BRITISH POTATO COUNCIL

Project Report

Development and initial validation of primers for the detection and quantification of *Polyscytalum pustulans*

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1. Summary

The aim of this work was to develop and validate a molecular test for the detection of the causal agent of skin spot (*Polyscytalum pustulans*). An eye plug test can be used to identify infected stocks prior to planting, however the test takes 5 days or more to complete and assessments can be difficult because the plugs can become overgrown with common contaminants. Development of a molecular test would provide a more rapid method for the detection and quantification of *P. pustulans*.

Prior to this project, none of the genetic data needed to develop a molecular test existed for *P. pustulans*. During the course of the project the data (gene sequence) was generated. The wood-decaying soft rot fungi (*Cadophora* spp.) and a group of common soil fungi (*Rhexocercosporidium* spp.) had sequence that was most similar to that of *P. pustulans*. The sequence data was used to develop a molecular assay based on the real-time polymerase chain reaction (PCR). Cultures of related fungal species were tested to check the specificity of the assay and no cross-reactions were observed. *P. pustulans* was detected in symptomatic and asymptomatic potato tubers, proving the test has the potential to detect the pathogen prior to skin symptoms being visible. A mobile testing system was set-up at SBEU and a range of potato stocks tested. The real-time PCR assay was successful in discriminating between low and moderate levels of skin spot based on the quantity of pathogen-specific DNA detected in tuber peel.

2. Experimental Section

2.1 Introduction

Polyscytalum pustulans causes a serious but intermittent blemish disease of potatoes in store (Wale et al., 2005). The fungus is capable of infecting stem bases and potato tubers (Hide & Read, 1991). However, symptoms of the blemish disease on tubers, known as skin spot, normally become visible many weeks after harvest. This is a problem because the pathogen is primarily seed-borne and it is important that seed suppliers and purchasers can make an early assessment of the risk of stocks being infected. Ideally, infected stocks would be identified prior to storing so that timely control measures can be put in place. However, no suitable early disease prediction test currently exists, and there are no rapid molecular tests for this pathogen. Nevertheless, from the 1960's onwards, batches of seed potatoes have been screened for the presence of *P. pustulans* using an eye-plug test (Hide et al., 1968). This test is often used to identify infected stocks prior to planting but Hide *et al.* (1968) admit that this method of quantification is not ideal, primarily because overgrowth of common contaminants can make assessments difficult. The eye-plug test entails incubating excised plugs of tissue around tuber eyes in a humid chamber for 5 days or more at 15°C then examining microscopically. Pustules resembling those of skin spot can be plated onto selective media to enable the causal pathogen to be identified. Testing for the presence of the fungus in soil can be achieved by planting disease-free bait plants or by plating soil dilutions onto semi selective media (Carnegie & Cameron, 1990). Real-time PCR has proved useful for the detection and quantification of other potato pathogens including *Rhizoctonia solani* (Lees *et al*., 2002), *Helminthosporium solani* (Cullen *et al*., 2001), *Colletotrichum coccodes* (Cullen *et al*., 2002), TRV and PMTV (Mumford *et al*., 2000) and *Spongospora subterranea* (Ward *et al*., 2004). Real-time PCR has been proven to be more sensitive than antibody based methods and conventional PCR (Ratti *et al*., 2004).

The purpose of the work presented here was to develop and validate a real-time PCR assay for the detection and quantification of the target pathogen

2.2 Material and methods

2.2.1 DNA extraction

DNA was extracted using the Wizard® Magnetic DNA Purification System for Food (Promega, FF3750) in conjunction with a Kingfisher ML magnetic particle processor (Thermo Electron Corporation). For culture extractions, mycelium was scraped from the surface of an actively growing culture and placed into a 2 ml screw cap tube containing 0.5 ml of 1 mm zirconia beads and 1 ml Lysis Buffer A containing 10% Antifoam B emulsion (Sigma). Samples were shaken at full speed for 30 s on a mini-beadbeater (BioSpec Products, Inc.) before following the standard Promega extraction protocol. The extractions were completed using the gDNA program including the optional heating stage on the Kingfisher ML. Samples were eluted into 0.2 ml molecular grade water and stored at –30°C until required. DNA was extracted from potato samples using the same process, except potato tissue was placed 1:10 (w/v) in CTAB based lysis buffer and ground using a homex grinder.

At SBEU, potato peel samples were placed in a 400 ml blender bag (Separator 400, Grade Packaging Ltd) and ground using a manual roller extractor. Sap was transferred into labelled 10 mL capped test tubes and the sediment left to settle overnight. A 500 µl volume of supernatant was aliquoted into 3 replicate 1.5 ml microcentifuge tubes per sample and centrifuged at 7000 rpm for 8 minutes (Sigma 1-14 microfuge; Sigma, Osterode, Germany). The supernatant was discarded and the pelleted samples were stored at -20°C. Primary extractions were performed using mini DNeasy® plant DNA extraction kits (Qiagen Ltd, UK). The 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 Sigma 1-14 microfuge was used at a speed of 4000 rpm for 4 minutes. Samples were stored at -20°C until required.

2.2.2 ITS PCR and sequencing

The Internal Transcribed Spacer (ITS) regions of two cultures of *P. pustulans* (PP03 and PP14) were amplified using the primer pair ITS5 and ITS4 which amplifies ITS1, 5.8S rDNA, ITS2 with flanking regions of 18S and 28S rDNA (White *et al.*, 1990). The reaction mixture contained 5 pmol of each primer, 37.5 mmol $MgCl_2$, 5 mmol dNTP, 0.75 U of BIO-X-ACT Long DNA Polymerase (Bioline, BIO-21049) and 1 µl of template DNA. Samples were cycled at 95°C for 4 min followed by 35 cycles of 95°C for 1 min, 52°C for 1 min and 72°C for 1 min with a final extension phase at 72°C for 2 mins using a GeneAmp PCR System 9700. PCR products were purified using the Promega Wizard SV gel and PCR clean-up system (Cat no. A9282) following the manufacturer's instructions. Purified products were quantified on a gel and diluted to 1.3 ng/ul prior to sending to the University of Dundee for sequencing.

2.2.3 Assay design and specificity testing

ITS1, 5.8S rDNA and ITS2 sequences from 5 different fungal species were aligned using clustal V within the MEGALIGN package (DNAstar, Lasergene6) and areas of *P. pustulans* specific sequence identified. Sequences aligned were: *Cadophora* sp. (DQ317329); *Cadophora* sp. (AY371506); *Cadophora luteo-olivacea* (DQ317327); *Phomopsis quercella* (AJ293878); *Rhexocercosporidium* sp. (DQ303121). Primers PP03_Unique_32F/ PP03_Unique_112R and probe PP03_Unique_54T were selected to amplify a *P. pustulans* specific fragment within the 18S ribosomal RNA (Accession AF087480) using Primer Express software (Applied Biosystems, Branchburg, New Jersey, USA). Fluorogenic probe PP03_Unique_54T probe was modified 3' with TAMRA (tetra-methylcarboxyrhodamine) and 5' with FAM (6-carboxyfluorescein).

Assay specificity was determined by testing against nucleic extracts of several fungal cultures including: *Phomopsis sp.* from *viticola*, *Phomopsis sp.* from *acer*, *Phomopsis sp.* from *Quercus robor*, *Rhexocercosporidium* (sp. DSE48.1b) and *Cadophora gregata* (biotype B). In addition, a healthy potato control was prepared by extracting from the internal surface of an apparently healthy potato. Finally, primer and probe sequences were checked for specificity against all published sequences on the EMBL database using the blast search algorithm.

2.2.4 Testing potato stocks - CSL

Potato stocks showing symptoms of skin spot were selected from various regions of the UK. Skin from apparently diseased potatoes was peeled and the resulting DNA extract tested using the *P. pustulans* real-time PCR assay as described previously. DNA was extracted from 4 asymptomatic potatoes selected from one batch of potatoes showing low levels of skin spot. Also, a DNA extract was prepared from a diagnostic sample showing unusual skin symptoms. All DNA extracts were tested using the *P. pustulans* real-time PCR assay.

2.2.5 Testing potato stocks – SBEU

Twenty potato tubers showing no, few (one, two and four pustules), and moderate (nine pustules) levels of skin spot were selected from experimental material (BPC R&D project reference R251) during early February 2007. Skin from the sample potatoes was peeled and DNA extracts prepared as described previously. In addition, a ten-fold dilution series of *P. pustulans* conidia, from 10^2 to 10^6 conidia/ml, was prepared in Purite water. All DNA extracts were tested using the *P. pustulans* real-time PCR assay.

2.3 Results

2.3.1 ITS PCR and sequencing

A single PCR product of 1100 bp was obtained from both isolates of *P. pustulans* (Figure 1). A contiguous (contig) sequence of DNA was constructed by assembling both the forward and reverse sequence reads for both isolates using SeqMan version 6 (DNAstar). An alignment of the contigs from PP03 and PP14 revealed both 1061 bp sequences were 100% identical. A search of the EMBL database using the blastn algorithm revealed the most related sequences were from *Cadophora* and *Rhexocercosporidium* spp.

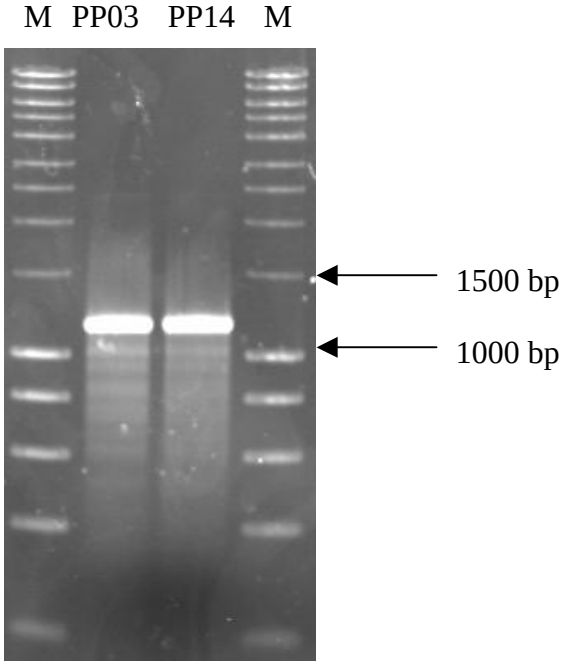

FIG. 1 AGAROSE GEL ELECTROPHORESIS OF THE PCR PRODUCTS OBTAINED USING PRIMERS ITS5/ITS4.

A clear single band of approximately 1100 bp is visible for both *P. pustulans* isolates PP03 and PP14. M represents the Hyperladder I (Bioline).

2.3.2 Assay design and specificity testing

The contig sequence from PP03 was used to design primer set PP03_Unique_32F/PP03_Unique_112R and probe PP03_Unique_54T. The real-time PCR assay did not cross-react with any of the related fungal species tested. In addition, no reaction was seen when testing a DNA extract from healthy potato.

2.3.3 Testing potato stocks - CSL

All the DNA extractions prepared from potatoes showing skin spot symptoms tested positive using the real-time PCR assay for *P. pustulans*. The majority (3/4) of the DNA extracts prepared from asymptomatic potatoes, selected from a batch showing a low level of skin spot, tested positive for *P. pustulans*. The DNA extract prepared from a diagnostic sample showing unusual skin symptoms also tested positive for *P. pustulans*.

2.3.4 Testing P. pustulans *conidia – SBEU*

A standard curve was produced from a dilution series of *P. pustulans* conidia using the real-time PCR assay for *P. pustulans* (Figure 2). The standard curve was used to estimate the equivalent number of spores in future peel extractions from the C_T value. This was converted to pg of target DNA by estimating the quantity of DNA in the original conidial suspensions, using a Qubit™ Quantitation Fluorometer (Invitrogen Ltd, Paisley, UK), to provide pg/spore, and then multiplied by the number of conidia to provide pg of *P. pustulans* DNA per sample. Finally all peel samples were weighed and the resulting concentration of *P. pustulans* DNA expressed as pg /g of peel.

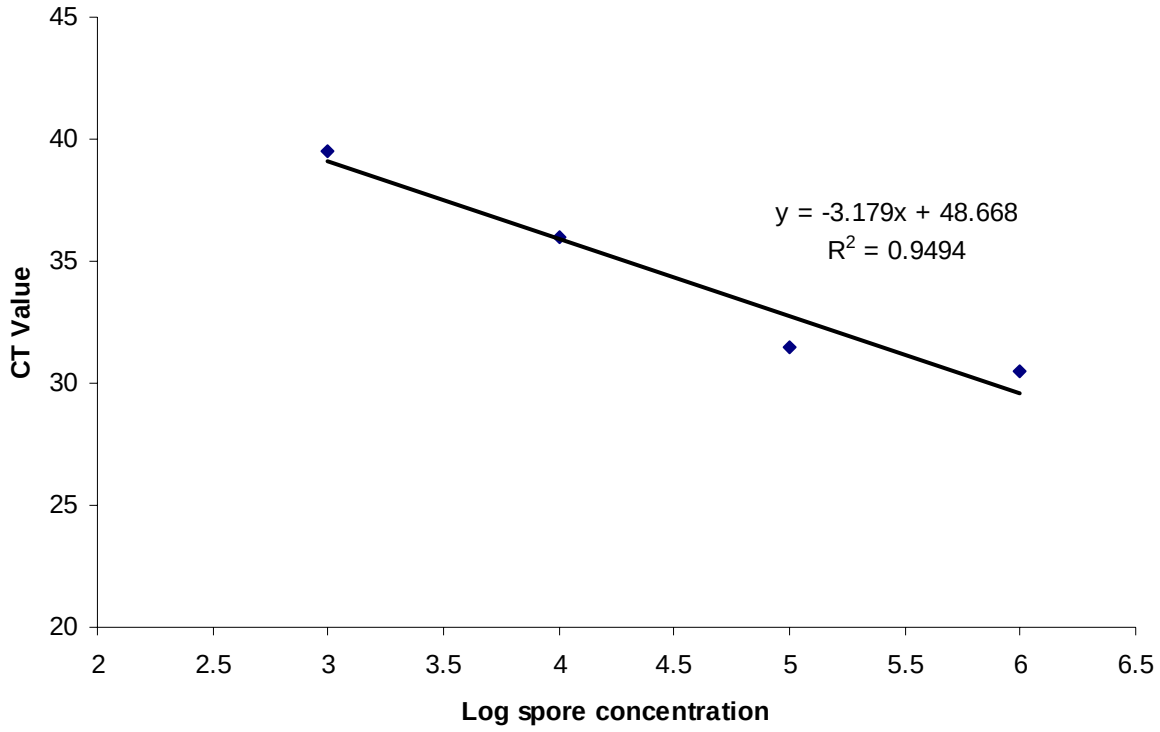

FIGURE 2. RELATIONSHIP BETWEEN THE C_T VALUE OBTAINED BY REAL-TIME PCR AND THE AMOUNT OF *POLYSCYTALUM PUSTULANS*-SPECIFIC DNA DETECTED IN A DILUTION SERIES OF *P. PUSTULANS* CONIDIA.

2.3.5 Testing potato stocks – SBEU

Twenty tubers from a number of stocks with varying degrees of skin spot were tested using the real-time PCR assay for *P. pustulans*. An asymptomatic tuber from a 'clean'stock (i.e. with no recorded skin spot) tested negative for *P. pustulans*. Six asymptomatic tubers from a stock (Stock P) with a 2% incidence of skin spot pustules tested negative for *P. pustulans* (Table 1). Four out of six asymptomatic tubers from a stock (Stock M) with 8.5% incidence of skin spot pustules tested positive for *P. pustulans*. All three tubers from a stock (Stock C), which had relatively high levels of visual symptoms of skin spot, tested positive for *P. pustulans.*

TABLE 1. COMPARISON OF VISUALLY ASSESSED SKIN SPOT INCIDENCE WITHIN THREE TUBER STOCKS AND FREQUENCY OF POSITIVES FROM REAL-TIME PCR ASSAY USING P. PUSTULANS PRIMERS.

Stock	Skin spot incidence (%)[1]	Frequency PCR positives[2]
P	2.0	0/6
M	8.5	4/6
C	16.7	3/3

[1] Skin spot incidence assessed visually on >500 tubers per stock.
[2] The PCR assay was performed on asymptomatic tubers.

Four tubers with varying degrees of skin spot pustules were tested. In general, the level of *P. pustulans*-specific DNA per gram of peel increased with increasing numbers of skin spot pustules (on a per tuber basis) from low (one, two and four pustules) to moderate (nine pustules) (Figure 3).

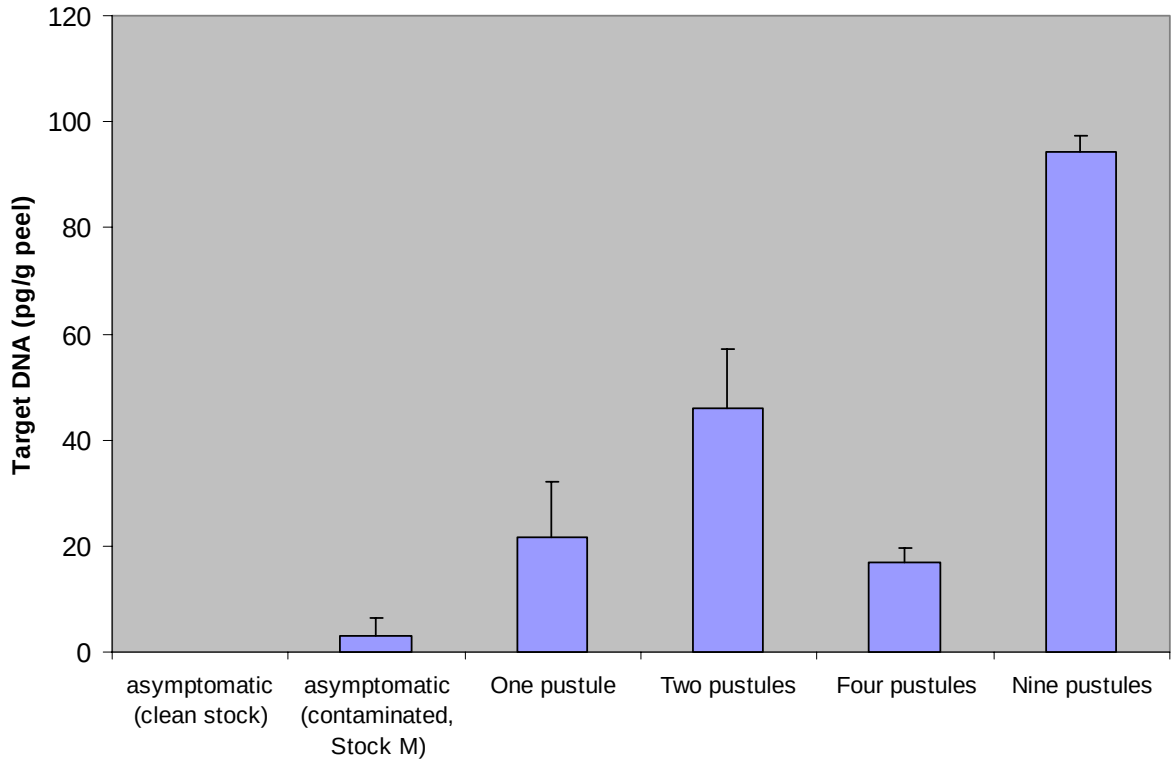

FIGURE 3. THE AMOUNT OF *P. PUSTULANS*-SPECIFIC DNA DETECTED IN THE PEEL OF SINGLE TUBERS AND DEGREE OF VISIBLE SKIN SPOT SYMPTOMS ON SINGLE TUBERS.
Error bars are standard errors of the mean (P=0.05)

2.4 Discussion

Nucleic acid sequence was generated from two isolates of *P. pustulans*. The ITS region was several hundred bp longer than closest related species. This is the first record of such data being generated for *P. pustulans*. The sequence data from both isolates were identical, suggesting little variation between the two sequenced isolates. A survey of a larger number of isolates is required to determine the genetic variation in the *P. pustulans* population.

A real-time PCR test was developed using this sequence data. The assay proved to be specific when testing against a range of closely related fungi. All symptomatic tubers tested positive using the *P. pustulans* assay. Moreover, the real-time PCR assay was able to differentiate between low levels (i.e. one to four pustules) and a moderate level (i.e. nine pustules) of skin spot on the basis of the quantity of pathogen-specific DNA. Interestingly, some asymptomatic tubers tested positive for *P. pustulans* using real-time PCR. These tubers came from stocks which had tubers with low to high levels of skin spot symptoms. Such a result suggests the assay could be used to predict the risk from this pathogen prior to the onset of symptoms.

The ability of the diagnostic assay to detect latent infections is of importance to the potato industry because, since skin spot is mainly a tuber-borne disease, this test would allow seed suppliers and purchasers to identify at-risk stocks prior to planting. However, the capability of the real-time PCR assay to detect latent *P. pustulans* in tubers at, or around, harvest time is not known. Nor is there information on which levels of *P. pustulans*-specific DNA pose potential risks to seed or tuber health. However, if this test was able to detect pathogen prior to commercial storage, it is envisaged that store managers could use this information to tailor the

curing regime to the risk of skin spot as determined by the level of latent pathogen. In addition, fungicide applications could be targeted at the highest risk stocks.

2.5 Conclusions

Clearly this project has developed a useful diagnostic tool for detecting and quantifying *P. pustulans* DNA. The results described in this report suggest that the real-time PCR assay is capable of differentiating samples on the basis of skin spot severity. Importantly, the test was able to detect *P. pustulans* DNA in asymptomatic tubers. However, further development is required to convert this into a useful test for potato growers to predict the risk skin spot poses to stored potatoes.

2.6 References

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3. Summary of knowledge transfer

14/12/2006 - Project included in a potato blemish disease workshop at the Cambridge University Potato Growers Research Association (CUPGRA) conference in Cambridge. Workshop co-presented by Giles Budge (CSL) and Jeff Peters (SBEU).

17/01/2007 –Skin Spot Diagnosis. Poster presented at the 4th EAPR/UEITP/FNK Potato Processing Conference and Engineering and Utilisation Section Meeting, Grantham by Jeff Peters (SBEU), Giles Budge (CSL).