Spongospora subterranea f. sp. subterranea: molecular variation, PCR detection and isolation in vitro

X. Qu, J. A. Kavanagh and D. Egan, Department of Environmental Resource Management, National University of Ireland, Belfield, Dublin 4, Ireland

Introduction
The plasmodiophorid Spongospora subterranea f. sp. subterranea is a soil-borne obligate parasitic pathogen that causes powdery scab of potato, and also transmits potato mop-top virus (PMTV), the cause of “spraing” in potato tubers. Both these diseases cause major problems in potato production in Ireland and are of great concern to the seed and ware potato industry as there are no acceptable chemicals or highly resistant cultivars available for the control of these diseases. Although a bioassay (Flett, 1983) and an ELISA system (Harrison et al., 1993) have recently been developed for the detection of this pathogen, problems relating to their use for the rapid and sensitive identification of this pathogen in host tissues and soil, still exist. Also, the existence of physiological races or pathotypes of S. subterranea has not yet been reported.

The aims of this study are to 1) investigate variation within S. subterranea isolates; 2) develop a PCR assay for the detection of S. subterranea in host tissues and soil; 3) develop a competitive PCR assay for the quantification of S. subterranea in soil; 4) to verify by PCR whether the amoebae isolated in vitro from S. subterranea infected tuber tissues are in fact S. subterranea.

Materials and methods
Tubers showing powdery scab symptoms were collected from ten potato cultivars originating from the Republic of Ireland, Northern Ireland and Scotland, and clean sporeballs of S. subterranea were prepared from these tubers using the method of Qu et al. (1998). Amoebae were isolated from S. subterranea infected tubers of potato cvs. Saturna and Kerr’s Pink with powdery scab lesions containing sporeballs using the method of Diriwächter et al. (1979). S. subterranea infested field soil samples were collected from Co. Donegal.

DNA was extracted from sporeballs, tubers and amoebae using the CTAB method (Doyle & Doyle, 1990). Soil DNA was extracted using a method based on a bead-beating technique (Yeates et al., 1997).

Standard PCR amplifications were performed in a Hybaid omniGene thermal cycler with 35 cycles of 95°C for 0.5 min, 60°C for 0.5 min, and 72°C for 1 min. The final step was extension at 72°C for 7 min. Amplification products were separated by gel electrophoresis in 1.5% agar containing ethidium bromide and visualized with a UV transilluminator. For competitive PCR, a control DNA template was prepared from DNA.

PCR products were sequenced with an ABI PRISM(tm) DNA Sequencing Kit (Perkin Elmer Applied Biosystems) and the DNA sequences were determined using a Perkin Elmer Applied Biosystems Model 373A DNA Sequencer. Sequence data were compared with listings in the Genbank nucleotide sequence database. Primers were designed based on the sequence data and synthesised by Genosys Biotechnologies (Europe) Ltd.
Results

Molecular variation
A single fragment of c. 600 bp was generated from DNA extracted from all thirty *S. subterranea* isolates by PCR with the universal primer pair ITS4/5. The results of sequencing and alignments revealed that the ITS1 region of *S. subterranea* consists of 151-156 bp, the ITS2 region 154-152 bp and the 5.8s rDNA 159 bp. Multiple alignments of the ITS1 and ITS2 regions of the thirty *S. subterranea* isolates revealed that there were two ITS types, A and B. The ITS1 sequence type A differed from ITS1 sequence type B by having three nucleotide differences and at one position an insertion of five nucleotides. The ITS2 sequence type A had six nucleotide differences from those in type B and two positions where single base deletions occurred. These ITS types may identify two groups within *S. subterranea*.

PCR detection
A 20 base region in ITS1 region and a 21 base region in ITS2 region specific to *S. subterranea*, with little or no homology to the other fungi, were identified and used to design the internal primer pair SsF and SsR, respectively.

When the designed primer pair SsF/R was used to amplify DNA extracted from clean sporeballs and zoospores of *S. subterranea* isolated from 4 potato cvs. Saturna 1, Saturna 2, Kerr’s Pink and Cara, a single predicted 434 bp fragment was amplified from all samples. This monomorphic primer pair SsF/R was subsequently used to amplify DNA from healthy potatoes, a wide range of other filamentous fungi and taxonomically related pathogens and no product was obtained. These results indicated that this primer pair was specific for *S. subterranea*. The sensitivity of the primer pair SsF/R was assessed by amplifying DNA from serial dilutions of sporeballs of *S. subterranea*. Successful amplification, with a clear visible 434 bp product on ethidium bromide-stained agarose gels, was achieved at 1 sporeball.

The primer pair SsF/R were used to amplify DNA from symptomatic potato tubers of eight cultivars, an asymptomatic but *S. subterranea* infected potato tuber and a healthy micro-tuber. A 434 bp fragment was amplified from DNA from all symptomatic and asymptomatic tubers, but not from DNA of healthy micro-tuber.

When this primer pair was used for the detection of *S. subterranea* in soil by PCR, a 434 bp fragment was amplified from DNA extracted from spiked soil containing one sporeball per 1 g soil, indicating that this PCR assay was very sensitive for detection of *S. subterranea* in soil. *S. subterranea* was also detected in all infested field soils by PCR with this primer pair. For quantitative applications, a heterologous internal control template was constructed for use in competitive PCR. By co-amplification of *S. subterranea* DNA and the internal template in soil extracts, the numbers of sporeballs of this pathogen was quantified accurately. This is a rapid, sensitive and precise technique for the quantification of *S. subterranea* in soil and will be of immense value in powdery scab disease management.

Isolation in vitro
Amoebae with bacteria were isolated from surface sterilized *S. subterranea* infected potato tubers on 1% water agar at 18°C, encysted after 5 to 7 days, and could be subcultured indefinitely in the presence of bacteria. These cysts were morphologically distinct from the resting spores of *S. subterranea*. Amoebae and cysts from primary and subsequent subcultures derived from these failed to infect tomato roots but plasmodia and zoosporangia were found in tomato roots inoculated with sporeballs of *S. subterranea*. When DNA extracted from amoebae
and cysts was PCR amplified with *S. subterranea* specific primer pair SsF/R, no products was generated. These results indicate that the amoebae isolated from *S. subterranea* infected potato tubers are not vegetative or reproductive forms of *S. subterranea*.

**References**


