European Journal of Plant Pathology **109:** 589–597, 2003.

Detection and quantification of *Spongospora subterranea* in soil, water and plant tissue samples using real-time PCR

Pieter van de Graaf, Alison K. Lees, Danny W. Cullen and James M. Duncan
Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, UK (Phone: +441382562731; Fax: +441382562426; E-mail: pvande@scri.sari.ac.uk)

Accepted 18 March 2003

Key words: bait plants, internal transcribed spacer regions, plasmodiophorids, powdery scab, Potato mop top virus, TaqMan®

Abstract

A sensitive real-time polymerase chain reaction (PCR) assay was developed for the quantification of *Spongospora subterranea*, the cause of powdery scab and root galling in potato, and the vector of Potato mop top virus. A specific primer pair and a fluorogenic TaqMan® probe were designed to perform a quantitative assay for the detection of *S. subterranea* in soil, water and plant tissue samples. The assay was tested using DNA from cystosori, zoospores, plasmodia and zoosporangia of the pathogen. DNA was extracted directly from cystosori suspended in water and from clay soil with varying levels of added cystosori. DNA obtained from zoospores released into nutrient solution by cystosori in the presence of tomato bait plants was also tested, as was DNA from plasmodia and zoosporangia in infected tomato roots. In many cases, detection was successful even at low inoculum levels. This specific quantitative assay could therefore be a useful tool for studying the biology of *S. subterranea*, and for the optimisation of disease avoidance and control measures.

Abbreviations: C_t – threshold cycle; cv. – cultivar; ITS – internal transcribed spacer; PMTV – Potato mop top virus; RES – Rothamsted Research; SAC – Scottish Agricultural College; SCRI – Scottish Crop Research Institute; SDW – sterile distilled water; SE – standard error.

Introduction

Spongospora subterranea, the cause of powdery scab of potato, is a pathogen belonging to the plasmodiophorids. Visible symptoms of infection by *S. subterranea* include galls on roots and cankers and scabs on tubers. Powdery scab regularly leads to extensive losses in both seed and ware potato crops throughout Europe, especially in cool and wet climates (Wale, 2000). Scabs on tubers contain masses of cystosori (sporeballs) which are resting structures capable of surviving in soil for many years. Under moist conditions, these cystosori release primary zoospores, which can infect potato and a wide range of other plant hosts including many common weed species (Kole, 1954; Würzer, 1964; Janke, 1965; Jones and Harrison, 1969; Hims and Preece, 1975). Following infection of suitable hosts, plasmodia are formed in the roots and they can develop into zoosporangia, which release secondary zoospores when mature. *Spongospora subterranea* is also the vector of Potato mop top virus (PMTV), one of the causes of spraing (Stevenson et al., 2001).

No effective methods are currently available for the control of powdery scab or spraing and many aspects of the biology of *S. subterranea* are poorly understood. This is partly due to difficulties with the detection and quantification of the pathogen in soil and plant tissues.

The quantification of *S. subterranea* DNA in soil, water and plant tissue is essential to allow detailed studies into the epidemiology and control of powdery

scab and spraing. An enzyme linked immunosorbent assay (ELISA) for quantifying *S. subterranea* is available, but is not sensitive enough to detect low levels of inoculum in soil (Walsh et al., 1996). Quantification of *S. subterranea* DNA is possible by co-amplification of competitor and target DNA in a conventional polymerase chain reaction (PCR) assay (Bell et al., 1999; Qu et al., 2000). By comparison of the intensity of the amplification product produced by the competitor and target DNA, the amount of target DNA in the original sample can be estimated. However, a more rapid and accurate method is needed for processing of large sample numbers.

Real-time PCR is a technique in which DNA of a specific target organism can be quantified by measurements of the intensity of fluorescence with time during the exponential phase of DNA amplification. Fluorescence is generated by cleavage by Taq DNA polymerase of a TaqMan® probe specific to the organism and released during amplification of the target DNA by specific primers. The fluorescence is measured by specialist equipment, and can be translated into DNA quantities by inclusion in the assay of a standard dilution series of target DNA which is used to create a standard curve (Anonymous, 1998). Real-time PCR using a TaqMan® probe is a quick and reliable method with which large numbers of samples can be tested easily. This method has been used with success for the detection and quantification of a range of plant pathogens including fungi (Bates et al., 2001; Cullen et al., 2001), bacteria (Hyman et al., 2000; Weller et al., 2000) and viruses (Boonham et al., 2000; Eun et al., 2000).

The objective of this work was to develop a sensitive and quantitative real-time PCR assay specific to *S. subterranea* that could be used as a reliable tool for the detection and quantification of this important potato pathogen and virus vector in plant tissue, water and soil.

Materials and methods

Preparation of inoculum

Potato tubers with powdery scab symptoms, harvested in Aberdeenshire, Scotland in 2000, were obtained from S.J. Wale (SAC) and stored in the dark at 4 °C until use. Tubers were washed with tap water and left to dry overnight at room temperature before scabs containing cystosori were scraped off the tuber surface with a scalpel and ground with a pestle and mortar. The resulting powder was passed through a 53-μm mesh sieve. For preparation of low inoculum concentrations (<100 cystosori), single cystosori were counted and collected using a dissecting microscope and suspended in sterile distilled water (SDW). For higher inoculum concentrations, cystosori were suspended in SDW and the concentration established using a microscope and an haemocytometer.

Preparation of soil

Silty clay soil from Aberdeenshire, Scotland was obtained from S.J. Wale (SAC), air dried, passed through a 1-cm mesh sieve and stored at room temperature. SDW (20 ml per sample) containing varying numbers of cystosori (0, 1, 3, 5, 10, 30, 50, 100 and 500) was added to samples (10 g each) of the dried clay soil and the soil was then air dried again before DNA extraction.

Bait plant test

Tomato seeds (cv. Moneymaker) were planted in seed trays containing sterile sand and nutrient solution and kept at 15 °C until germinated. The nutrient solution was prepared as described by Merz (1989) and did not contain zinc. Individual tomato seedlings were transferred to 60 ml plastic pots wrapped in aluminum foil, which contained 50 ml of nutrient solution. The tomato seedlings were placed through small holes in the aluminum foil with their root system suspended in the nutrient solution and then incubated in a growth room with a 16-h light regime at a constant temperature of 15 °C.

One week after transfer of the tomato seedlings to individual pots, different numbers of cystosori (0, 5, 15, 25, 50, 150, 250, 500 or 2500 cystosori per 50 ml) were added to the nutrient solution. One bait plant was used per replicate, with five replicates per treatment. Following two weeks of baiting, the tomato plants were removed from their pots and their root systems were washed in SDW and air dried overnight. The root samples were then frozen before DNA extraction. In order to assess the ability of the assay to detect zoospores, the nutrient solution remaining in each pot was filtered using a sterile syringe and a 5-μm syringe filter. The nutrient solution samples

were frozen to kill any zoospores present before DNA extraction.

DNA extraction

Cystosori in soil and water
DNA was extracted from cystosori in soil and water using the method described by Bell et al. (1999). For DNA extraction from cystosori in water, extraction buffer (19 ml) was added to different numbers (0, 2, 6, 10, 20, 60, 100, 200 and 1000) of cystosori in SDW (1 ml). In all cases, there were five replicates per treatment.

Zoospores in nutrient solution
DNA was extracted from zoospores present in nutrient solution using a Nucleon® PhytoPure plant DNA extraction kit (Tepnel Life Sciences, Wythenshawe, Manchester, UK) according to the manufacturer's instructions. After defrosting, samples were centrifuged at 13 400*g* for 10 min, the supernatant was removed and the remaining pellet subjected to the PhytoPure DNA extraction kit protocol, using only one-third of the amounts of the chemicals prescribed for 0.1 g plant tissue samples.

Plasmodia and zoosporangia in bait plant roots
Dry tomato root samples were weighed, cut into small pieces and ground in 600 µl of Reagent 1 of a Nucleon® PhytoPure plant DNA extraction kit. The DNA extraction kit protocol was then followed.

Primer design

The internal transcribed spacer regions (ITS1 and ITS2) of *S. subterranea* were accessed from the Genebank and EMBL databases. The Primer Express® software (Applied Biosystems) was used to design forward and reverse primers SsTQF1 (5′-CCG GCA GAC CCA AAA CC-3′) and SsTQR1 (5′-CGG GCG TCA CCC TTC A-3′) and TaqMan® probe SsTQP1 (5′-CAG ACA ATC GCA CCC AGG TTC TCA TG-3′) for use in a real-time quantitative PCR assay. The fluorogenic probe was labelled at the 5′ end with the fluorescent reporter dye FAM (6-carboxy-fluorescein) and modified at the 3′ end with the quencher dye TAMRA (6-carboxy-tetramethylrhodamine). The specificity of the designed primer sequences was confirmed before synthesis following a database (BLAST and FASTA programs) search of DNA sequences.

Real-time PCR amplification

Real-time quantitative PCR was performed in MicroAmp® optical 96-well reaction plates with optical caps using an ABI Prism 7700 Sequence Detection System (Applied Biosystems). For each sample, 1 µl template DNA was added to 24 µl reaction mix consisting of 12.5 µl TaqMan® Universal PCR Master Mix, 9.5 µl sterile HPLC water, 0.75 µl each of the primers SsTQF1 and SsTQR1 (10 µM), and 0.5 µl of the TaqMan® probe SsTQP1 (5 µM). The universal thermal cycle protocol recommended by Applied Biosystems (Anonymous, 1998) was used for PCR amplification: 50 °C for 2 min, followed by 95 °C for 10 min, then 45 cycles at 95 °C for 15 s and 60 °C for 1 min.

A range of standards containing different amounts of *S. subterranea* DNA was included in the real-time PCR assay. DNA was extracted from a known number of cystosori following the method of Bell et al. (1999) and diluted with TE buffer to obtain a dilution series of DNA equivalent to 100 000, 10 000, 1000, 100, 25 and 10 cystosori per ml. The C_t value, which is the number of PCR cycles needed to reach a minimum level of reporter fluorescence associated with an exponential increase in PCR product, was calculated for each unknown sample by the software (version 1.6) of the ABI Prism Sequence Detection System and automatically compared with the C_t values of the standard series. By comparison with the standard curve of the C_t values against the logarithm of the standard amount of cystosori DNA, the amount of *S. subterranea* DNA in each unknown sample was expressed in cystosori equivalents (from now on referred to as 'units') based on the C_t value of the sample (Figure 1).

DNA was extracted from cystosori from potato tubers with powdery scab collected by SAC from 16 different locations in Northern Britain (Table 1). The DNA extracts were tested for amplification in the real-time PCR assay using the primer/probe set designed for the detection of *S. subterranea*. The specificity of the primers and TaqMan® probe was tested in a separate assay using genomic DNA from a range of potato pathogens and soil borne micro-organisms (Table 2).

The DNA samples extracted from cystosori, zoospores and plasmodia/zoosporangia were tested in further assays. A non-template control with 1 µl TE buffer instead of DNA was always included and all samples were tested in duplicate.

Results

Assay sensitivity and specificity

As only 1 µl of the DNA standard dilution series was added per reaction, the amounts of DNA used in the real-time PCR assay were equivalent to 100, 10, 1.0, 0.1, 0.025 and 0.01 cystosori. This standard dilution series of *S. subterranea* DNA was amplified consistently in the real-time PCR assay with primer pair SsTQF1 and SsTQR1 and probe SsTQP1. Only DNA equivalent to 0.01 cystosori was not consistently amplified. The average C_t value obtained when an equivalent of 0.025 cystosori was amplified in the real-time PCR assay was 37 cycles, compared with an average C_t value of 25 with DNA equivalent to 100 cystosori (1×10^5 cystosori per ml TE buffer) (Figure 1). As expected, the non-template control had a C_t value >45 cycles. The correlation coefficient of the standard curves used for calculating the amount of DNA in the unknown samples was always greater than 0.94.

The real-time PCR assay was able to detect DNA of *S. subterranea* from all 16 sources listed in Table 1.

Table 1. Origin of samples of *S. subterranea* tested for amplification with specific primers and TaqMan® probe

Origin	Potato variety
Wooler, Northumbria, England	Estima
Skeen, Scotland	Penta
Aberlady, Scotland	Unknown
Coupar Angus, Scotland	Cara
Coupar Angus, Scotland	Estima
Kirrimuir, Scotland	Nadine
Forfar, Scotland	Estima
Arbroath, Scotland	Maris Piper
Arbroath, Scotland	Red Cara
Arbroath, Scotland	Estima
Montrose, Scotland	Estima
Montrose, Scotland	Nadine
Inverbervie, Scotland	Estima
Aberdeen, Scotland	Maris Bard
Balnakettle, Scotland	Nadine
Portmahomack, Scotland	Maris Piper

Table 2. Micro-organisms tested for amplification with specific primers and TaqMan® probe designed for the detection of *S. subterranea*

Species	Original host	Supplied by
Colletotrichum coccodes	Potato	SCRI, UK
Helminthosporium solani	Potato	SCRI, UK
Fusarium avenaceum	Potato	SCRI, UK
Fusarium coeruleum	Potato	SCRI, UK
Fusarium culmorum	Potato	SCRI, UK
Fusarium sulphureum	Potato	SCRI, UK
Olpidium brassicae	Lettuce	RES, UK
Phoma clematidina	Clematis	ADAS, UK
Phoma exigua var. *foveata*	Potato	SCRI, UK
Phytophthora erythroseptica	Potato	SCRI, UK
Plasmodiophora brassicae	Cabbage	SAC, UK
Polymyxa betae	Sugar beet	RES, UK
Polymyxa graminis	Barley	RES, UK
Polyscytalum pustulans	Potato	SCRI, UK
Pythium aphanidermatum	Unknown	SCRI, UK
Pythium ultimum	Potato	SCRI, UK
Rhizoctonia solani	Potato	RES, UK
Streptomyces scabies	Potato	SCRI, UK

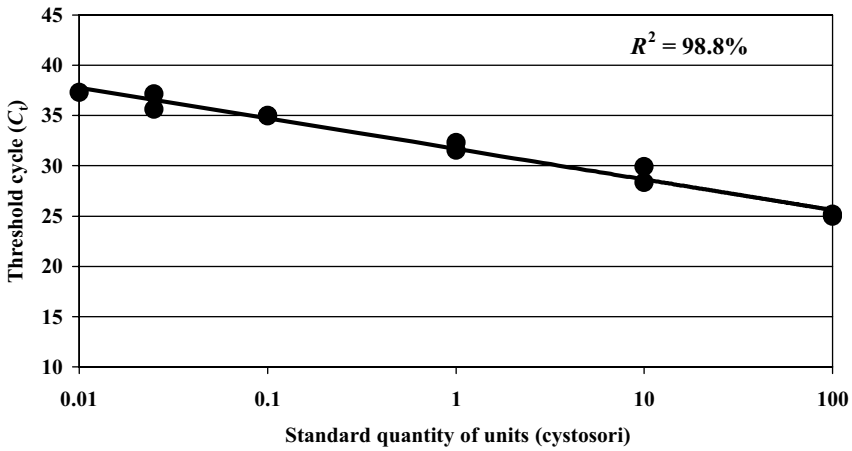

Figure 1. Standard curve used in the real-time PCR assay for the quantification of *S. subterranea* in DNA samples extracted from soil, water and plant tissues.

DNA of the micro-organisms listed in Table 2 was not amplified in the real-time PCR assay and always had C_t values >45 cycles.

Detection and quantification of cystosori

The real-time PCR assay could detect and quantify cystosori suspended in water, or mixed with clay soil. The average amount of DNA detectable from samples originating from cystosori in water was comparable with the numbers added to the original sample (Figure 2). Only at concentrations lower than 1 cystosorus per ml was the discrimination between inoculum levels less successful. The majority of samples at these low concentrations tested negative, while detection was much more consistent at concentrations >1 cystosorus per ml (Table 3).

The number of positive samples was high at most inoculum levels in clay soil, possibly due to natural contamination of the soil used (Table 3). Quantification of cystosori in soil was less accurate than of cystosori in water, but significant inhibition took place in only two of the eight inoculum levels tested (5 and 10 cystosori per ml) (Figure 2).

Detection and quantification of plasmodia, zoosporangia and zoospores

Plasmodia and zoosporangia of *S. subterranea* that formed in the roots of tomato plants during a bait test, were readily detected by the real-time PCR assay (Table 4). PCR amplification of DNA extracted from bait plants inoculated with low inoculum levels was not consistent in the real-time PCR assay, possibly due to low infection rates. Many of the DNA samples taken from bait plants inoculated with less than 0.3 cystosori per ml (15 cystosori per plant) did not test positive. However, *S. subterranea* was detected in almost all plant samples inoculated with >1 cystosorus per ml nutrient solution.

The average quantity (units) of *S. subterranea* DNA detected by the real-time PCR assay in inoculated plants was always higher than the quantity present in

Table 3. Detection of DNA of *S. subterranea* by real-time PCR assay after direct extraction from cystosori in water and clay soil ($n = 5$)

Amount of inoculum added*	Positive samples (%)	
	In water	In clay soil
0	0	80
0.1	20	80
0.3	40	40
0.5	40	80
1	60	60
3	100	80
5	80	60
10	100	100
50	100	100

*Cystosori per ml liquid or g soil.

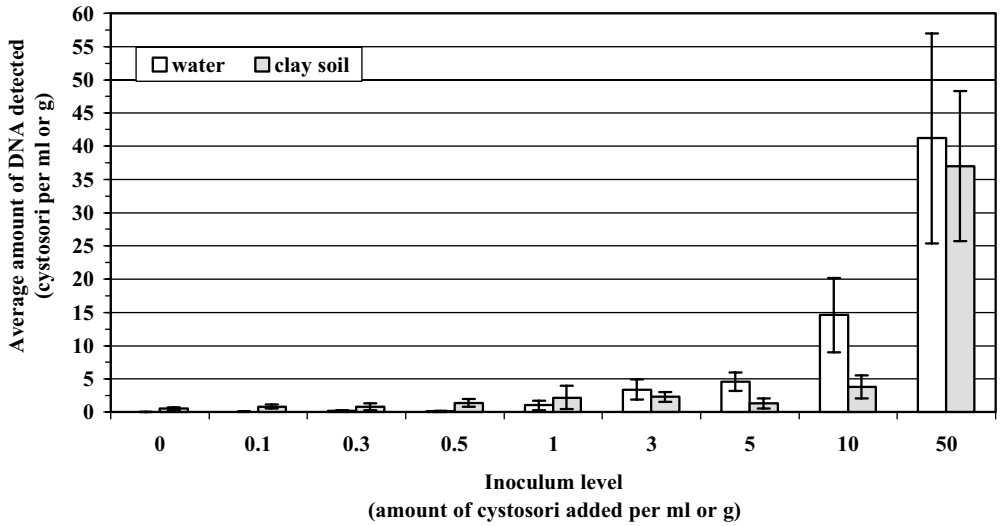

Figure 2. Quantification of DNA of *S. subterranea* by real-time PCR assay after direct extraction from cystosori in water and clay soil ($n = 5$). SE-bars.

Table 4. Detection and quantification of DNA of *S. subterranea* by real-time PCR assay after extraction from plasmodia and zoosporangia in tomato roots and zoospores in nutrient solution after a bait plant test ($n = 5$)

Amount of inoculum added (cystosori per ml nutrient solution)	From plasmodia and zoosporangia in bait plant roots		From zoospores in bait plant nutrient solution	
	Positive samples (%)	Average amount of DNA detected (units* per mg dry weight) ± SE	Positive samples (%)	Average amount of DNA detected (10^{-3} units per ml) ± SE
0	0	0 ± 0	0	0 ± 0
0.1	40	1.3 ± 0.8	20	0.6 ± 0.6
0.3	60	1.5 ± 1.2	40	1.9 ± 1.2
0.5	80	4.8 ± 1.5	60	2.1 ± 0.9
1	60	8.1 ± 5.1	20	1.4 ± 1.4
3	80	88.5 ± 31.3	60	7.7 ± 4.5
5	100	1855.6 ± 607.7	100	118.8 ± 105.9
10	100	1683.2 ± 671.8	100	114.0 ± 51.7
50	100	1564.4 ± 822.8	60	30.3 ± 24.9

*One unit equals the amount of DNA extracted from a single cystosorus of average size.

the initial inoculum, showing that the pathogen had multiplied within the host tissues. For example, to create an inoculum concentration of 5 cystosori per ml of nutrient solution, 250 cystosori were initially added to each plant as inoculum. The average dry weight of the plant roots was 10 mg, and thus the average quantity of DNA detected per bait plant at this inoculum level (5 cystosori per ml) was 18 556 units, almost 75 times the 250 units originally added.

The real-time PCR assay was also able to detect zoospores of *S. subterranea* in the bait plant nutrient solution at all levels of added inoculum. However, the amounts of DNA detected were very low and the results were less consistent than for the bait plant roots (Table 4).

In the bait plant test, the quantity of *S. subterranea* DNA detected as plasmodia and zoosporangia in the plant roots and as zoospores in the nutrient solution was greater in samples treated with inoculum concentrations of 5 or 10 cystosori per ml than at a concentration of 50 cystosori per ml. However, due to the large variation within the treatments, this effect was not statistically significant.

Discussion

It was demonstrated that the real-time PCR assay developed for the detection and quantification of *S. subterranea* could detect the pathogen in water, soil and plant tissue. Specificity of the primers and probe was confirmed by a lack of amplification of DNA from 18 other potato pathogens and soil fungi. In addition, DNA of *S. subterranea* from 16 different locations in Northern Britain was detected as expected demonstrating consistency of detection across populations.

The real-time PCR assay was very sensitive with consistent detection of standard DNA quantities equivalent to 0.025 cystosori (in 1 μl) and occasional detection of lower DNA quantities. These results are similar to the minimum detection levels for conventional PCR (Bell et al., 1999) and ELISA (Walsh et al., 1996).

The method used for the extraction of DNA from cystosori of *S. subterranea* was developed specifically for this purpose by Bell et al. (1999). The extraction protocol includes taking a 1-ml sample from 20 ml extraction buffer containing the cystosori either with or without soil. As the cystosori are not broken up until after this step, theoretically only inoculum concentrations of ≥1 cystosorus per ml can be detected consistently. This agrees with our findings since the real-time PCR assay reliably detected cystosori in water at concentrations ≥1 cystosorus per ml. Similar results have been reported by Qu et al. (1998) and Bell et al. (1999) for conventional PCR. The real-time PCR assay was able to detect as few as 0.1 cystosori per ml but this was not consistent due to the small chance of collecting cystosori when sampling 1 ml from 20 ml at such low inoculum concentrations.

The real-time PCR assay was able to detect and quantify *S. subterranea* both in artificially and naturally contaminated soil samples. Using the protocol of Bell et al. (1999), the detection limit should be 2 cystosori

per g soil (10 g soil in 20 ml buffer). In our assay, DNA of *S. subterranea* was detected at concentrations <2 cystosori per g soil and was also detected in the uninoculated control. This was probably due to natural contamination of the clay soil. The detection and quantification of cystosori in clay soil was less consistent than in water possibly due to binding of DNA to soil particles or inhibition of the PCR by co-extracted soil chemicals. Such effects are a common problem with soil extractions (e.g. Bell et al., 1999; Cullen et al., 2001). In addition to the extraction method, the detection of low levels of inoculum in soil is dependent on the sampling technique and the likelihood of including cystosori in the small sample used for the DNA extraction. Taking multiple 1 ml samples from one tube containing 10 g of soil in 20 ml of extraction buffer would increase the chance of detection and provide more accurate quantification.

According to Bell et al. (1999), conventional PCR was able to detect ≥5 cystosori per g soil consistently, but these workers did not attempt to quantify concentrations <10 cystosori per g soil. Qu et al. (2000) were able to quantify ≥4 cystosori per g soil using a different primer set. In contrast, ELISA can only detect and reliably quantify very high levels of cystosori in soil (>2000 cystosori per g) (Walsh et al., 1996). Due to the natural contamination of the soil used in the experiments described here, the exact detection limit of cystosori in soil using the real-time PCR assay could not be established.

Bait plants can be used to test soil or other substrates for the presence of *S. subterranea* and are generally thought to provide more sensitive detection than methods that involve direct DNA extraction from the soil. Detection of *S. subterranea* at concentrations as low as 0.1 cystosori per ml nutrient solution has been reported (Flett, 1983; Burnett, 1991). However, detection and quantification have always relied on examination of stained bait plant roots by microscope, a laborious and unreliable process. With this technique, stages before the formation of zoosporangia are usually not detected and no distinction can be made between zoosporangia of *S. subterranea* and those formed by other root pathogens. Due to the painstaking nature of examination by microscope, only a part of the root system can be tested, increasing the chance of false negative results. These problems can be overcome by combining the bait plant test with a PCR assay. Conventional PCR has been used for the detection of *S. subterranea* in bait plant roots, but without any quantification of the level of infection of individual plants (Bouchek-Mechiche et al., 2000; van de Graaf et al., 2000). The real-time PCR assay was able to reliably detect and quantify *S. subterranea* in bait plant roots at initial inoculum concentrations ≥0.5 cystosori per ml, and regularly detected even lower concentrations, and would therefore be a very useful technique to combine with the bait plant test.

The average amount of DNA detected in the bait plant root samples by the real-time PCR assay was always more than the initial amount added due to multiplication of the pathogen in the host. It is speculated that at 5 cystosori per ml, the bait plant roots were fully colonised by *S. subterranea*, since no increase in the amount of pathogen DNA was detected at higher inoculum levels. Similarly, Burnett (1991) was unable to find significant differences in infection levels between inoculum concentrations of ≥1 cystosorus per ml when zoosporangia in potato bait plant roots were counted. Thus it appears that *S. subterranea* can only be quantified reliably by the bait plant method if inoculum levels in the sample are very low.

Zoospores were successfully detected using the real-time PCR assay at all levels of added inoculum, but the results were not as consistent as those for the bait plant roots. This could be due to the fragile nature of the zoospores or to the fact that the majority of zoospores were already attached to the host roots at the time of sampling. At 15 °C, most secondary zoospores are not released until three weeks after inoculation (van de Graaf et al., 2000), but some of the DNA detected in the real-time PCR assay could have come from secondary zoospores released from early matured zoosporangia in the bait plant roots.

The real-time PCR assay described in this paper is a specific, sensitive and reliable method for the detection and quantification of the different life stages of *S. subterranea* in a range of sample types. With this PCR assay, a large number of samples can be processed quickly and easily. Real-time PCR assays are less labour-intensive and provide less chance of contaminations compared with conventional PCR assays. In addition, real-time PCR has no need for the use of ethidium bromide, which is toxic to humans. The detection limits of the real-time PCR assay are similar to or better than with the existing detection methods such as visual examination of bait plant roots, ELISA and conventional PCR. Unlike some of these other techniques, the real-time PCR assay allows reliable quantification

in samples with varying levels of *S. subterranea* DNA, either after DNA extraction directly from field samples or in combination with a bait plant test.

Real-time PCR is a technique that could be applied in practice for diagnostics and disease avoidance. In addition, use of the real-time PCR assay can simplify epidemiological and ecological studies of *S. subterranea* (van de Graaf et al., 2002). Quantification of cystosori in soil or on seed tubers could help to identify the relative importance of different sources of inoculum or to determine the likelihood of PMTV transmission. The assay could also be used to assess the susceptibility of potato varieties and alternative hosts to root infection by *S. subterranea* by quantification of plasmodia and zoosporangia in root tissue. Latent and immature infections of seed tubers could be detected and the effect of environmental factors on the release of zoospores or other important stages in the disease cycle could be quantified. In short, the real-time PCR assay would be a valuable tool in any future studies on the epidemiology and control of powdery scab caused by *S. subterranea* or spraing caused by PMTV.

Acknowledgements

Funding of this work by the Scottish Executive Environment and Rural Affairs Department and the British Potato Council is gratefully acknowledged. The authors would like to thank Louise Sullivan (SCRI) for technical assistance and Dr. Alex Hilton and Dr. Stuart Wale (SAC Aberdeen) for the supply of soil, inoculum and tuber samples.

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