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Detection and quantification of *Spongospora subterranea* f. sp. *subterranea* in soils and on tubers using specific PCR primers

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Abstract

PCR-based methods were developed for the detection and quantification of the potato pathogen *Spongospora subterranea* f. sp. *subterranea* (*S. subterranea*) in peel, tuber washings and soil. A partial sequence was obtained for *S. subterranea* ribosomal DNA and specific PCR primers (*Sps*1 and *Sps*2) were chosen from the internal transcribed spacer regions. These primers amplified a 391 bp product from *S. subterranea* DNA but did not amplify DNA from potato or a range of soil-borne microbes, including related species. Diluted *S. subterranea* DNA was detected at a concentration equivalent to 25×10^{-5} cystosori or 1 zoospore per PCR. Amplification was detected from peel and washings of infected and apparently healthy tubers, but not from peel of Scottish classified seed potatoes or axenically micropropagated potatoes. A rapid method for extracting *S. subterranea* DNA from soils was developed. This yielded DNA pure enough for PCR within 3 h and facilitated the detection of 1–5 cystosori per gram of soil. A PCR quantification technique was developed involving comparison of product ratios obtained after co-amplification of *S. subterranea* DNA along with an internal standard (competitor DNA fragment). This quantitative technique was also adapted for use in soil. PCR detection of *S. subterranea* in soil was considerably more sensitive than previously reported immunoassays and was quicker and easier than conventional bait plant bioassays. Such an assay could be useful for developing disease risk assessments for field soils and seed potato stocks and for future studies on the ecology and control of *S. subterranea.*

Abbreviations: dNTPs – deoxynucleoside triphosphates; EtBr – ethidium bromide; ITS – internal transcribed spacer; PCR – polymerase chain reaction; SAC – Scottish Agricultural College; SCRI – Scottish Crop Research Institute; SDW – sterile distilled water.

Introduction

Spongospora subterranea (Wallroth) Lagerheim f. sp. *subterranea* Tomlinson (subsequently referred to as *S. subterranea*) is the causative agent of powdery scab, a blemish disease of potato tubers (*Solanum tuberosum* L.). It falls in the plasmodiophorid group of organisms, which show some similarities to fungi and protozoans but differ significantly from both (Braselton, 1995). Powdery scab infections are manifested as disfiguring lesions, each of which contains a mass of brown powdery cystosori (resting spore balls or sporosori). These are sponge-like aggregations, 19–85 μm across (Jones, 1978), of many thick-walled cysts. Cystosori can survive for many years in soil (Kole, 1954) before germinating to release

1 zoospore per cyst. Disease inoculum may be seed- or soil-borne. Powdery scab can lead to serious losses in the yield of marketable tubers and it is a major disease problem for growers in many areas of the world (Wale, 1987; Harrison et al., 1997; Falloon et al., 1999). Infection on seed tubers can also reduce yields in the subsequent crop (Falloon et al., 1998). Furthermore, *S. subterranea* acts as a vector of potato mop-top furovirus (Jones and Harrison, 1969), which can be seriously detrimental to some potato cultivars. Although powdery scab was first reported in 1841 (Wallroth, 1842), our understanding of its pathology remains poor, partly due to its obligate biotrophic nature.

Powdery scab is difficult to control (Burgess and Wale, 1994), although some recent progress has been made (Falloon et al., 1999). Minimising soil- and seed tuber-borne inoculum should reduce disease risk and for this, methods to detect and quantify *S. subterranea* levels are required. Polyclonal antiserum in an enzyme-linked immunosorbent assay (ELISA) has been used to quantify low levels of tuber contamination, but when subsequent batches of antisera were tested, the method was much less sensitive (Harrison et al., 1993). Detection of cystosori in soil is much more difficult. Walsh et al. (1996) did not detect fewer than about 100 cystosori per gram of soil using polyclonal antiserum in ELISA, so a more sensitive technique is required. Bioassays, using tomato or potato microplants as bait plants have been used successfully to detect or quantify *S. subterranea* in soil (Flett, 1983; Wale et al., 1993). These are more sensitive than ELISA-based methods but are slow, laborious, require experience to recognise infection and may be unable to differentiate high inoculum levels.

Species-specific PCR tests are available for a number of plant pathogens, including some plasmodiophorids (Mutasa et al., 1995; Buhariwalla et al., 1995).Variations in the internal transcribed spacer (ITS) sequences of the ribosomal RNA operon were used to differentiate species and isolates of *Polymyxa* (Ward et al., 1994). Coupling PCR with suitable DNA extraction methods can allow direct detection of target microbes in soil and plant material. PCR can be modified to quantify the target DNA by the co-amplification of a competitor DNA fragment as an internal standard (Wang et al., 1989). This has been employed in quantitative detection of microbes in environmental samples (Möller and Jansson, 1997). The aim of this work was to develop a rapid and species-specific PCR-based assay for the detection and quantification of *S. subterranea* in soil and on tubers.

Materials and methods

S. subterranea *samples: sources, isolation and counting of cystosori*

Three different samples of scabbed tubers were used as a source of *S. subterranea*: cv. Erntestolz, harvested 1995, Aberdeenshire, UK; cv. Duke of York, harvested 1996, Arthrath, Aberdeenshire (both supplied by S.J. Wale) and cv. Maris Piper, harvested 1996, Gourdie, Dundee, UK. All were stored in darkness at 4 °C for up to 6 months, prior to isolation of cystosori. Tubers were washed in tap water and dried on absorbent tissue at room temperature overnight. Powdery scab lesions were cut from tubers using a razor blade and passed through a 53 μm mesh sieve. Sieved cystosori were counted using a previously described method (Arif et al., 1995), i.e. they were suspended in 10 ml SDW and centrifuged at 3000*g* for 1 min, the pellet was resuspended in 10 ml SDW and the centrifugation and resuspension were repeated 5 times. The pellet was finally resuspended in 5 ml SDW and transferred to a glass 5 cm Petri dish under a stereoscopic microscope. Up to 100 cystosori were counted out using a finely drawn Pasteur pipette directly into sterile 1.5 ml microtubes. When more than 100 cystosori were required, numbers were estimated by suspending cystosori in 1 ml SDW and counting 5 replicate samples using a haemocytometer, diluting where appropriate and evaporating the water at 37 °C.

DNA from a sample of *S. subterranea* with an atypical ITS sequence ('Inverness' sample; Bulman and Marshall, 1998) was supplied by S. Bulman.

Samples of 2 other isolates of *S. subterranea* were supplied by L. Torrance and one of these – isolate I – had previously been described as carrying the potato mop-top virus (Arif et al., 1995).

Release of S. subterranea *zoospores*

Zoospores were released from cystosori from tubers of cv. Duke of York (Wallace et al.,1995), except that incubation of cystosori was in sterile soil leachate rather than SDW. Zoospore concentrations were estimated with a haemocytometer and DNA was extracted from the pellet obtained after centrifugation of the zoospore suspension at 3000*g* for 10 min.

Samples of other obligate parasites

Resting spores of *Plasmodiophora brassicae* were isolated from galls of cabbage roots (supplied by S.J. Wale) as described by Arnold et al. (1996). Resting spores of *Ligniera* sp., *Polymyxa betae*, *Polymyxa graminis* and *Olpidium brassicae* (supplied by M.J. Adams) (Table 1) were passed through a 53 μm mesh sieve to remove large fragments of host root debris. DNA extractions were performed on approximately 10^5 resting spores of each pathogen. DNA from each pathogen (except *Ligniera* sp.), extracted as described by Mutasa et al. (1993), was also supplied by E. Mutasa-Göttgens. Samples of watercress that had been inoculated with *S. subterranea* f. sp. *nasturtii* and had displayed symptoms of crookroot were supplied by G. Down and DNA was extracted from the infected tissue.

Culture of soil-borne fungi

Various soil-borne fungi were obtained from the SCRI culture collection (Table 1) and grown in 20 ml of a defined sucrose/asparagine/mineral salts broth (Elliot et al., 1966) at 25 °C for 2–7 days. Mycelia of each isolate were washed with SDW and approximately 0.1 g (fresh weight) was used for DNA extraction.

Preparation of potato tissue and tuber washings for DNA extraction

Duplicate DNA extractions were performed on approximately 0.1 g tissue samples from each of the following: leaves and mini-tubers from axenically micropropagated potatoes (cvs. Russet Burbank and Saturna respectively, supplied by S. Millam); a scabbed tuber (cv. Duke of York, Arthrath, Aberdeenshire, UK, supplied by R. Clayton); one apparently healthy tuber of each of cvs. Desiree, Maris Bard and Estima (purchased from a supermarket, washed prior to purchase) and two apparently healthy Scottish classified seed tubers, cv. Swift (grade EEC3, purchased from a garden store). Thin slices of peel (up to 0.5 mm deep) were cut using a razor blade from 2 widely separated areas of each tuber and chopped into small pieces prior to DNA extraction. Washings were prepared

Table 1. Origin of microbes tested for amplification with universal primers, ITS4 and ITS6 and *S. subterranea*-specific primers, *Sps*1 and *Sps*2

	Isolate No.	Host	Location	Isolation year
Microbes isolated from potato				
Colleteotricum coccodes (Wallr.) S. Hughes	CLC77	cv. Maris Piper	SCRI	1988
Gliocladium roseum Bainier	GLR172	cv. Maris Piper	SCRI	1983
Fusarium avenaceum (Fr.) Sacc.	FAVA2	cv. Desiree	Dalgety, Angus, UK	1992
Fusarium oxysporum Schlecht.	FOX150	cv. Maris Piper	SCRI	1984
Fusarium sulphureum Schlecht.	FSU163	cv. Maris Piper	SCRI	1987
Monodyctis levis (Wiltshire) S. Hughes	MDL203	cv. Maris Piper	SCRI	1985
Pythium ultimum Trow	85r/80	cv. Maris Piper	SCRI	1990
Rhizoctonia solani Kuhn	RZS374	cv. Maris Piper	SCRI	1984
Trichoderma viride Pers.	TDV402	cv. Maris Piper	SCRI	1986
Verticillium tricorpus Isaac	VTR447	cv. Maris Piper	SCRI	1982
Streptomyces scabies	ATCC49173	Not known	ATCC	Not known
Other microbes				
Fusarium equiseti (Corda) Sacc.	FEQ134	Soil isolate	SCRI	1985
Ligniera sp.	F69V0F*	Small sugar pumpkin	California, USA	Not given
Olpidium brassicae (Wor.) Dang.	F61V0B*	Lettuce	California, USA	1992
Phoma chrysamthemicola Hollos	PHC85	Chrysanthemum	Dundee, UK	1985
Plasmodiophora brassicae Woronin	PBJC1	Cabbage	Aberdeen, UK	1996
Polymyxa betae Keskin	41*	Sugar beet	Norfolk, UK	1992
Polymyxa graminis Ledingham	45*	Barley	Oxfordshire, UK	1992
Spongospora subterranea f. sp. *nasturti*	Not given	Water cress	Bath, UK	1998
Verticillium lecanii (A.W. Zimmerm.) Viegas	VLE430	Raspberry	SCRI	1984

*Isolate numbers supplied by Dr. M.J. Adams, Rothamsted Experimental Station, Harpenden, UK.

from tubers, which were not visibly soiled (supplied by R. Clayton). Five of these showed no powdery scab symptoms and came from a crop not known to be infected (cv. Maris Piper, Ellon, Aberdeenshire), and 5 were moderately scabbed (cv. Desiree, Peterhead, Aberdeenshire). Washings were prepared by vigorously rubbing the tubers by hand in separate, fresh polythene bags along with 15 ml SDW. The liquid was centrifuged at 3000*g* for 10 min, the supernatant discarded and DNA extracted from the pellet.

DNA extraction

DNA was extracted from microbial and potato material using a Nucleon® PhytoPure DNA Extraction Kit (Nucleon Biosciences, Coatbridge, UK). DNA extracted from *S. subterranea* with this kit amplified more consistently with *Sps*1 and *Sps*2 than did DNA extracted using two other techniques [Kolar et al., 1988; Raeder and Broda, 1985 (adapted by D.C. Guy, SCRI)], suggesting lower levels of compounds inhibitory to PCR. A few grains (approximately 0.03 g or 50 µl) of sterile sand were added to 1.5 ml microtubes along with fungal or potato material, and the contents were ground briefly with a Treff homogeniser (Scotlab, Coatbridge, UK). Cell lysis and DNA extraction were then performed as recommended in the PhytoPure kit instructions. DNA pellets were washed twice in 70% ethanol, air dried for 30 min, dissolved in 10 µl (extractions from up to 100 cystosori) or 100 µl SDW (all other extractions) and stored at 4 °C. For quantitative PCR, greater care was taken to standardise extraction conditions; in particular samples were ground for 5 min and DNA was precipitated overnight at room temperature. DNA in extracts was quantified by electrophoresis alongside a lambda DNA (Pharmacia Biotech, St. Albans, UK) dilution series in 1.0% (w/v) agarose gels with Tris–borate–EDTA buffer with EtBr staining, according to standard methods (Sambrook et al., 1988). For PCR sensitivity tests, serial dilutions of DNA extracted from 10^5 cystosori or 2×10^5 zoospores were used.

DNA extraction from soil samples

A rapid method was developed to extract DNA from *S. subterranea* in soil samples. Samples of soil (10 g) were placed in sterile universal bottles with 20 ml of extraction buffer [2% hexadecyltrimethyl ammonium bromide (CTAB), 1.5 M NaCl, 120 mM Na_2HPO_4, pH 8.0]. These were vortex mixed and repeatedly inverted for 1 min to give a slurry and then sonicated in a water bath for 30 min. The slurry was mixed again and 1 ml aliquots were transferred (using cut-ended pipette tips) into sterile 2 ml screw cap vials containing 0.1 g of 1 mm glass beads. Tubes were shaken at 5000 rpm on a Biospec Mini Bead Beater (Stratech Scientific, Luton, UK) for 1 min to rupture cystosori. Solid debris was pelleted by centrifugation for 5 min at 2900*g* and the supernatant (approximately 700–800 µl) was transferred to a fresh microtube containing 750 µl of chloroform. This tube was shaken for 30 s and then centrifuged at 13 400*g* for 5 min. The upper aqueous phase was removed, mixed with 750 µl of isopropanol in a fresh microtube and allowed to stand for 1 h at room temperature. DNA was pelleted by centrifugation at 13 400*g* for 10 min. The supernatant was removed and the tubes were air dried for 5 min before dissolving the pellet in 100 µl of TE buffer, pH 8.0. The DNA extracts were further purified by passage through Sephadex spin columns. These were prepared by addition of 700 µl of a slurry of pre-swollen Sephadex G 75 beads to a sterile Micro Bio-Spin column (Bio-Rad, Hemel Hempsted, UK) held in a capless 1.7 ml microtube. Excess buffer was removed from the columns by centrifugation at 320*g* for 2 min, at which point eluate was discarded from the lower tube, followed by a further 1 min centrifugation and removal of eluate. DNA extracts were loaded on to the columns, which were spun for 3 min at 320*g*, and the eluates were transferred to fresh tubes.

An alternative extraction buffer (10 mM Tris, 1 mM EDTA, 2% CTAB, pH 8.0) was used in place of the phosphate extraction buffer in the above protocol to investigate the effects of buffer composition on extract purity.

Soil samples were spiked by the addition of known numbers of cystosori in order to determine the sensitivity of the test. Samples of soil without any added cystosori and extraction buffer without soil were also tested in these experiments as negative controls.

PCR conditions

Reaction mixtures (25 µl) contained 50–100 ng template DNA (where quantified) in 0.5 µl SDW, 250 µM of each dNTP (dATP, dTTP, dGTP and

dCTP) (Bioline, London, UK), 2.5 µl PCR reaction buffer (Bioline), 1 U *Taq* polymerase (Bioline) and 0.5 µM primers (Pharmacia). Primer pairs used were universal primers, ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990) and ITS6 (5′-GAAGGTGAAGTCGTAACAAGG-3′) (Cooke and Duncan, 1997); or *S. subterranea*-specific primers, *Sps*1 (5′-CCTGGGTGCGATTGTCTGTT-3′) and *Sps*2 (5′-CACGCCAATGGTTAGAGACG-3′), which were designed to yield an amplification product of 391 bp. For the production of a competitor DNA fragment for PCR quantification, primer *Sps* comp1 (5′-CACGCCAATGGTTAGAGACGTCGACGAAAGCGCAACTTGCGTTC-3′) was used in the PCR reaction with primer *Sps*1. The 24 bp sequence on the 3′ end of primer *Sps*comp1 was designed to anneal to a region 142 bp upstream from the annealing site of *Sps*2 to yield an amplification product of 249 bp with *Sps*1. The remainder of *Sps*comp1 was identical to *Sps*2 so that the competitor DNA could subsequently be re-amplified using *Sps*1 and *Sps*2. Positive (DNA extracted from 10^5cystosori) and negative (SDW) controls were included in all PCR tests. Problems with contamination were minimised by using dedicated pipettes and aerosol resistant tips. Furthermore, PCR reagents were prepared in isolation from areas where PCR products were handled, and were exposed to ultraviolet light (320 nm) for 10 min before the addition of template DNA (Sarker and Sommer, 1990).

A Techne Progene thermocycler (Techne Ltd., Cambridge, UK) and 200 µl thin walled PCR tubes (Bio-Rad) were used in all PCRs. The following thermal profiles were used for PCR: initial denaturation at 95 °C for 2 min, followed by 35 cycles (PCR with DNA extracted from zoospores or 100 or fewer cystosori or soil samples) or 30 cycles (all other PCRs) of melting (95 °C for 20 s), annealing (55 °C for universal primers or 64 °C for *S. subterranea*-specific primers for 25 s) and extension (72 °C for 50 s) with a final cycle of 72 °C for 10 min. Amplified DNA fragments were separated and visualised by electrophoresis and EtBr staining as described previously or with SYBR Gold (Flowgen, Lichfield, UK) staining (as recommended by the manufacturer) alongside standard size markers (100 bp ladders, Pharmacia). All PCR tests were repeated at least twice. Amplification products (10 µl) obtained with *Sps*1 and *Sps*2 were digested with 1 unit of the restriction endonuclease, *Cac*8I (New England Biolabs, Hitchin, UK) according to the manufacturer's instructions.

Sequencing of S. subterranea *PCR products*

Direct PCR product sequencing of *S. subterranea* ITS regions was carried out on an Applied Biosystems™ (Warrington, UK) Automated Sequencer using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit according to the manufacturer's instructions (Perkin Elmer, Warrington, UK) with a Perkin Elmer GeneAmp 9600 PCR thermocycler. The sequencing reaction contained approximately 120 ng of *S. subterranea* DNA and was initiated on both strands using the ITS4 and ITS6 primers. Sequence data were aligned with ITS1 and ITS2 data for *S. subterranea*, *Plasmodiophora brassicae*, *Polymyxa betae*, *Polymyxa graminis*, *Sorosphaera veronicae* Schroeter and *Spongospora subterranea* (Wallr.) Lagerh. f. sp. *nasturtii* Tomlinson. These sequences were supplied by S. Bulman, J. Marshall and R. Falloon, sequences were aligned using the computer package CLUSTAL V (Higgins et al., 1992), and primer sequences (*Sps*1 and *Sps*2) were chosen from species-specific regions.

PCR quantification using competitor DNA

S. subterranea DNA was amplified with *Sps*1 and *Sps*comp1 to yield a 249 bp competitor DNA fragment. This was purified by extraction with chloroform and precipitation with 2 volumes of ethanol, redissolved in dH_2O and quantified using a spectrophotometer (260 nm). The competitor DNA was added to PCR reaction mixes at concentrations of 1 fg per 25 µl reaction mix. Target DNA extracted from 5, 10, 50, 100, 500, 1000 or 5000 cystosori. Two sets of extracts were prepared on different occasions (each included triplicate samples of each number of cystosori) and a 0.5 µl aliquot of each 100 µl extract was used in PCRs in the presence of the competitor DNA. Three competitive PCRs were performed on each using different preparations of competitor DNA.

PCR products were visualised using 2% (w/v) agarose gels, and photographed using Polaroid 667 black and white instant film (Polaroid Ltd., St Albans, UK). Photographs were transferred to computer by scanning and stored as image files in TIFF format. The image files were imported to GelCompar™ Version 4.0 (Applied Maths, Kortrijk, Belgium), where measurements of band intensities were made as a measure of the amount of DNA. The ratio of the amount of amplified target DNA: amount of amplified competitor DNA was calculated for each reaction and was plotted against

the number of cystosori used for DNA extraction. For samples where only one amplified DNA product band could be visualised, that band was arbitrarily assigned a value of 95% of the total and the other product 5% so that a ratio could be calculated. This value was chosen because the faintest bands that could be quantified always had a value of at least 5% of total amplified DNA.

PCR quantification in soils

Soils from 5 different agricultural fields (at SCRI or supplied by S.J. Wale) were tested to ensure that no *S. subterranea* could be detected by PCR. Samples (10 g) were then spiked with 10 000, 1000, 100 and 10 cystosori per gram, extraction buffer (20 ml) was added and 0.5 pg of competitor DNA was added to each sample bottle prior to mixing and DNA extraction. The quantitative response obtained was measured by analysis of PCR product band intensity.

Results

Specificity of Sps*1 and* Sps*2 primers*

The universal primers ITS4 and ITS6 were used to amplify products (of varying sizes) from DNA extracts from all of the organisms listed in Table 1 (Figure 1a, data not shown for *S. subterranea* f. sp. *nasturtii* or *Streptomyces scabies*). Amplification from obligate parasites yielded multiple bands, possibly indicating multiple copies of varying sizes or the presence of contaminating DNA sequences and reflecting the low stringency of the amplification conditions for these primers.

Using the *S. subterranea*-specific primers *Sps*1 and *Sps*2, a single amplification product of the predicted size (391 bp) was obtained with DNA from 6 *S. subterranea* isolates (3 isolated by us; 2 supplied by L. Torrance and the 'Inverness' sample from S. Bulman) (Figure 1b, data shown only for *S. subterranea* from tubers of cv. Erntestolz). These PCR products were digested with the restriction endonuclease *Cac*8I to yield bands of the predicted sizes (results not shown), thus confirming the identity of the product. PCR with *Sps*1 and *Sps*2 failed to amplify extracts from each of the other organisms tested (Figure 1b, results not shown for *S. subterranea* f. sp. *nasturtii* or *Streptomyces* spp.).

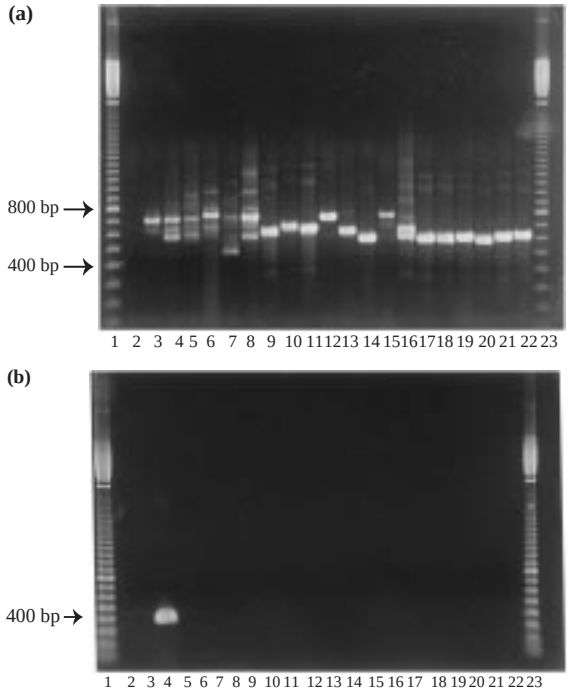

Figure 1. EtBr-stained agarose gel of PCR products obtained from DNA extracted from micropropagated potato and soil microbes using universal primers, ITS4 and ITS6 (a) and *S. subterranea*-specific primers, *Sps*1 and *Sps*2 (b). The *S. subterranea* amplification product shown is from cystosori isolated from tubers of cv. Erntestolz (Portsoy, Aberdeenshire, UK). Lane 2 = SDW, lane 3 = Micropropagated potato leaf, lane 4 = *S. subterranea*, lane 5 = *Polymyxa graminis*, lane 6 = *Polymyxa betae*, lane 7 = *Plasmodiophora brassicae*, lane 8 = *Ligniera* sp., lane 9 = *Verticillium tricorpus*, lane 10 = *Verticillium lecanii*, lane 11 = *Trichoderma viride*, lane 12 = *Rhizoctonia solani*, lane 13 = *Pythium ultimum*, lane 14 = *Phoma chrysamthemicola*, lane 15 = *Olpidium brassicae*, lane 16 = *Monodyctis levis*, lane 17 = *Fusarium sulphureum*, lane 18 = *Fusarium oxysporum*, lane 19 = *Fusarium equiseti*, lane 20 = *Fusarium avenaceum*, lane 21 = *Gliocladium roseum*, lane 22 = *Colletotrichum coccodes*. Lanes 1 and 23 are 100 bp DNA ladders (Pharmacia).

Sensitivity of PCR with a dilution series of S. subterranea *DNA*

PCR using *Sps*1 and *Sps*2 gave a visible 391 bp amplification product from *S. subterranea* DNA diluted to the equivalent of 25×10^{-5} cystosori (Figure 2a) and 1 zoospore (Figure 2b) per reaction but yields declined below 0.5 cystosori or 25 zoospores per reaction. There was no amplification from the equivalent of 0.5 or 0.1 zoospores. Similarly, positive

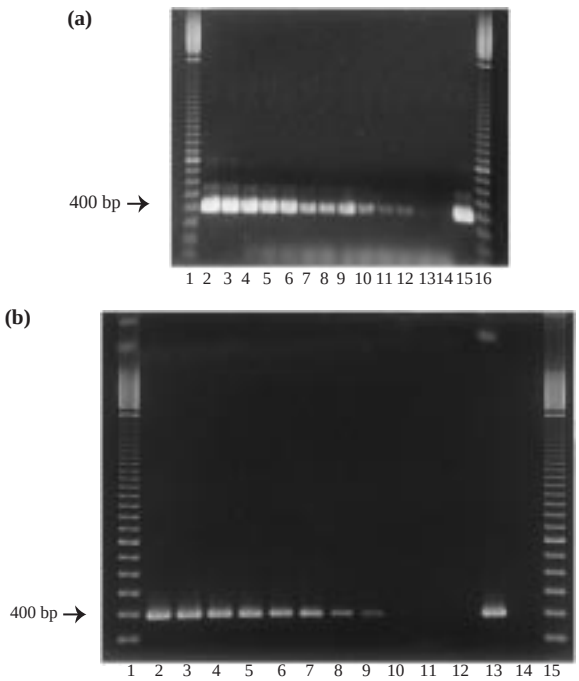

Figure 2. EtBr-stained agarose gel of PCR products obtained from a serial dilution of *S. subterranea* DNA extracted from ca. 10^5 cystosori (a) or 2×10^5 zoospores (b) with *Sps*1 and *Sps*2. The DNA concentration for each PCR reaction in (a) was equivalent to 500 cystosori (lane 2), 50 cystosori (lane 3), 5 cystosori (lane 4), 2.5 cystosori (lane 5), 0.5 cystosori (lane 6), 0.25 cystosori (lane 7), 0.05 cystosori (lane 8), 0.025 cystosori (lane 9), 5×10^{-3} cystosori (lane 10), 25×10^{-4} cystosori (lane 11) 5×10^{-4} cystosori (lane 12) and 25×10^{-5} cystosori (lane 13) per 25 µl PCR reaction. Lanes 14 and 15 in (a) are negative (water) and positive (DNA from 10^5cystosori) controls, respectively. Lanes 1 and 16 in (a) are 100 bp DNA ladders. The DNA concentration for each PCR reaction in (b) was equivalent to 500 (lane 2), 250 (lane 3), 100 (lane 4), 75 (lane 5), 50 (lane 6), 25 (lane 7), 10 (lane 8), 5 (lane 9), 1 (lane 10), 0.5 (lane 11) and 0.25 zoospores (lane 12) per 25 µl PCR reaction. Lanes 1 and 15 in (b) are 100 bp DNA ladders (Pharmacia). Lanes 13 and 14 in (b) are positive (DNA from 10^5cystosori) and negative (water) controls, respectively.

results (of decreasing intensity) were obtained with DNA extracted from 100, 50, 10, 5 and 1 cystosori (Figure 3).

Detection of S. subterranea *on potato tubers*

DNA from peel of scabbed tubers gave rise to 391 bp PCR products (Figure 4), as did DNA from the apparently healthy tubers (cvs. Desiree, Estima and Maris Bard), though the bands were of varying intensity,

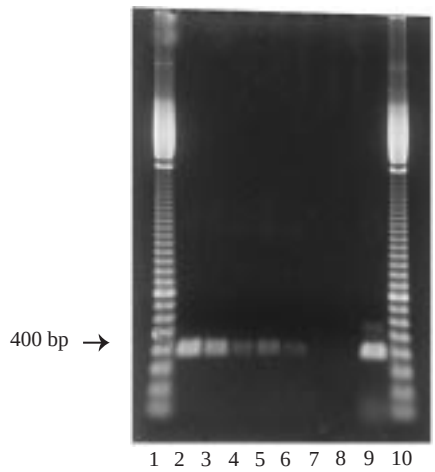

Figure 3. EtBr-stained agarose gel of PCR products obtained from 0.5 µl aliquots of *S. subterranea* DNA extracted from 100 (lane 2), 50 (lane 3), 10 (lane 4), 5 (lane 5), 1 (lane 6) and 0 (lane 7) cystosori with *Sps*1 and *Sps*2. Lanes 8 and 9 are negative (water) and positive (DNA from 10^5 cystosori) controls, respectively. Lanes 1 and 10 are 100 bp DNA ladders (Pharmacia).

particularly from the 2 samples from the cv. Desiree tuber. No amplification occurred from DNA from the Scottish classified seed potatoes or axenically micropropagated tubers or leaves (Figure 4). All potato DNA amplified successfully with primers ITS4 and ITS6 (data not shown).

Extraction of DNA from soils

Preliminary results with DNA extracted from soil using the Phytopure kit showed major problems with inhibition of PCR. The extraction method was consistently found to recover high molecular weight DNA of adequate purity for PCR from various agricultural soils (obtained from SCRI fields or supplied by S.J. Wale). The purity of the extracts varied in different soils (as assessed by the colour of the pellet after isopropanol precipitation), but in all cases the DNA was pure enough for PCR amplification after spin column clean up, which in some cases was not necessary. However spin column clean up was adopted as standard to avoid repeating failed tests. Use of the Tris–EDTA buffer gave extracts that were visibly far cleaner than those produced using the phosphate buffer. However, no DNA was visible in these extracts after electrophoresis. This is in contrast to previous results obtained elsewhere (Bell et al., in press) and suggests

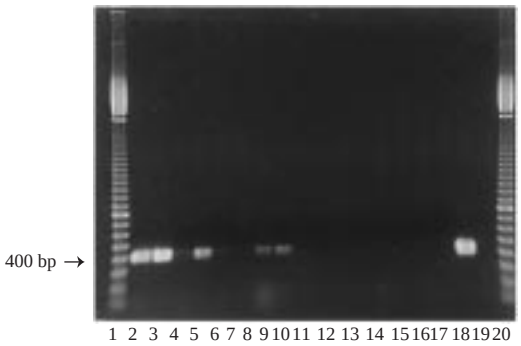

Figure 4. EtBr-stained agarose gel of PCR products obtained after amplification with *Sps*1 and *Sps*2 of an aliquot of DNA extracted from: 2 scabbed tuber peel samples, cv. Duke of York (lanes 2 and 3), 2 peel samples per cultivar of apparently healthy tubers, cv. Desiree (lanes 4 and 5), cv. Estima (lanes 6 and 7) and cv. Maris Bard (lanes 8 and 9), two peel samples from each of 2 tubers of Scottish classified seed potatoes cv. Swift (lanes 10–13), 2 samples of axenically micropropagated mini-tubers, cv. Saturna (lanes 14 and 15) and 2 samples of axenically micropropagated potato leaves, cv. Russet Burbank (lanes 16 and 17). Lanes 18 and 19 are positive (DNA from 10^5 cystosori) and negative (water) controls, respectively. Lanes 1 and 20 are 100 bp DNA ladders (Pharmacia).

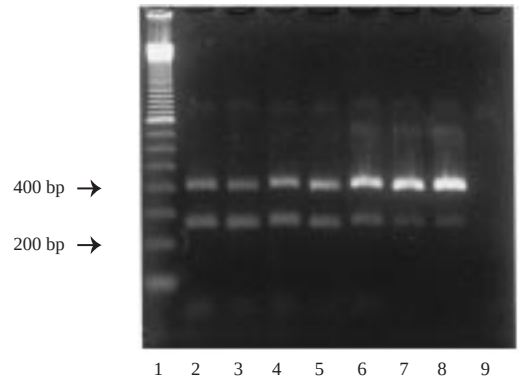

Figure 5. EtBr-stained agarose gel of PCR products obtained after co-amplification with *Sps*1 and *Sps*2 of an aliquot of *S. subterranea* DNA with 1 fg of competitor DNA in a 25 µl reaction. The 391 bp product is from *S. subterranea* and the 249 bp product is from the competitor. The aliquot of *S. subterranea* DNA was from 5 (lane 2), 10 (lane 3), 50 (lane 4), 100(lane 5), 500 (lane 6) 1000 (lane 7) or 5000 cystosori. Lane 1 is a DNA ladder (Pharmacia) and lane 9 a negative control (water).

that TE buffers are not suited to all soil types and that phosphate buffers of this type may be more robust, perhaps preventing excessive loss of DNA by adhesion to clay particles (Ogram et al., 1988).

Detection of S. subterranea *in soils*

S. subterranea was detected in 5 replicate soil samples that had been individually seeded with 5 and 10 cystosori per gram of soil, and in 2 of 5 replicate samples seeded at 1 and 3 cystosori per gram. SYBR Gold staining greatly improved the visualisation of PCR products from low numbers of cystosori relative to EtBr staining. Samples with fewer than 5 cystosori per gram did not give positive results when the sonication step was omitted.

PCR quantification using a competitor DNA fragment

A 249 bp product was obtained after amplification of DNA extracted from 10^5 *S. subterranea* cystosori with *Sps*1 and *Sps*comp1, which could be differentiated on an agarose gel from the 391 bp product from *Sps*1 and *Sps*2. Figure 5 shows the results when DNA extracted from a dilution series of cystosori (5, 10, 50, 100, 500, 1000 and 5000) was amplified in the presence of 1 fg of competitor DNA per 25 µl reaction and Figure 6 shows a standard plot constructed using data obtained from this and the other 5 replicate gels. This should allow estimation of the number of cystosori present in unknown samples.

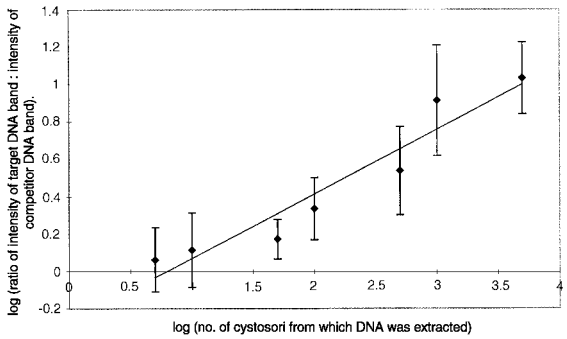

Figure 6. Relationship between the amount of *S. subterranea* cystosori from which DNA was extracted and the ratio of the amount of amplified target DNA: amount of amplified competitor DNA (as assessed by Gel Compar analysis of photographs of EtBr-stained agarose gels) after co-amplification of 0.5% of each extract with 1 fg of competitor DNA. Error bars are the standard deviation of the mean ratio of intensity for each cystosori number where $n = 6$.

DNA extracts from 5 tuber washings of cv. Maris Piper (symptomless) and Desiree (moderately scabbed)

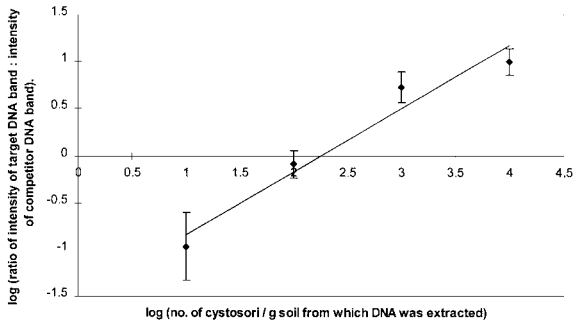

Figure 7. Relationship between the concentration of *S. subterranea* cystosori per gram of soil from which DNA was extracted and the ratio of the amount of amplified target DNA: amount of amplified competitor DNA (as assessed by Gel Compar analysis of photographs of EtBr-stained agarose gels) after coamplification of 0.5% of an extract prepared from a mixture of 10 g soil, 20 ml buffer and 0.5 pg of competitor DNA. Error bars are the standard deviation of the mean ratio of intensity for each cystosori number where $n = 5$.

were tested using quantitative PCR and the ratio of the products indicated that there were 10 cystosori per extract from the symptomless tubers and approximately 10^3 cystosori per extract from the moderately scabbed tubers (data not shown).

Quantification of S. subterranea *in soils*

Similar quantitative responses were obtained from the 5 agricultural field soils tested. Figure 7 shows the average results from the 5 soils after quantitative PCR on extracts prepared from soil that had been spiked with 10 000, 1000, 100 and 10 cystosori per gram of soil and 0.05 pg of competitor DNA per gram of soil.

Discussion

The first aim of this project was to develop a species-specific PCR test for *S. subterranea*. During the preparation of this manuscript another paper was published which described such a test, coincidentally using primers almost identical to those described here (Bulman and Marshall, 1998). Both studies confirm the diagnostic value of the ITS target sequences concerned. However, this paper includes important modifications of the basic PCR test not described previously, as it has demonstrated sensitive detection of *S. subterranea* in soil and tuber washings as well as skin material, and the assay has been adapted to allow quantification of seed- and soil-borne inoculum levels.

PCR testing using the specific primers described here gave positive results with *S. subterranea* isolates from different potato cultivars and locations. The available sequence data (Bulman and Marshall, 1998; Ward and Adams, 1998) shows that most *S. subterranea* isolates have ITS sequences identical to those from which our primers were chosen and so all such isolates will give positive results with our test. However, a second type of ITS sequence exists that has a single base mismatch to our forward primer near the 5′ end (Bulman and Marshall, 1998). We have shown that this mismatch does not prevent successful amplification from a sample of this type. The specificity of the primers *Sps*1 and *Sps*2 was demonstrated by negative test results obtained with DNA extracted from a number of other organisms and by the successful amplification of all such DNA by non-specific (ITS) primers. Additionally, ITS sequence data for related organisms, where available (Ward and Adams, 1988; GenBank accessions Y12824-31; Bulman and Marshall, unpublished sequences), suggests that the primers are suitably specific. The negative result with *Streptomyces scabies* demonstrates that our assay may be used to differentiate between powdery scab and common scab, which are sometimes confused in visual diagnosis.

The assay was sensitive, being able to detect a single cystosorus or 1–10 cystosori per gram of soil. This compares favourably with sensitivities in both a bait plant assay (10 cystosori per 150 ml nutrient solution; Flett, 1983) and an ELISA method (100 cystosori per gram of soil; Walsh et al., 1996). Merz (1993) found that highly infested soils had inoculum densities greater than 500 cystosori per gram of soil and so our quantitative assay is suitable for this level of infestation, more so than the bioassay of Wale et al., (1993) which could not discriminate levels above 3 cystosori per gram. The method for extraction of DNA from soil described here is quicker, simpler and cheaper than many others previously described and yielded high molecular weight DNA of sufficient purity for PCR assays. Bead beating alone is sufficient to rupture and release DNA from cystosori but the sonication step was included as it releases some DNA, although not as much as bead beating (data not shown), and it can be applied to bulk slurry samples. Thus, target DNA from low numbers of cystosori, which may still represent relatively large numbers of infectious zoospores, should be better dispersed and so is more likely to be present in subsamples used for bead beating and testing. Results can be obtained in 1–2 working days in contrast to bait tests, which take around 3 weeks (Wale et al., 1993) and there is no requirement

for traditional skills in pathogen identification or experimental animals for antibody production.

S. subterranea was also detected on diseased and apparently healthy tubers from peel and tuber washings. It is known that apparently healthy tubers can carry low levels of inoculum within intact lenticels (Diriwächter and Parbery, 1991; Harrison et al., 1997). Detection of *S. subterranea* in stocks of seed tubers without powdery scab symptoms will permit the identification of seed that is a potential source of inoculum in subsequent crops. For quantification, tuber sampling methods need to be developed, but tuber washings, which give an indication of infestation of the whole tuber surface, may be the most suitable sampling technique if infections are localised.

PCR quantification is problematic since small changes in amplification efficiency can greatly effect the final amount of product. However, if an appropriate competitor DNA sequence is co-amplified with the target DNA it functions as an internal standard. The template: product ratios should be equal for the target DNA and the competitor DNA and since the starting amount of competitor DNA is known, the amount of target DNA in the initial sample can be estimated. A competitor DNA was used with the same primer binding sites and a similar size to the target sequence to ensure similar amplification efficiencies for both. This gave a quantitative response that allowed an approximate estimation of *S. subterranea* numbers in unknown samples. Previous studies have used scintillation counting of radioactivity in radio-labelled DNA to quantify ratios of competitor and target fragments (Wang et al., 1989; Möller and Jansson, 1997), but the simpler technique of computer software analysis of band intensity ratios on photographs was found to be adequate. In soil tests, samples were spiked with competitor DNA prior to DNA extraction to compensate for variable recoveries from different soil types due to DNA-clay binding (Ogram et al., 1988). Similar quantitative responses were obtained from different soils, which should allow estimation of *S. subterranea* numbers within an order of magnitude. For a more detailed study of *S. subterranea* numbers, standard curves could be constructed for individual soils under investigation to improve the accuracy of the quantification. Competitor DNA also serves as a positive control, indicating failed reactions (perhaps due to the presence of inhibitory substances).

Although many factors influence the occurrence and severity of powdery scab, methods to detect and quantify *S. subterranea* inocula in field soils or seed tuber stocks could form an important part of disease risk assessment. This would allow more informed decision making, which is essential to help to reduce losses due to powdery scab. Risk assessments will influence management decisions on a number of issues such as application of biocides; crop rotation strategy; matching cultivars to fields according to disease risk, cultivar resistance rating and crop end use. A barrier to implementation of such strategies is lack of knowledge on distribution of *S. subterranea* in soil samples and seed stocks and, therefore, on appropriate sampling regimes. However, the methods described here offer a means of addressing the issue of pathogen distribution and should be a valuable tool in future studies on *S. subterranea* such as monitoring population changes following control measures or elucidating the relative importance of seed- and soil-borne inocula.

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