest that the HC method in combination with a PCR assay could replace the laborious PPS extraction and counting, and bioassays. In addition, Taqman real-time PCR have been demonstrated to be 10-fold more sensitive, which may stimulate implementation of this quantitative PCR assay at routine diagnostic laboratories. The threshold level for infection and wart expression was shown to be 0.2 sporangium per g soil in a bioassay experiment using 2.5 l pots filled with naturally infested soil and each planted with a single seed tuber (van Leeuwen et al., 2005). Documented data on average sporangial densities in infected potato fields are scarce and not in the public domain. In two Dutch potato fields, natural levels amounted to 0.23 and 0.19 sporangia per g soil 3–5 years after the first report of wart diseases (JGN Wander, Applied Plant Research, The Netherlands, pers. comm.). From the bioassay threshold data and average soil contamination levels, it is concluded that the HC extraction method in combination with a specific PCR assay have at least the potential to compete with the prescribed EPPO methods in terms of sensitivity and specificity.

Table 3. Microscopic counts and real-time PCR threshold cycle (Ct values) for wart sporangia of *Synchytrium endobioticum* in HC extracts of spiked and unspiked REF soil (average of 5 repetitions)

Number of sporangia added $(100\ g)^{-1}$	Average numbers recovered $(100\ g)^{-1}$	Average Ct values
None	0.0a	40.0a
10	5.0b	37.4b
100	39.8c	31.6c
1000	385.8d	26.8d

Data followed by different letters in each column are significantly different at $p > 0.05$ (ANOVA).

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