Diagnostics for skin blemish pathogens of potato

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The British potato industry suffers, on average, 1 million tonnes of waste annually through disease, damage, and failure to meet market specifications. The British Potato Council (BPC) estimated the annual cost of avoidable waste between leaving the field and reaching the consumer at £30 million[1]. Providing practical solutions to wastage problems is therefore regarded as a high priority throughout the whole industry. Tuber skin blemish diseases are a major cause of wastage and these are of increasing concern due to the demand for washed potatoes with a high quality appearance for the fresh 'pre-pack' market.

The bacterial disease common scab (*Streptomyces scabies*) and the three 'fungal' diseases, powdery scab (*Spongospora subterranea* f. sp. *subterannea*), silver scurf (*Helminthosporium solani*), and black dot (*Colletotrichum coccodes*), are the predominant blemish diseases in the UK. These diseases primarily reduce the market value of potato crops but can also affect the yield. Annual losses from the ware crop were estimated at £3 million for common scab, £1 million for powdery scab, and up to £5 million for silver scurf and black dot, and there are additional losses in the seed industry[1]. The use of disease-susceptible varieties, spread of inoculum on seed potatoes, and changing agricultural practices have all increased the incidence of blemish diseases.

Classical symptoms for common and powdery scab include the appearance of raised or pitted corky scabs on the tuber surface. Tubers infected with silver scurf and black dot show grey or silver patches, whilst black microsclerotia may form on the tuber surface to produce a 'sooty' appearance with the latter disease (Fig. 1). Infection by these pathogens can arise from both contaminated seed tubers and soil-borne inoculum. An improved understanding of the epidemiology of these diseases will assist in their control, thus improving the quality and efficiency of ware production and ensuring supplies of healthy seed. The BPC has funded the development of molecular diagnostics for powdery scab, and MAFF has funded a similar project to study the epidemiology of the soil-borne phase of common scab, black dot, and silver scurf. The objectives were to develop rapid assays to allow specific detection and quantification of these pathogens in potatoes and soil. The diagnostic method selected was the polymerase chain reaction (PCR), which allows the exponential amplification of specific DNA fragments from complex DNA samples by *in vitro* DNA synthesis[2]. This highly sensitive procedure requires a DNA template containing the region to be amplified and two oligonucleotide 'primers' flanking this target region. The amplification process is automated by the use of a thermocycler and a thermostable DNA (*Taq*) polymerase. Another key requirement for both projects was the development of a method to extract and purify microbial DNA from soil.

Figure 1 Classical symptoms of common scab, silver scurf, black dot, and powdery scab.

This substrate presents problems because microbes can be protected from lysis in the soil matrix and co-extracted humic compounds are potent inhibitors of PCR.

Common Scab, Silver Scurf, and Black Dot The key step in the development of a successful PCR assay is the identification of DNA sequences that are characteristic for those species under study. These are used to design specific primers. For fungal pathogens, regions in the nuclear rDNA gene unit are often targeted[3]. Genus and species-specific primers have been designed in the non-coding, variable internal transcribed spacer (ITS) regions 1 and 2 of rDNA. ITS1 and ITS2 sequences of *C. coccodes* reference isolates are present in the databases, whereas these regions had to be sequenced for several UK isolates of *H. solani*. Primers were then designed to unique sequences within these ITS regions for specific detection of *H. solani* and *C. coccodes*. In the case of *S. scabies*, primers were designed to detect the proposed pathogenicity gene (*nec1*), recently described[4]. *Nec1* confers the necrogenic potential when acquired by non-pathogenic strains of *Streptomyces*.

Two sets of primers (outer and nested) were designed for *H. solani*, *C. coccodes*, and *S. scabies*. Nested PCR was used to increase specificity and sensitivity of single-round PCR, and specific product was detected for each organism when 10 fg (10^{-15} g) DNA was included per reaction, the equivalent of one genome. Positive results were obtained when 25 different *H. solani* and *C. coccodes* isolates were tested. Preliminary results also indicated that the *nec1* primers amplified product from other pathogenic but not from non-pathogenic isolates of *Streptomyces* spp. Comparisons between each primer to DNA and protein databases of other fungi and bacteria revealed no significant levels of similarity. The specificity of primers was confirmed as no PCR products were amplified when testing DNA from a range of different fungal and bacterial plant pathogens in the SCRI collection.

A simple and rapid procedure for direct extraction of DNA from soils[5] was modified and recovered high molecular weight DNA of a suitable purity for PCR within 3 hours. The key steps of the protocol were the physical disruption of soil microbes in a Mini-Beadbeater, the reduction of humic contamination by the use of an alkaline sodium phosphate + CTAB buffer, and purification of DNA extracts *via* polyvinylpolypyrrolidone (PVPP) or Sephadex G-75 spin-column chromatography (Fig. 2).

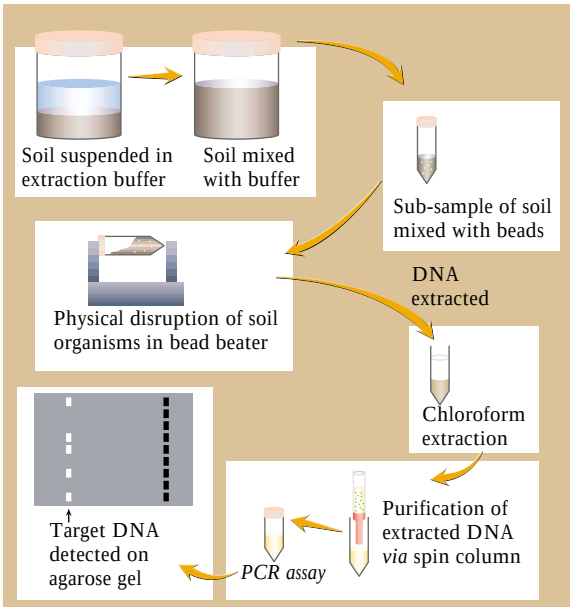

Figure 2 Extraction of DNA from soil.

Primers were designed to operate under the same conditions to ensure adaptability for a rapid multiplex PCR assay (*i.e.* incorporation of the three primer sets in one reaction), in which all three pathogens could be detected simultaneously. The sensitivity of PCR for the specific detection of *H. solani*, *C. coccodes*, and *S. scabies* in seeded soils was tested in parallel for both the single primer sets and in multiplex PCR. Soil samples were seeded with each organism, singly and in combination, and the level of sensitivity for both PCR systems was set at 3 spores per gram of soil, the lowest level of inoculum added (Fig. 3). However, multiplex PCR had the advantage of reducing the time and cost of the procedure. Work is underway to set limits of PCR detection for each organism in plant material. An automated and quantitative PCR system to eliminate the need for gel electrophoresis also will be developed.

Powdery Scab As with *H. solani*, the rDNA unit of *S. subterranea* f. sp. *subterranea* was partially sequenced and the ITS regions were compared to related organisms in the databases in order to design species-specific primers. Selected primers were shown to be highly specific as they amplified the predicted size of product (391 bp) from *S. subterranea* f. sp. *subterranea* but not from related organisms or other potato pathogens. *S. subterranea* was detected in peel and washings of diseased and apparently healthy tubers, but not in Scottish classified seed potatoes or axenically micropropagated tubers. It was possible to detect *S. subterranea* in soil at levels of 1-10 spore balls

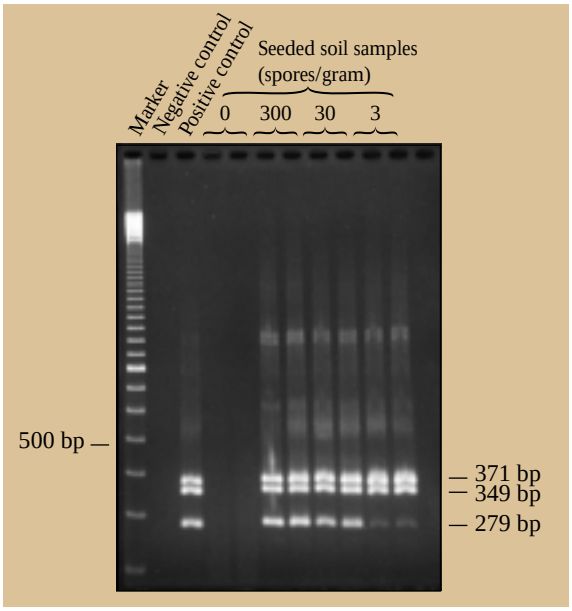

Figure 3 Detection of *H. solani* (371 bp), *C. coccodes* (349 bp), and *S. scabies* (279 bp) in spiked soil by nested multiplex PCR.

per gram with the PCR assay and aforementioned soil DNA extraction protocol. A quantitative PCR assay was also developed using an internal control (competitor) based on a DNA fragment of a smaller size (249 bp) to the target but with the same primer binding sites. A fixed concentration of 'competitor DNA' was co-amplified with DNA extracted from a dilution series of *S. subterranea* spore balls, and the ratio of the amount of both products was estimated from the intensity of the bands on a gel (Fig. 4). This data was used to generate a standard curve to estimate the DNA concentration from unknown numbers of sporeballs amplified under the same conditions, and hence the numbers of target organisms could be determined in a test sample.

PCR-based tests are useful tools for rapid and accurate assessment of tuber and soil contamination by plant pathogens, and will assist epidemiological studies. Quantitative results can be obtained in a day, in contrast to several weeks for glasshouse-based bait tests. Results will supplement our current knowledge on methods to control these pathogens (*e.g.* early harvesting, maximum levels of seed health, fungicide seed treatments, and strict hygiene in seed and ware stores) and will provide a basis for the development of new integrated management strategies. Whilst control of soil-borne diseases is inherently difficult, there are possible strategies to minimise economic loss. Quantitative data on inoculum levels would be an important component of disease risk assesments to assist in decision making on control issues such as the application of biocides; avoiding high risk fields; matching cultivars to fields according to their respective resistance rating and disease risk; crop rotation strategy; irrigation regime; and prioritising land according to the crop end use.

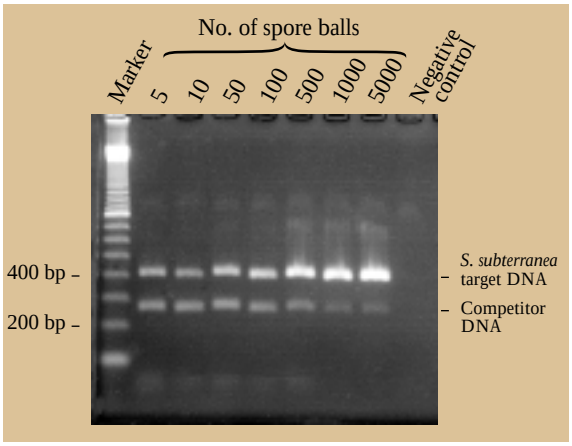

Figure 4 PCR products obtained after using primers Sps1 and Sps2 to coamplify 1fg of competitor DNA and *S. subterranea* DNA extracted from varying amounts of spore balls.

References

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