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Conventional PCR and real-time quantitative PCR detection of *Helminthosporium solani* in soil and on potato tubers

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Abstract

Silver scurf is an economically important blemish disease of potato caused by the fungus *Helminthosporium solani*. Two sets of PCR primers, Hs1F1/Hs2R1 (outer) and Hs1NF1/Hs2NR1 (nested) were designed to unique sequences of the nuclear ribosomal internal transcribed spacer (ITS1 and ITS2) regions of *H. solani*. Nested PCR was used to increase the specificity and sensitivity of single round PCR. Each primer set amplified a single product of 447 bp and 371 bp respectively, with DNA from 71 European and North American isolates of *H. solani*, and the specificity of primers was confirmed by the absence of amplified product with DNA from other fungal and bacterial plant pathogens. A simple and rapid procedure for direct extraction of DNA from soils and potato tubers was modified and developed to yield DNA of a purity and quality suitable for PCR within 3 h. The sensitivity of PCR for the specific detection of *H. solani* in seeded soils was determined to be 1.5 spores g^{-1} of soil. *H. solani* was also detected by PCR in naturally infested soil and from peel and peel extract from infected and apparently healthy tubers. Specific primers and a TaqMan™ fluorogenic probe were designed using the original primer sequences to perform real-time quantitative (TaqMan™) PCR. The same levels of sensitivity for specific detection of *H. solani* in soil and tubers were obtained during first round TaqMan-based PCR as with conventional nested PCR and gel electrophoresis. This rapid and quantitative PCR assay allows an accurate estimation of tuber and soil contamination by *H. solani*, thus providing a tool to study the ecology of the organism and to serve as a crucial component for disease risk assessments.

Abbreviations: BSA – bovine serum albumin; CTAB – hexadecyltrimethylammonium bromide; cv – cultivar; dNTPs – deoxynucleoside triphosphates; ITS – internal transcribed spacer; PCR – polymerase chain reaction; PVPP – polyvinylpolypyrrolidone; SASA – Scottish Agricultural Science Agency; SCRI – Scottish Crop Research Institute; TBZ – thiabendazole.

Introduction

Helminthosporium solani Dur. & Mont. is the causal agent of silver scurf, an economically important fungal blemish disease of potato tuber periderm (Jellis and Taylor, 1974). Tubers infected with silver scurf develop tan to grey lesions that have a characteristic silvery appearance when moist. Silver scurf primarily reduces the market value of potato crops and is of increasing importance due to the demand for washed potatoes with a high quality appearance for the fresh 'pre-pack' market. Tuber skin blemish diseases (i.e., silver scurf, black dot, powdery scab, common scab) are a major cause of wastage to the British potato industry and potential cost savings by reducing their incidence were estimated at £9 million per year (British Potato Council, EYEwitness, 1998). Control strategies for *H. solani* are limited due to the development of insensitivity to thiabendazole (TBZ) and other benzimidazole fungicides applied as post-harvest tuber and

seed treatments (Hide et al., 1998; Kawchuck et al., 1994; Merida and Loria, 1990). Wastage due to silver scurf has thus increased throughout Europe and North America.

Tuber infection by *H. solani* may occur during the growing season from both contaminated seed tubers (Jellis and Taylor, 1977) and soil-borne inoculum (Firman and Allen, 1995; Jellis and Taylor, 1977; Merida and Loria, 1994), and the severity of disease on tubers can increase rapidly during long-term storage due to successive cycles of sporulation and infection (Jellis and Taylor, 1977; Rodriguez et al., 1996). The anamorphic fungus *H. solani* belongs to the family Pleosporaceae, is slow growing in culture and has been described only as a pathogen of potato (Jellis and Taylor, 1974). However, knowledge about the ecology of *H. solani* and the disease epidemiology of silver scurf is incomplete. An improved understanding of the epidemiology of silver scurf will assist in its control, thus improving the quality and efficiency of ware production and ensuring supplies of healthy seed. Methods are therefore required to detect and quantify *H. solani* in potatoes and soil to permit the screening of seed stocks and allow predictions on the probability of infection from field soils.

The polymerase chain reaction (PCR) allows the exponential amplification of specific DNA fragments (diagnostic amplicons) from complex DNA samples by *in vitro* DNA synthesis and has been used widely to detect plant pathogenic fungi (Miller, 1996). A PCR assay for the detection of *H. solani* was developed by Olivier and Loria (1998) using primers based on ITS regions. However, the detection of this fungus was only possible in seeded soil when a nested PCR was combined with at least a 20-fold dilution of soil DNA extract to reduce soil inhibitors. Furthermore, the sensitivity of this PCR assay was set at only 1000 spores of *H. solani* g^{-1} soil. Specific primers for the β-tubulin gene of *H. solani* were also reported for the identification of thiabendazole-resistant isolates (McKay and Cooke, 1997). Although these primers are specific for *H. solani*, they were not tested for detection of the organism directly in soil or from plant material. In addition, this PCR assay may be less sensitive than one based on the detection of target in a multi-copy ribosomal gene unit, as the β-tubulin gene is likely to be present in a low copy number in the genome of fungi (Luck et al., 1994; Smith et al., 1988).

Real-time quantitative PCR uses two primers and an additional dual-labelled fluorogenic probe to allow the continuous monitoring of amplicon synthesis during thermocycling and requires no post-PCR sample handling for target quantification (Orlando et al., 1998). Our aim was to develop rapid and robust PCR diagnostic (PCRD) systems for potato pathogens, and this study presents the development of specific primers for both conventional PCR and real-time quantitative (TaqMan™) PCR to allow rapid and sensitive detection and monitoring of *H. solani* on potato tubers and in soil.

Materials and methods

Design of H. solani*-specific PCR primers*

The internal transcribed spacer regions (ITS1 and ITS2) of the rDNA gene repeat of two UK isolates of *H. solani* (strains H2 [TBZ sensitive] and H6 [TBZ resistant]) were amplified with the universal primers ITS5/ITS4 (White et al., 1990), and PCR products were purified using the Wizard™ DNA Clean Up System (Promega) as recommended by the manufacturer. Both strands of each PCR product were sequenced on two separate occasions using primers ITS4 and ITS5, and an Applied Biosystems™ (Warrington, UK) Automated Sequencer and the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit, according to the manufacturer's instructions (PE Applied Biosystems, UK). ITS 1/2 and 5.8 S rDNA regions were subjected to a database search using both the BLAST and FASTA programs, and related sequences were aligned using the CLUSTAL V package (Higgens et al., 1992). The majority of related DNA sequences were for teleomorphs and associated anamorphs of genera in the Pleosporaceae, including isolates of *H. solani*, *H. asterinum*, *H. chlorophorae*, and *H. velutinum*, and other related fungi (i.e., *Alternaria*, *Bipolaris*, *Cercospora*, *Cochliobolus*, *Corynespora*, *Curvularia*, *Drechslera*, *Exserohilum*, *Leptosphaeria*, *Mycosphaerella*, *Ophiosphaerella*, *Phaeosphaeria*, *Pleospora*, *Pyrenophora*, *Rhizopycnis*, *Sarcoscypha*, *Stagonospora*, *Stemphylium*). Suitable ITS 1 and 2 regions were selected based on sequence disparities for the design of *H. solani*-specific primers, and two sets of 20-mer primers (outer and nested) were designed (Table 1) using the Primer! Lite program from the world wide web (http://www.williamstone.com/primers/). Expected amplicon sizes for the outer and nested PCR primers were 447 bp and 371 bp, respectively. The Primer Express® software (PE Applied Biosystems) was used

Table 1. Helminthosporium solani-specific PCR primers

Primers	Sequence (5′ to 3′)	Target DNA	Size of product (bp)
Conventional PCR			
First-Round PCR (Hs1F1 + Hs2R1)			447
Hs1F1	*GTTTCAGCGGCCGCAAGG CT	ITS1	
Hs2R1	CCAGGGCTTCAAGAAGCGCA	ITS2	
Nested PCR (Hs1NF1 + Hs2NR1)			371
Hs1NF1	*GAACCCTCTGTCTACCTGTA	ITS1	
Hs2NR1	ACGAGAAGCTGGCACGACCG	ITS2	
Real-Time Quantitative PCR			
First-Round PCR (HsTqF1 + HsTqR1)			131
HsTqF1	*GTTTCAGCGGCCGCAAG	ITS1	
HsTqR1	TTCAGATACAAGGGTTTAAGGGATTC	ITS1	
TaqMan™ Probe			
HsTqP1	*TCGGAACCCTCTGTCTACCTGTACCACTTGTT	ITS1	

*The underlined sequences denote those used in the design of both conventional PCR primers and TaqMan™ primers/probes.

to design primers and TaqMan™ probes based on the original primer sequences (Table 1) to develop real-time quantitative PCR. The original primers developed were not used because real-time PCR requires small amplicons of 50–150 bp in length to yield consistent results (PE Applied Biosystems). The fluorogenic probe (HsTqP1) was labelled at the 5′ end with the fluorescent reporter dye FAM (6-carboxy-fluorescein), and the 3′ end was modified with the quencher dye TAMRA (6-carboxy-tetramethylrhodamine) (PE Applied Biosystems). The starting concentration of target sequence present in each reaction was calculated by comparing Ct values of unknown samples to the Ct values of standards with known amounts of *H. solani* DNA, where the Ct value is defined as the cycle number at which a statistically significant increase in the reporter fluorescence can first be detected (i.e., exceeds the threshold), and is dependent upon the input of starting copies of target. Ct values were plotted versus the log of the initial concentration of *H. solani* DNA to produce a standard curve (Figure 5).

Extraction of microbial DNA from pure cultures, soils, and potato tubers

Mycelium was scraped from the surface of 7 day old colonies of *H. solani* grown on Potato Dextrose Agar (PDA; Difco Labs, UK) incubated at 15 °C, and was used to inoculate 50 ml of Potato Dextrose Broth (PDB; Difco Labs). Cultures were incubated at ambient temperature on an orbital shaker (100 rpm) in darkness for up to 14 days, and DNA extraction was performed by the method of Nicholson et al. (1996). For the inoculation of soils, spores were removed from the surface of colonies on agar plates, resuspended in sterile distilled water (dH_2O) and the concentration was determined by direct microscopic counting. Spore suspensions were made in sterile dH_2O and used to inoculate 4 kg soil to a concentration of 10^6 spores of *H. solani* per kg. This soil was serially diluted with virgin soil to produce 4 kg samples that contained 1000, 300, 100, 30, 10, 3, 1.5 spores of *H. solani* g^{-1} or the equivalent volume of dH_2O as an unseeded control. DNA was quantified using a TKO 100 Mini-Fluorometer (Hoefer Scientific Instruments, San Fransisco, USA) following the manufacturer's instructions and the quality was checked by agarose gel electrophoresis. Serial dilutions (10 ng μl^{-1} to 1 fg μl^{-1}) of genomic DNA in ultrapure dH_2O (HPLC-grade, Sigma, UK) were used as template to assess the sensitivity of PCR.

Soil samples

Samples were collected from three sites in Scotland representing two soil types. Sites were located at Gogar Bank and SASA Farm, both clay loam soils, and Pentland Hill, a silty clay loam, in which potatoes had not been planted for up to 13, 20, and 50 years, respectively. Duplicate or triplicate soil samples (10 g each) were resuspended in 20 ml extraction buffer (SPCB: 120 mM sodium phosphate, 2% CTAB (hexadecyltrimethylammonium bromide),

1.5 M NaCl; pH 8.0), sonicated for 15 min, and shaken (5 min) in the presence of 1.5 g sterile glass beads (1.0 mm diameter) on a flask shaker. Soil suspensions were allowed to settle for 1 min before triplicate 1.5 ml aliquots (equivalent to 0.75 g soil) were transferred to 2-ml screw-cap tubes containing 0.2 g each of 0.1 mm diameter zirconia/silica beads and 1.0 mm diameter glass beads, and were blended in a Mini-BeadBeater (Bio-Spec Products, Bartlesville, OK., USA) at 5000 rpm for 60 s. Samples were centrifuged (2460 $\times$ g for 5 min) and the supernatant was extracted with an equal volume of chloroform, mixed and re-centrifuged (11, 550 $\times$ g for 5 min). DNA in the aqueous phase was precipitated with 0.3 M sodium acetate (pH 5.2) and an equal volume of isopropanol for 1 h at room temperature. The DNA was pelleted by centrifugation (11, 550 $\times$ g for 5 min), washed in 70% ethanol, re-pelleted, resuspended in 75 µl TE (10 mM Tris–HCl and 1 mM EDTA, pH 8.0) and stored at $-80\,^{\circ}$C until required. Soil DNA extracts were purified through a Micro Bio-Spin column (0.8 ml capacity; Bio-Rad Laboratories, UK) that contained water-insoluble polyvinylpolypyrrolidone (PVPP; Sigma) (Cullen and Hirsch, 1998) except that a Biofuge 13 (Heraeus Instruments GmbH, Osterode, Germany) microcentrifuge was used at a speed of 5000 rpm (2200 $\times$ g). A combined spin column with both PVPP and Sephadex G-75 to purify DNA extracts that contained a high concentration of humic compounds, was prepared as follows: 0.8 ml G-75 slurry was added (height of 13–14 mm) to a spin column and residual buffer was collected in a 1.5 ml microcentrifuge tube after two successive centrifugation steps at 350 $\times$ g for 1 min. Dry PVPP powder was added on top of the Sephadex G-75 column to a height of 5–7 mm. Columns were conditioned by two sequential additions of 100 µl dH_2O, each followed by a 3 min centrifugation at 350 $\times$ g. DNA (75 µl) was added to the top of the PVPP powder and columns were centrifuged for 1 min (350 $\times$ g) and then allowed to stand for 2 min (to permit DNA to migrate into Sephadex pores) followed by two successive spins at 350 $\times$ g for 5 and 3 min. Purified eluate was collected in a new sterile 1.5 ml tube.

Tuber samples

Cultivars of Shula and Shelagh, which are classified as resistant and susceptible to silver scurf (Jellis and Plumb, 1991) were obtained from field trials at SCRI. Tubers were washed to remove excess soil, and 2 mm thick peel strips were removed from across the length of single tubers from the rose end to heel end with a hand-held potato peeler. Two methods of preparation for the extraction of DNA from tubers were compared to determine the most efficient and reliable for PCR analysis. Peel strips were stored at $-80\,^{\circ}$C for subsequent use or immediately passed through a roller press (Meuk, E. Pollähne, Wennisgen, Germany) for the collection of extract in a 30 ml tube containing the antioxidant, dithiothreitol (final concentration at 0.075%). The roller press was washed with 1 M NaOH, 70% ethanol, followed by water to prevent cross-contamination between samples. Peel extracts were stored at $-80\,^{\circ}$C before 0.5 ml aliquots were transferred to 2-ml screw-cap tubes containing 0.2 g each of 0.1 mm diameter zirconia/silica beads and 1.0 mm diameter glass beads and 1.0 ml SPCB. DNA was extracted from peel extract and purified by PVPP spin column chromatography as described for soil samples. Alternatively, frozen peel tissue was diced and then ground in liquid nitrogen using a mortar and pestle to produce a powder, and duplicate 0.5 g samples were transferred to 2-ml screw-cap tubes containing two 4.0 mm diameter steel ball bearings and 1.0 ml SPCB. Samples were blended in a Mini-BeadBeater at 4600 rpm for 60 s and processed as for peel extracts. Percentage disease cover of potato tubers was estimated by the method of Hilton et al. (2000).

PCR methods

PCR amplification of samples was based on an initial denaturation at 95 °C (2 min), followed by 35 cycles of denaturation at 95 °C for 45 s, annealing at 66 °C (Hs1F1/Hs2R1) or 60 °C (Hs1NF1/Hs2NR1) for 1 min, extension at 72 °C for 90 s, and a final elongation at 72 °C for 5 min in a reaction volume of 25 µl using a GeneAmp PCR System 9600 thermal cycler (PE Applied Biosystems). Optimal conditions for PCR (single round and nested) contained a master mix of the following components: 1 $\times$ reaction buffer (16 mM $[NH_4]_2SO_4$, 67 mM Tris–HCl pH 8.8, 0.1% Tween-20; Bioline UK Ltd), 200 µM each dNTPs (Bioline), 0.3 µM each primer (MWG-BIOTECH UK Ltd), 5.0 mM $MgCl_2$, 250 µg ml^{-1} BSA (Boehringer Mannheim, UK), 1U or 2U Biotaq Diamond (Bioline); the latter amount was used for soil and potato DNA extracts. One µl of undiluted or 1/10 diluted DNA (representing 10–100 ng) was used as template and 1 µl of single round PCR product was used for nested PCR.

H. solani DNA (10 ng) was used as positive control in the PCR assay; negative controls were carried out with PCR reagents and 1 µl dH_2O or non-target DNA. PCR products were analysed by electrophoresis on 2% agarose gels (2% NuSieve 3 : 1 agarose, Flowgen, UK) in 1 × TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.0), stained with ethidium bromide (0.5 mg l^{-1}), and photographed under UV illumination (Sambrook et al., 1989).

Real-time quantitative (TaqMan™) PCR was performed in MicroAmp optical 96-well plates using the automated ABI Prism 7700 sequence detector (PE Applied Biosystems). Reaction components were obtained from the TaqMan™ Universal PCR Master Mix (PE Applied Biosystems) and 25 µl and 50 µl reaction volumes were compared using 2 µl of undiluted and diluted (1/10, 1/20, 1/40, 1/50, 1/100) template DNA to determine the optimum concentrations for reliable quantification. Primers HsTqF1/HsTqR1 were included at a final concentration of 300 nM per reaction, and the Taqman™ probe (HsTqP1) was used at 100 nM. The manufacturer's recommended universal thermal cycle protocol (PE Applied Biosystems) was used for PCR amplification: stage 1 (50 °C for 2 min; AmpErase® uracil-N-glycosylase [UNG] digestion); stage 2 (95 °C for 10 min; denaturation of UNG and activation of AmpliTaq Gold DNA polymerase); and stage 3 (45 cycles at 95 °C for 15 s, 60 °C for 1 min). The Ct values for each PCR reaction were automatically calculated and analysed by the ABI prism sequence detection systems software (version 1.6).

The specificity of all primer sets was tested against genomic DNA from 71 European and North American isolates of *H. solani* (Table 2) and also from a range of other plant pathogens in the SCRI culture collection (Table 3). To exclude false negative results with *H. solani*-specific primers, all template DNA samples were tested for PCR amplification using universal primers (ITS5/4 for fungal DNA; V3 region primers 341F/534R for 16S bacterial rDNA following the method of Muyzer et al., 1993).

Results

Design and specificity/sensitivity of H. solani*-specific primers for conventional PCR*

A single PCR product of identical sequence (ITS1/2 and 5.8S rDNA) and size (550 bp) was amplified with the universal primers ITS4/ITS5 from DNA of both *H. solani* isolates H2 (TBZ^S) and H6 (TBZ^R) tested. Two sets of PCR primers (Hs1F1/Hs2R1 and Hs1NF1/Hs2NR1) were subsequently designed to unique sequences within the ITS1/ITS2 regions of isolates H2 and H6 (Table 1) after the alignment of related DNA sequences from other fungi (see Materials and methods). The sensitivity of PCR for the detection of *H. solani* was tested, and a specific product (371 bp) was reliably detected after a nested PCR (primers Hs1NF1/Hs2NR1) when genomic DNA was included at concentrations down to and including 1 fg per reaction (data not shown). Both outer and nested sets of primers amplified product of the correct size during a first round PCR from the corresponding genomic DNA of all 71 isolates of *H. solani* tested (Table 2; Figure 1). Comparisons between each primer with DNA and protein database sequences of other plant pathogenic fungi and bacteria revealed no significant levels of similarity, apart from 100% homology to ITS sequences in GenBank for 17 other isolates of *H. solani*. Primer specificity was confirmed in each case by the absence of PCR product when testing genomic DNA from a wide range of fungal and bacterial plant pathogens (Table 3; Figure 2). The quality of each DNA preparation for amplification by PCR was confirmed by the detection of product using either universal eukaryotic (ITS4/5) or prokaryotic (341F/534R) primers (Figure 2).

Detection of H. solani *from potato tubers and soil by conventional PCR*

Following a nested PCR using primers Hs1F1/Hs2R1 and Hs1NF1/Hs2NR1, the assay detected a single product of the expected size (371 bp) when tested against DNA extracted from tubers with no visible disease symptoms or with either 1% or 50% cover of silver scurf (Figure 3). No amplification products were detected using DNA extracts obtained from tubers showing symptoms of black dot or common scab, confirming both the specificity of the primers to detect *H. solani* and the absence of *H. solani* on the tubers tested (data not shown). There was no difference in the sensitivity of detection by PCR when DNA was extracted from either peel extract or diced and frozen tuber peel, or when undiluted and diluted (1/10) DNA extracts were tested.

H. solani was consistently detected by first round PCR (primers Hs1F1/Hs2R1) in soil samples seeded with conidia down to a level of 3 spores g^{-1} using two different soil types (see Materials and methods),

Table 2. Isolates of *Helminthosporium solani* from potato listed according to location and date of isolation

Code	Origin[a]	Date of isolation	Code	Origin[a]	Date of isolation
H1	Scotland, SASA Farm 3	1996	H37	Rock Springs, Pennsylvania, USA	—
H2	Scotland, SASA Farm 5	1996	H38	British Columbia, Canada	—
H3	Scotland, SASA Farm 4	1996	H39	Lake Placid, New York, USA	—
H4	Scotland, SASA Farm 1	1994	H40	North Dakota, USA	—
H5	Scotland, SASA Farm 6	1994	H41	Russia	1997
H6	Scotland, SASA Farm 5	1996	H42	Rothamsted Farm, England	1996
H7	Scotland, SASA Farm 5	1994	H43	Rothamsted Farm, England	1996
H8	Lincolnshire, England, BPC	1994	H44	Rothamsted Farm, England	1996
H9	Lincolnshire, England, BPC	1994	H45	Rothamsted Farm, England	1996
H10	Dundee, Scotland, SCRI	1990	H46	Rothamsted Farm, England	1996
H11	Dundee, Scotland, SCRI	pre-1990	H47	Rothamsted Farm, England	1997
H12	Scotland, SASA Farm 1	pre-1990	H48	Rothamsted Farm, England	1997
H13	Dundee, Scotland, SCRI	1993	H49	Rothamsted Farm, England	1997
H14	Scotland, SASA	—	H50	Rothamsted Farm, England	1997
H15	Scotland, SASA	—	H51	Rothamsted Farm, England	1997
H16	Scotland, SASA	—	H52	Rothamsted Farm, England	1997
H17	Scotland, SASA	—	H53	Rothamsted Farm, England	1997
H18	Dept. Agriculture, N. Ireland	1996	H54	Rothamsted Farm, England	1997
H19	Dept. Agriculture, N. Ireland	1996	H55	PBI, Cambridge, England	—
H20	Dept. Agriculture, N. Ireland	1996	H56	PBI, Cambridge, England	—
H21	Dept. Agriculture, N. Ireland	1996	H57	PBI, Cambridge, England	—
H22	Scotland, SASA	1998	H58	PBI, Cambridge, England	—
H23	Huntly, Scotland, SASA	1998	H59	PBI, Cambridge, England	—
H24	Aberdeenshire, Scotland,	1998	H60	PBI, Cambridge, England	—
H25	Forfar, Scotland, SASA	1998	H61	PBI, Cambridge, England	—
H26	Newmill of Balgavies, Scotland	1998	H62	PBI, Cambridge, England	—
H27	St.Andrews, Scotland, SASA	1998	H63	PBI, Cambridge, England	—
H28	Ross-shire, Scotland, SASA	1998	H64	PBI, Cambridge, England	—
H29	Unknown	1998	H65	PBI, Cambridge, England	—
H30	Unknown	1998	H66	PBI, Cambridge, England	—
H31	Eskhill, Scotland	1998	H67	PBI, Cambridge, England	—
H32	Eskhill, Scotland	1998	H68	PBI, Cambridge, England	—
H33	Eskhill, Scotland	1998	H69	PBI, Cambridge, England	—
H34	Dundee, Scotland, SCRI,	1998	H70	PBI, Cambridge, England	—
H35	Dundee, Scotland, SCRI,	1998	H71	PBI, Cambridge, England	—
H36	Minnesota, USA	—			

—: Unknown.
[a]BPC, British Potato Council; SASA; Scottish Agricultural Science Agency; SCRI, Scottish Crop Research Institute; PBI, Plant Breeding International.

and no signals were detected from unseeded controls (Figure 4). However, stronger signals were achieved at this concentration following nested PCR using primers Hs1NF1/Hs2NR1 (Figure 4). In addition, product of the correct size after nested PCR was also detected from soil samples (usually 2/3 replicates) seeded with the equivalent of 1.5 spores g^{-1}; the detection limit of the PCR system. *H. solani* was also detected by PCR in naturally infested field soil collected from Gogar Bank Farm, that had not been planted with potatoes for 13 years. The presence of viable propagules of *H. solani* was also detected in the same soil samples by a mini-tuber baiting test (unpublished results).

Real-time quantification of H. solani *DNA extracted from soil samples and potato tubers*

Real-time quantitative PCR was directly performed with an automated ABI Prism 7700 sequence detector using the primers (HsTqF1/HsTqR1) and the fluorogenic probe (HsTqP1) developed in this

Table 3. Fungal and bacterial species used in this study to test the specificity of *Helminthosporium solani* primers

Fungal species	Bacterial species
Alternaria brassicae[a]	*Enterobacter aerogenes*
Botrytis cinerea	*Erwinia carotovora* subsp. *atroseptica*
Colletotrichum acutatum[b]	*Erwinia carotovora* subsp. *betavasculorum*
Colletotrichum coccodes	*Erwinia carotovora* subsp. *carotovora*
Colletotrichum gloeosporioides[b]	*Erwinia carotovora* subsp. *odorifera*
Erysiphe graminis f.sp. *avenae*	*Erwinia carotovora* subsp. *wasabiae*
Erysiphe graminis f.sp. *hordei*	*Erwinia chrysanthemi*
Fusarium coeruleum	*Erwinia rhapontici*
Fusarium oxysporum[a]	*Erwinia stewartii*
Fusarium sulphureum	*Pseudomonas syringae* pv. *morsprunorum*
Helminthosporium solani	*Pseudomonas syringae* pv. *syringae*
Leptosphaeria maculans[a]	*Ralstonia solanacearum*
Nectria haematococca[a]	*Streptomyces acidiscabies*[c]
Neurospora crassa[a]	*Streptomyces scabies*[c]
Phoma exigua var. *foveata*	*Xanthomonas albilineans*
Phytophthora cactorum	*Xanthomonas campestris* pv. *campestris*
Phytophthora cryptogea	*Xanthomonas campestris* pv. *phaseoli*
Phytophthora erythroseptica	*Xanthomonas campestris* pv. *phaseoli* var. *fuscans*
Phytophthora fragariae	*Xanthomonas campestris* pv. *vesicatoria*
Phytophthora infestans	
Polyscytalum pustulans	
Puccinia recondita f.sp. *tritici*	
Pyrenopeziza brassicae[a]	
Pythium ultimum	
Rhizoctonia solani	
Sclerotinia sclerotiorum[a]	
Spongospora subterranea	
Tapesia yallundae[a]	
Verticillium dahliae[a]	
Verticillium lecanu	

[a]DNA samples were obtained from S. Foster, IACR-Rothamsted, Harpenden, UK.
[b]DNA samples were obtained from K. Hughes, Central Science Laboratories, York, UK.
[c]Isolates were obtained from R. Loria, Cornell University, Ithaca, NY, USA.

study. Based on three sample replications (each 45 cycles), the ABI Prism fluorescence detection system automatically calculated the starting concentration of *H. solani* DNA by a comparison of the Ct values from unknown samples with those of the standard curve (Figure 5). The linear correlation coefficient of the standard curve was $r = 0.969$ (Figure 5), demonstrating the accuracy of PCR-based quantification. During the optimisation of real-time PCR, it was determined that 2 µl of 1/20 diluted soil DNA extracts or potato tuber DNA extracts in a reaction volume of 50 µl, provided the optimal conditions for accurate quantification of *H. solani* DNA. No signals were obtained for the same undiluted DNA extracts in a reaction volume of 50 µl or from undiluted and diluted DNA extracts in 25 µl volumes.

Soil samples and tuber extracts tested by conventional PCR were also analysed and quantified by real-time PCR (Tables 4 and 5). The same level of sensitivity (1.5 spores g^{-1}) was achieved for soil samples and tuber extracts (symptomless) by real-time PCR after a first round of 45 cycles as during conventional nested PCR. *H. solani* target DNA was detected in apparently healthy potato tubers during real-time PCR. The lowest concentration of target DNA was detected in frozen peel from symptomless tubers at concentrations of 142 fg and 260 fg for cultivars Shula and Shelagh, respectively (Table 4). A higher concentration of target DNA was usually recovered when DNA was extracted from peel extract rather than frozen peel tissue (Table 4) and, as expected, higher levels of target DNA were detected as the percentage of disease cover increased. No signals specific for *H. solani* were detected in control tubers or those showing symptoms of black dot or common scab (data not shown).

It was possible to detect the equivalent of 1.5 *H. solani* spores g^{-1} in 2 out of 9 replicate seeded samples of Pentland Hill soil by real-time PCR. The quantity of target DNA detected from this soil at this level of spores, based on a comparison with the standard curve, was 90 fg (equivalent to 4.5 fg per PCR reaction at a 1/20 DNA dilution). However, the quantity of DNA template extracted from Pentland Hill soil samples was approximately 10-fold lower than the concentration recovered from the corresponding seeded soils from the SASA Farm (Table 5). This was most likely due to differences in soil type, mixing and dilution of soils, as the efficiency of DNA extraction between replicates from the same soils was consistent (Tables 4 and 5). The concentration of *H. solani* target DNA in the unseeded soil sample of Gogar Bank Farm was estimated at 132 fg (equivalent to 6.6 fg per PCR reaction at a 1/20 dilution), equivalent to ≤ 3 spores g^{-1} when compared to the amounts estimated for seeded soil samples (Table 5).

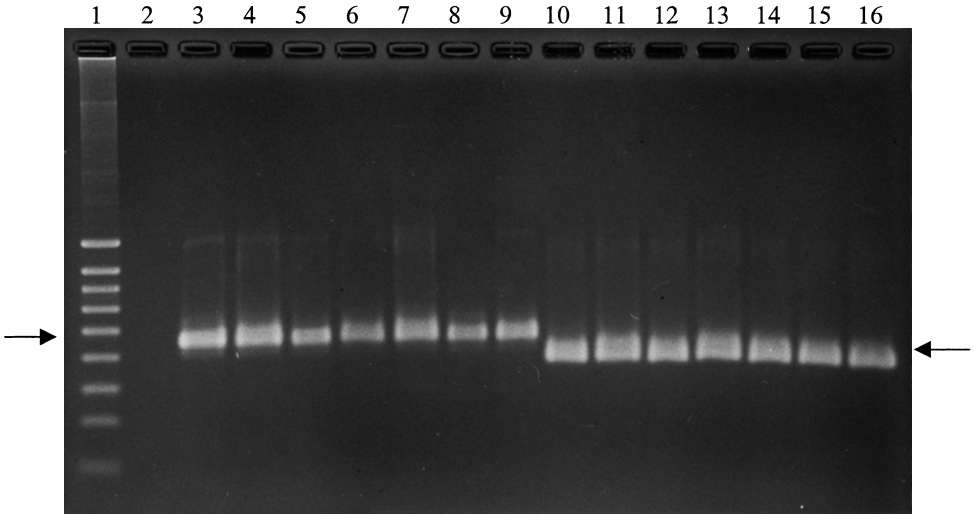

Figure 1. PCR amplification of genomic DNA from different isolates of *Helminthosporium solani* using primers Hs1F1/Hs2R1 and Hs1NF1/Hs2NR1. Lanes: 1, DNA marker; 2, negative control (dH_2O); 3–9 (amplification with *Hs1F1/Hs2R1) *H. solani* isolates, H2, H6, H8, H11, H18, H20, H24; 10–16 (amplification with *Hs1NF1/Hs2NR1) *H. solani* isolates, H36, H37, H38, H39, H40, H41, H55. *Both sets of PCR primers amplified a single product of the correct size from all isolates of *H. solani* tested (Table 2). Arrow markers on the left and right indicate the 447 bp (Hs1F1/Hs2R1) and 371 bp (Hs1NF1/Hs2NR1) products, respectively.

Discussion

In this study, we designed two sets of primers (outer and nested) for conventional PCR and one primer/probe set for real-time quantitative (TaqMan™) PCR for the specific detection of *H. solani* from soil and potato tubers. The specificity of both PCR systems was confirmed by testing against 71 isolates of *H. solani* and 49 other plant pathogenic fungi and bacteria (Tables 2 and 3). The PCR assay was of a high sensitivity to allow the detection of *H. solani* in seeded soil down to, and including, 1.5 spores g^{-1} and on potato tubers in the absence of visible symptoms of silver scurf. Unlike conventional PCR, real-time PCR has the advantage of calculating the absolute quantity of starting target DNA in tuber or soil samples within 3 h. This is in contrast to the 6 h taken by a conventional nested PCR, which, in this and previous cases, can only indicate the presence or absence of the target organism. Reproducibility among replicates was high (Tables 4 and 5), demonstrating that real-time PCR was accurate in terms of DNA extraction and recovery rates from all tuber and seeded soil samples. However, real-time quantitative PCR was more prone to inhibition by an inhibiting excess of DNA or co-extracted soil compounds as it was not possible to amplify signals from undiluted extracts in 25 µl or 50 µl reaction volumes, whereas this was achieved in 25 µl reaction volumes during conventional PCR. The choice between both PCR systems will be dictated by cost as the ABI Prism 7700 sequence detector system (PE Applied Biosystems) is more expensive than the equipment used for conventional PCR, even following the further development of this latter system to allow quantification, e.g., based on the use of competitor template fragments (Bell et al., 1999; Hyman et al., 2000).

The methods developed were more rapid and more sensitive than those of either Olivier and Loria (1998) or McKay and Cooke (1997), i.e., a detection limit of 1000 *H. solani* spores g^{-1} soil after nested PCR in the case of Olivier and Loria (1998) compared to 1.5 spores g^{-1} soil in this study. This contrast in sensitivity was probably due to the difference in DNA purity following DNA extraction, as Olivier and Loria (1998) found that a 20-fold DNA dilution in combination with a nested PCR was necessary to detect a PCR product, whereas undiluted samples could be used for conventional first round PCR in this study. Detection levels for *H. solani* in soil and on potato tubers were not recorded by McKay and Cooke (1997). The sensitivity of the PCR assay described here is further highlighted when the number of spores included per PCR reaction is considered; assuming a 100% recovery, for 3 spores g^{-1} (2.25 spores per 0.75 g sampled; DNA resuspended in

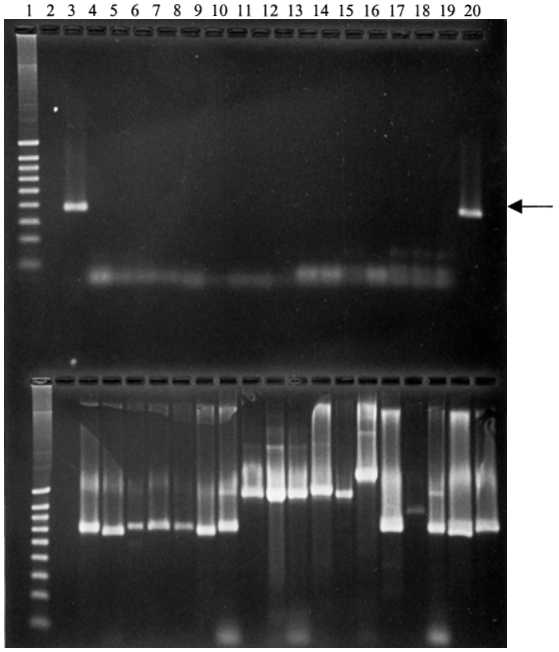

Figure 2. Specificity testing of *Helminthosporium solani*-specific primers against genomic DNA from a wide range of plant pathogens. Upper row (Hs1NF1/Hs2NR1), lanes: 1, DNA marker; 2, negative control (dH_2O); 3–20 (amplification with *Hs1NF1/Hs2NR1) isolates of *H. solani* H2, *Botrytis cinerea*, *Colletotrichum coccodes*, *Erysiphe graminis* f.sp. *avenae*, *Fusarium coeruleum*, *Fusarium sulphureum*, *Phoma exigua* var. *foveata*, *Phytophthora cactorum*, *Phytophthora cryptogea*, *Phytophthora erythroseptica*, *Phytophthora fragariae*, *Phytophthora infestans*, *Polyscytalum pustulans*, *Pythium ultimum*, *Rhizoctonia solani*, *Spongospora subterranea*, *Verticillium dahliae*, *H. solani* H6. Lower row (ITS4/ITS5), lanes 2–20 show amplification results using primers ITS4/ITS5 with the same fungal DNA extracts as tested in lanes 2–20, upper row. *No amplification products were generated with either set of primers (Hs1F1/Hs2R1 and Hs1NF1/Hs2NR1) when testing DNA samples from 49 other fungal and bacterial plant pathogens (Table 2). Arrow marker on the right indicates the 371 bp (Hs1NF1/Hs2NR1) product.

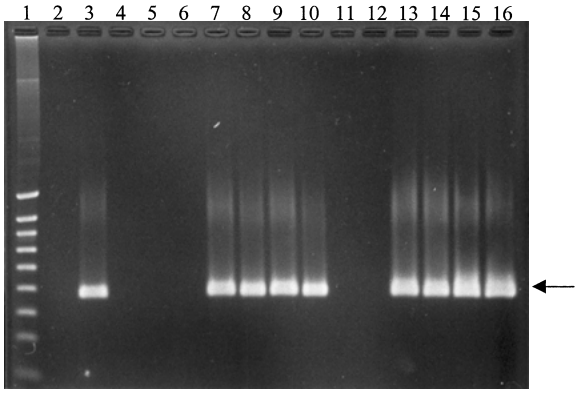

Figure 3. PCR detection of *Helminthosporium solani* from potato tubers. Lanes 2–16 show results from potato peel extracts amplified by nested PCR with primers Hs1F1/Hs2R1 followed by amplification with Hs1NF1/Hs2NR1. Lanes: 1, DNA marker; 2, negative control (dH_2O); 3, positive control (*H. solani* H2); 4–16 are undiluted DNA extracts from cvs. Shelagh (lanes 4–10) and Shula (lanes 11–16): 4, symptoms of black dot; 5–6, 0 control; 7–8, no visible symptoms; 9–10, 1% disease cover; 11–12, 0 control; 13–14, no visible symptoms; 15–16, 50% disease cover. Arrow marker on the right indicates the 371 bp (Hs1NF1/Hs2NR1) product.

75 µl) 1 µl of undiluted soil DNA extract would contain the equivalent of 0.03 spores in a reaction. In addition to the increased purity of extracted DNA, this study demonstrated the value of designing species-specific primers to ribosomal ITS regions, as their presence in multiple copies in the genome allows the detection of low concentrations of target DNA (Table 5; Figure 4). Primers specific to *H. solani* based on the β-tubulin gene (McKay and Cooke, 1997) would, in comparison, be present at a lower copy number in the genome of fungi (Luck et al., 1994; Smith et al., 1988), thus potentially reducing the sensitivity of the PCR assay.

The ITS1 and ITS2 regions that were sequenced for the two UK isolates of *H. solani* (H2 and H6) were 100% identical to the same regions deposited in GenBank of 6 North American isolates (Olivier and Loria, 1998) and 11 other isolates of *H. solani*. Therefore, as the *H. solani*-specific primers were designed from ITS regions and, as product was generated from DNA extracted from a wide range of European and North American isolates of *H. solani* (Table 2), detection of the fungus from an even wider geographic origin should be possible. Furthermore, although the blemish diseases silver scurf and black dot produce similar symptoms on potato, the PCR assay was able to distinguish *H. solani* from *Colletotrichum coccodes* (black dot), and can thus be used to differentiate symptoms of silver scurf from black dot. The detection of *H. solani* by PCR and mini-tuber baiting in the naturally infested field soil from Gogar Bank Farm, that had not been planted with potatoes for 13 years, further demonstrated the organism's ability to survive for long periods (Firman and Allen, 1995; Jellis and Taylor, 1977; Merida and Loria, 1994). The infection of mini-tubers by *H. solani* confirmed that viable propagules existed,

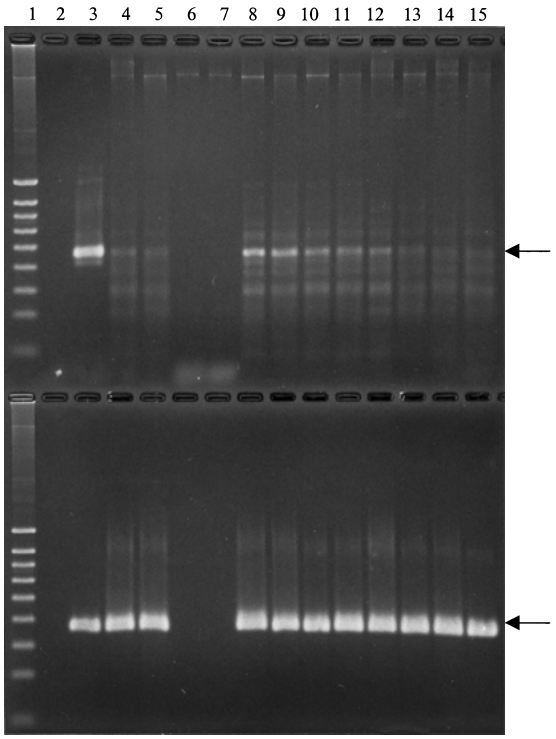

Figure 4. PCR detection of *Helminthosporium solani* in seeded soils and naturally infested soils. Upper row, lanes 2–14 show results after first-round amplification (primers Hs1F1/Hs2R1) of undiluted soil DNA extracts. Lanes: 1, DNA marker; 2, negative control (dH_2O); 3, positive control (*H. solani* H2); 4–5, unseeded Gogar Bank soil; 6–7, unseeded Pentland Hill soil; lanes 8–15 are seeded (*H. solani* spores g^{-1} soil) Pentland Hill soil samples as follows; 8–9, 300 spores; 10–11, 30 spores; 12–13, 3 spores; 14–15, 1.5 spores. Lower row, lanes 2–15 show amplification results of nested PCR (primers Hs1NF1/Hs2NR1) following the amplification of (1 μl) first-round products shown in lanes 2–15, upper row. Arrow markers on the top right and bottom right indicate the 447 bp (Hs1F1/Hs2R1) and 371 bp (Hs1NF1/Hs2NR1) products, respectively.

and highlighted the potential threat of disease development in potato crops if planted in this soil. Such a PCR test may thus be crucial in crop management decisions regarding suitable field sites for planting potatoes.

The success and reliability of any PCR assay will largely depend upon obtaining high yields and representative samples of target DNA from environmental samples. The extraction method used in this study is simple and inexpensive with a small number of efficient lysis and purification steps but can maximise the yields and quality of recovered DNA to allow rapid processing of many samples. The extraction procedure is based on the physical disruption of microbes within the sample using a beadbeater, to produce the highest yields of DNA and to lyse the majority of cell types within a sample (Borneman et al., 1996; Cullen and Hirsch, 1998; Miller et al., 1999; Yeates et al., 1997). High molecular weight (23 kb) DNA of a suitable purity for PCR was recovered from soil and potato tubers within, at most, 3 h by the modified extraction method (Cullen and Hirsch, 1998). The key steps of the protocol for the removal of co-extracted humic material/phenolic compounds from soil and plant material were the use of alkaline sodium phosphate + CTAB buffer and the purification of extracted DNA by either PVPP or a combined PVPP/Sephadex G-75 spin column chromatography. We recommend the use of potato peel extracts for the extraction and PCR detection of plant pathogens, as this method was less laborious and time-consuming than extracting DNA from diced and frozen peel tissue. In the case of real-time PCR, a higher yield of target DNA was recovered from peel extract than from frozen and diced peel tissue (Table 4). It was possible to detect *H. solani* in soil when spiked with as little as $\leq$3 spores g^{-1} in a single day, with the DNA extraction and PCR protocols developed. The same DNA extraction methods have been used successfully to detect other potato blemish pathogens such as *Colletotrichum coccodes* and *Streptomyces scabies* (Cullen et al., 1999), and *Spongospora subterranea* (Bell et al., 1999) in soil and plant material. PCR diagnostics thus offer the potential to allow rapid and accurate identification of diseases, and to quantify the presence of a pathogen in pre-symptomatic potato stocks and in soil. However, it is important that such sensitive methods are used to determine the threshold levels that are necessary for disease development and this will be crucial in the control of crop diseases. The molecular tools developed are currently being used to measure the inoculum levels of *H. solani* in several field soils and potato tubers as part of an epidemiological survey involving crop rotation with an aim to determine the threshold levels that are necessary for the development of silver scurf.

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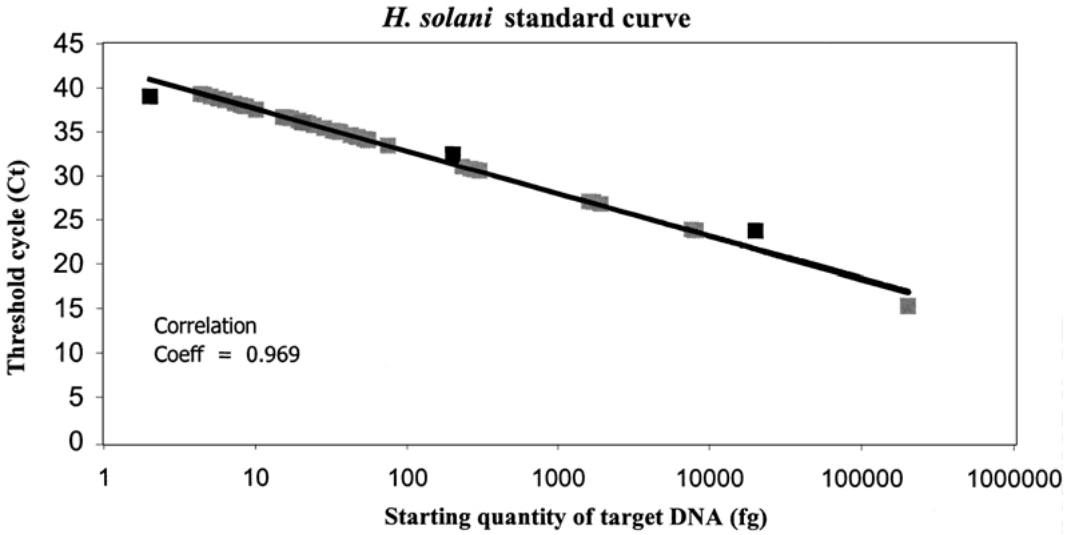

Figure 5. Calibration graph used for the absolute quantification of *Helminthosporium solani* target DNA during real-time PCR.

Table 4. Quantification of the starting concentration of *Helminthosporium solani* template DNA in naturally infected potato tubers

Cultivar/ sample tested[a]	% cover of disease[b]	Concentration of *H. solani* DNA[c]
Shelagh		
Peel extract	Control(0)	0
Peel extract	0	260 ± 3.6 fg
Peel extract	0	846 ± 11.1 fg
Frozen peel	1	446 ± 9.8 fg
Peel extract	1	932 ± 5.3 fg
Shula		
Peel extract	Control (0)	0
Frozen peel	0	142 ± 8.6 fg
Peel extract	0	386 ± 4.5 fg
Frozen peel	50	157 ± 5.0 pg
Peel extract	50	34.6 ± 3.0 pg

[a]DNA was extracted from frozen peel or peel extract as described in Materials and methods.
[b]Disease cover as estimated by Hilton et al. (2000).
[c]Mean concentration ± standard deviation from 3 replications.

Table 5. Quantification of the starting concentration of *Helminthosporium solani* template DNA in soil samples

No of spores added to soil g^{-1}	Concentration of *H. solani* DNA[a] (fg)
*Gogar Bank Farm**	
0	132 ± 9.0
*SASA Farm**	
0	0
3	168 ± 3.0
30	1060 ± 10.8
300	5500 ± 26.6
*Pentland Hill***	
0	0
1.5	90 ± 14.0
3	124 ± 21.2
30	168 ± 12.4
300	872 ± 17.0

[a]Mean concentration ± standard deviation from 6* or 9** replications as indicated.

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