Final Report

Skin Spot Management -

Polyscytalum pustulans qPCR assay

Ref: R413

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1. WORK UNDERTAKEN BETWEEN JULY 2008 AND JULY 2010

1.1. Summary

Polyscytalum pustulans causes a serious but intermittent blemish disease of potatoes in store. Symptoms of the blemish disease on tubers, known as skin spot, normally become visible many weeks after harvest. Store managers, seed suppliers and purchasers have difficulty, at present, in making an early assessment of the risk of stocks being infected at harvest. Conventional and real-time assays for the detection of *P. pustulans* DNA were developed as part of a previous PCL-funded project (R285). Preliminary data from a follow-on PCL-funded project, R294 (2007-08, see page 20 onwards), demonstrated that the real-time assay had potential to enable the early prediction of skin spot levels during storage. The work presented in this report (pages 4-19) for project (R413, 2008-10) confirms that there was a good relationship between levels of *P. pustulans* DNA in peel at harvest and skin spot development in tubers following a 20-week storage duration.

The conclusions are:

- Over two years of trials involving the testing of 106 sets of tuber samples, there was a good relationship between *P. pustulans* DNA levels in tuber peel at harvest and skin spot incidence on tubers after storage. This relationship promises to enable skin spot risk assessment and management strategies to be made prior to storage.
- During the 2008-09 season, at the end of a 20-week storage period, 51% of 53 tuber samples developed visible symptoms resembling skin spot and 87% had detectable levels of *P. pustulans* DNA. During the 2009-10 season, 100% of a further 53 samples collected developed visible skin spot after storage and all samples had detectable levels of *P. pustulans* DNA.
- It is suggested that for samples tested at or around harvest, *P. pustulans* DNA levels of $<10^3$ pg DNA/g peel represents low skin spot risk; 10^3 to 10^4 pg DNA/g peel represents moderate skin spot risk; and $> 10^4$ pg DNA/g peel represents high skin spot risk.
- There was broad agreement between results presented in this report and those reported in a previous PCL-funded project (R294) for samples collected at or around harvest
- The discrimination between low and high risk samples, in terms of skin spot development, was good in progeny tubers collected at harvest. However, this disagrees with the findings of R294 where better discrimination was achieved when testing tubers at the end of a 20-week storage period. A likely reason for this is that tubers investigated in R294 developed higher skin spot severity than was found on tubers in this report.
- This work indicates that crop duration is a significant component of the disease epidemiology. This, as a factor, has been under-investigated.
- Future sampling should involve the removal and testing of peel from the entire surface of the tuber, or as a minimum include cores taken from around the stolon and eyes.
- It is recommended that further work is done to refine the relationship between pathogen DNA levels in peel and skin spot levels by considering factors such as cultivar, crop duration and geographical location of crop.

1.2. 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 considered to be 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. Never-the-less, 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 polymerase chain reaction (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).

Previous Potato Council-funded research (Project reference R285 'Development and initial validation of primers for the detection and quantification of *Polyscytalum pustulans'*) resulted in the development of a molecular assay based on real-time PCR assay (Budge *et al*., 2007). Cultures of related fungal species were tested to check the specificity of the assay and no cross-reactivity was observed. 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. The PCL-funded project R294 ('Determining the ability of a novel quantitative PCR assay to detect latent infections of *Polyscytalum pustulans*') tested the ability of the real-time PCR assay developed during R285 to detect and quantify *P. pustulans* in experimental samples with different levels of skin spot infection (Peters *et al*., 2008). This work demonstrated that the PCR assay was able to detect latent infections at harvest, three weeks after harvest and at the end of a c. 20-week storage period.

The purpose of the work presented here was to validate the real-time PCR assay in quantifying *P. pustulans* DNA at harvest and during storage using commercial tuber samples. In addition, the relationship between pathogen DNA at harvest and skin spot development after storage was measured.

1.3. Material and methods

1.3.1. Commercial Sample acquisition (2008-09)

Fifty three samples of tubers of c. 25 kg from a list of preferred skin spot susceptible varieties were collected from the main potato-growing regions in GB at harvest. Each supplier was asked to provide the crop history for each sample. The number and variety of each sample is listed in Table 1. Each sample was divided into two random sub-samples: one sub-sample of 50 tubers for immediate pathogen DNA quantification in tuber peel at harvest; and the other stored at Sutton Bridge Experimental Unit (SBEU. From 1st August 2010 onwards the unit changed its name to Sutton Bridge Crop Storage Research [SBCSR]) with minimal curing to encourage skin spot development. The stored samples were assessed for visual symptoms of skin spot and for pathogen DNA quantification in tuber peel.

Variety	Number of samples
Desiree	2
Hermes	2
Kerrs Pink	3
King Edward	19
Lady Rosetta	4
Maris Piper	13
Melody	1
Pentland Dell	2
Sante	2
Saturna	5

TABLE 1. POTATO VARIETIES AND NUMBERS OF SAMPLES ACQUIRED FOR TRIALS 2008-2009

1.3.2. Commercial Sample acquisition (2009-10)

Three sets of tuber samples were acquired and tested for *Polyscytalum pustulans*. Set one consisted of 14 samples of c. 25 kg from a list of preferred skin spot susceptible varieties. These were collected from the main potato-growing regions in GB at harvest (collected early to mid- November 2009). The levels of *P. pustulans* DNA detected in tuber peel of Set 1 samples were low (<log 2.5 pg DNA/g peel) so were not sent to SBEU for storing as it was considered unlikely that sufficient skin spot would develop to inform the relationship between inoculum DNA and disease. Therefore, further samples, Set 2, were collected and tested in mid-December 2009.
Set 3 consisted of 24 samples of c. 25 kg tubers from the harvested progeny from the fungicide trial (R413 Experiment 2). The number and variety of each sample is listed in Table 2. Each sample was divided into two random sub-samples: one sub-sample of 50 tubers for immediate pathogen DNA quantification in tuber peel at harvest; and the other stored at SBEU with minimal curing to encourage skin spot development. The stored samples were assessed for visual symptoms of skin spot and for pathogen DNA quantification in tuber peel.

Variety	Number of samples
Set 1	
King Edward	10
Maris Piper	2
Rooster	2
Set 2	
Cultra	2
Desiree	6
King Edward	8
Maris Piper	4
Set 3	
Desiree	19[†]

[†] Five of the 24 tuber samples were rotted on receipt so were not processed.

TABLE 2. VARIETIES AND NUMBERS OF SAMPLES ACQUIRED FOR TRIALS 2009-2010

1.3.3. Sample preparation, storage and assessment

Between 11 September and 18 November 2009, freshly harvested samples were held overnight at ambient hangar conditions at SBEU prior to dispatch to Fera, Sand Hutton. In 2009-10, samples were delivered directly to Fera from 5 November to 17 December 2009. Samples were delivered to SBEU after extractions were performed.

On receipt at SBEU, tubers were hand-graded to remove <35 mm and >85 mm sized tubers, as well as those with obvious defects. A 50 tuber sub-sample was sent as soon as practicable to Fera for harvest time-point testing of *P. pustulans* DNA. The remaining tubers from each sample were placed into labelled plastic trays (approximately 15 kg per tray) and loaded into an experimental store at 6°C and 95 % RH.

After 20 weeks, 50 tubers from each sample were washed for 2 minutes and rinsed in clean tap water and visually assessed for typical skin spot symptoms. Skin spot was classified within the following surface area categories; zero, >0- 2%, >2-5%, >5-10%, >10-25%, and >50%. During assessment, every batch of 5 tubers were placed in paper bags, labelled 1-10 consecutively and sent as soon as practicable to Fera for final storage time-point testing of *P. pustulans* DNA.

1.3.4. DNA extraction

A strip of peel was removed from each tuber (from rose to stolon end) at Fera. Peel strips from each sub-sample of five tubers were weighed and placed into an ELISA grinding bag (Bioreba Ltd) containing 7.5 mL PB7 (2ml Tetrasodium pyrophosphate in 100mL Phosphate Buffer pH7). The contents of each ELISA bag was pulverised using a large sample grinder (Homex Ltd). The supernatant from each bag was transferred into a labelled 5mL sample tube (to c. 6.5mL). Sample tubes were spun on the Sigma 4K15 centrifuge at 1000rpm for 10 minutes at 4^{o}C and supernatant transferred to a clean, labelled 5mL tube and spun at 6200rpm for 15 minutes at 4^{o}C to pellet any sediment. DNA was extracted from each pellet using the Wizard® Magnetic DNA

Purification System for Food (Promega, FF3750) in conjunction with a Kingfisher ML magnetic particle processor (Thermo Electron Corporation). The extractions were completed using the gDNA program including the optional heating stage on the Kingfisher ML. amples were eluted into 200 uL TE buffer and stored at –30°C until required.

1.3.5. PCR quantification

All real-time PCR reactions were performed in 96-well reaction plates using TaqMan Universal PCR MasterMix (Applied Biosystems). For each reaction, 1 μl DNA, which had been diluted 1/5 with TE buffer, was added to 24 μl of mastermix in the appropriate well. Assays were performed using either an Applied Biosystems 7900HT Sequence Detector or Cepheid Smartcycler systems. The PCR mix contained 200 μM d-ntp mix, 0.3 μM primers, 0.1 μM fluorogenic probe and 0.125 U AmpliTaq Gold DNA polymerase in a 25 μl reaction volume. Reaction conditions were an initial 95°C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. These conditions are generic to other Potato Council-funded disease diagnostic assays. An internal PCR control assay used existing TaqMan primers and probe to amplify a fragment of the potato cytochrome oxidase (COX) gene. This provides a check that the quality of DNA following the extraction process is suitable for analysis.

1.3.6. Statistical analysis

Exponential curves were fitted using Genstat version 11 (VSN International). Comparisons of survey factors were analysed using the accumulated ANOVA routine within the Genstat curve fitting procedure.

1.4. Results

1.4.1. Skin spot development (2008-09)

At the end of a 20-week storage period, 51% and 100% of the 2008-90 and 2009-10 tuber samples, respectively, developed visible symptoms resembling skin spot. Correspondingly, 87% and 100% of the 2008-90 and 2009-10 tuber samples, respectively, had detectable levels of *P. pustulans* DNA. The incidence of skin spot varied from 0 to 64%. The percentage surface area (or severity) of sample tubers affected by the disease was low: all samples with skin spot had less than 1% severity, except for one sample which had a mean severity of 1.3%. As the severity of skin spot was low, all analyses were carried out on incidence data.

1.4.2. PCR quantification: the relationship between DNA levels in tuber peel at harvest and skin spot development (2008-09)

At harvest, the amount of detectable pathogen DNA increased with increasing incidence of skin spot detected at the end of a 20-week storage period (P<0.001, Fig. 1).

The relationship between pathogen DNA levels and skin spot incidence was consistent with an exponential model or 'asymptotic regression' (Equation 1).

$$y = \alpha + \beta r^{x} \quad (1)$$

Where α, is the upper asymptote; β, is the range of curve between the value x=0 and the asymptote; and r, is the rate of exponential increase.

The goodness of fit for the exponential regressions, R^2, was reasonable, 0.65.

Two samples of tubers (one cv King Edward, the other cv Maris Piper) were excluded from the analysis because some tubers had *P. pustulans* DNA associated with sunken lesions that were not recognised as typical skin spot pustules. These are indicated in Fig. 1 as ○.

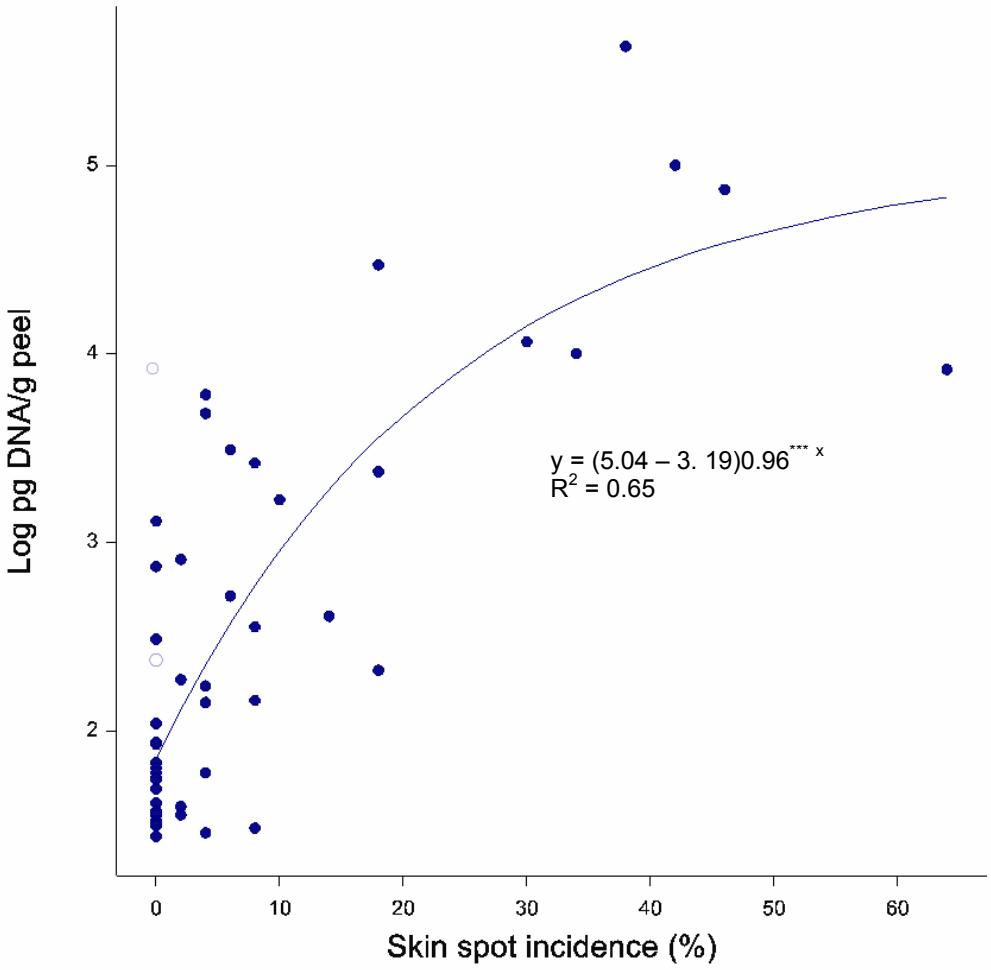

FIGURE 1. THE RELATIONSHIP BETWEEN *POLYSCYTALUM PUSTULANS* DNA LEVEL (LOG10 PG DNA/G PEEL) DETECTED ON PROGENY TUBERS AT HARVEST AND SKIN SPOT INCIDENCE ON PROGENY TUBERS AFTER STORAGE DURING SEASON 2008/09.

The significance of the rate of exponential increase (r) is denoted by ***, P ≤ 0.001. Data indicated by ○ were not included in the regression because samples had atypical symptoms associated with *P. pustulans*.

1.4.3. The effect of cultivar and harvest date on skin spot

In general, the incidence of skin spot increased with later harvests (*P*=0.011; Table 3). Crops that were harvested between 11 September to 16 October and 20 to 28 October had a mean skin spot incidence of 3.6% and 6.5%, respectively. Crops harvested in November had a mean skin spot incidence of 14.7%.

Harvest date	Skin spot incidence (%) after 20 weeks storage at 6 °C	Standard error
September to early October	3.6	2.74
Mid to late October	6.5	1.72
November	14.7	2.55

TABLE 3. EFFECT OF HARVEST DATE ON SKIN SPOT INCIDENCE.

Cultivar had a large effect on the incidence of skin spot developing on sampled crop (*P*<0.001; Table 4). Kerrs Pink and Lady Rosetta had higher incidence levels than the other varieties tested. In addition, cultivar had an influence on the amount of *P. pustulans* DNA detected in tuber peel (*P*=0.021; Fig. 2): Lady Rosetta tended to have low levels of target DNA detected; Saturna had high levels of DNA detected.

Cultivar	Skin spot incidence (%)	Standard Error
Dell	2.9	6.08
Desiree	8.2	6.40
King Edward	7.0	2.06
Hermes	5.9	6.08
Kerrs Pink	28.7	5.10
Melody	4.3	8.82
Maris Piper	0.6	2.41
Lady Rosetta	21.1	4.23
Sante	0.0	6.06
Saturna	14.8	3.88

TABLE 4. SKIN SPOT INCIDENCE IN DIFFERENT CULTIVARS.

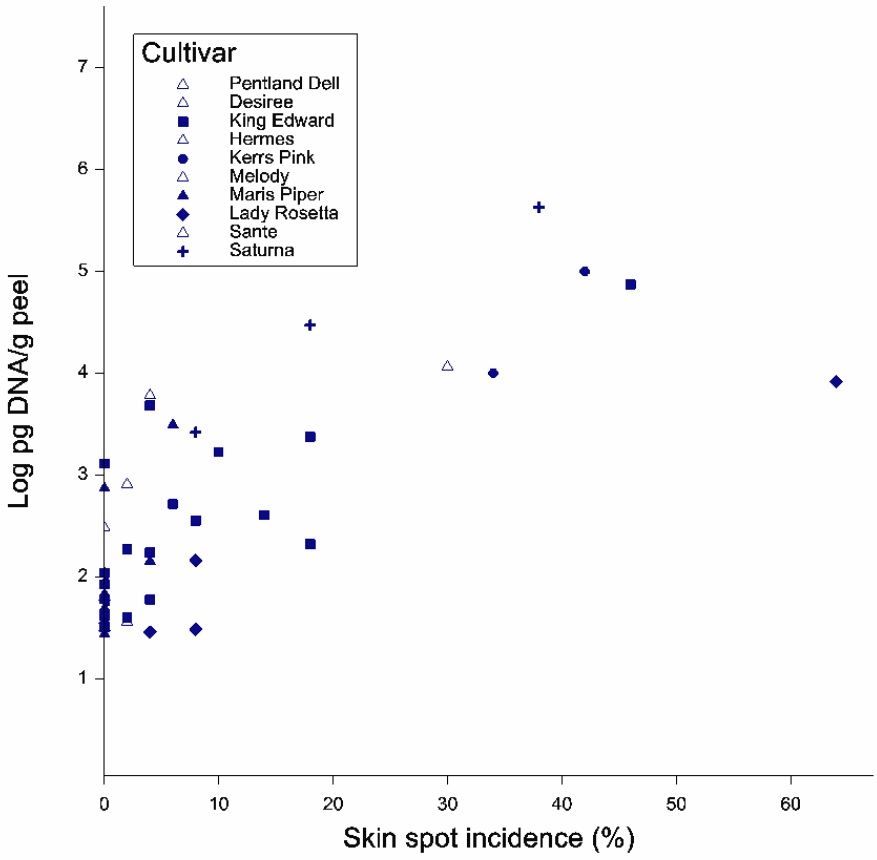

FIGURE 2. THE EFFECT OF CULTIVAR ON THE RELATIONSHIP BETWEEN *POLYSCYTALUM PUSTULANS* DNA LEVEL (LOG_{10} PG DNA/G PEEL) DETECTED ON PROGENY TUBERS AT HARVEST AND SKIN SPOT INCIDENCE ON PROGENY TUBERS AFTER STORAGE DURING SEASON 2008/09.

Cultivars indicated by Δ provided only one or two samples each and were amalgamated for the purposes on the analysis.

1.4.4. PCR quantification: the relationship between DNA levels in tuber peel after 20 weeks in store and skin spot development

At the end of a 20-week storage period at 6°C, the amount of detectable pathogen DNA increased with increasing incidence of skin spot (P<0.001, r^2 = 48%, Fig. 3). Unlike the results from the previous project (R294), the amount of DNA detected after storage was no different to that found in tuber peel at harvest (P=0.169).

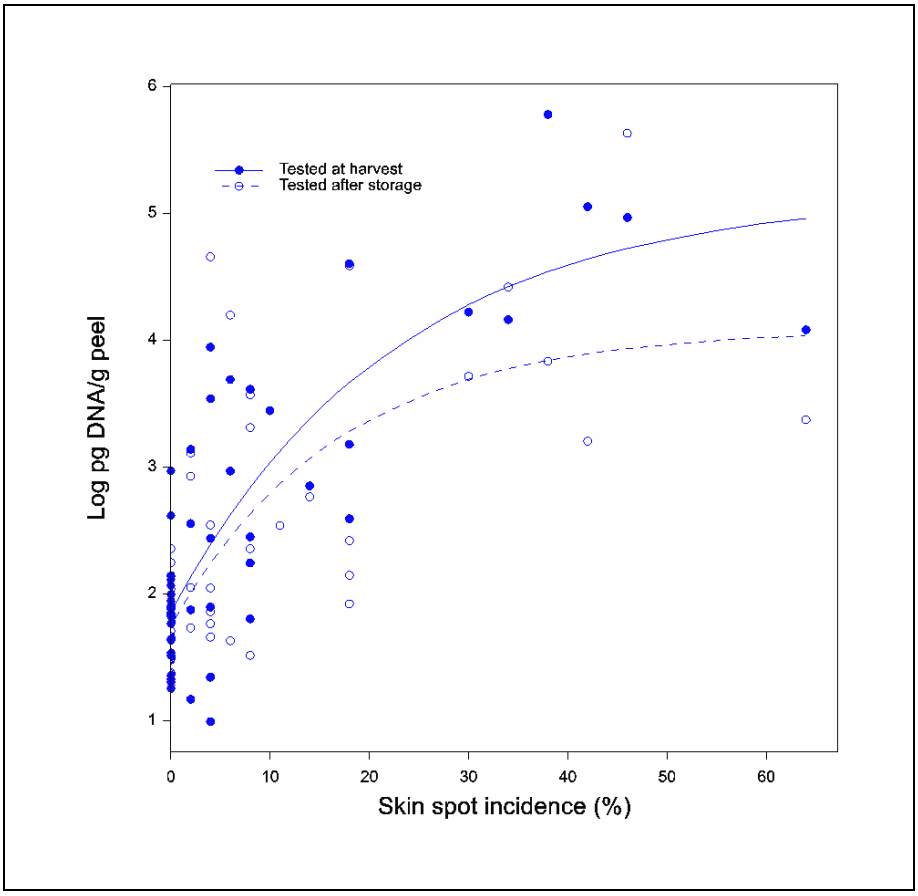

FIGURE 3. SEASON 2008/09. THE RELATIONSHIP BETWEEN *POLYSCYTALUM PUSTULANS* DNA LEVEL (LOG_{10} PG DNA/G PEEL) DETECTED ON PROGENY TUBERS AT HARVEST AND AFTER 20-WEEKS STORAGE AT 6°C, AND SKIN SPOT INCIDENCE ON PROGENY TUBERS AFTER STORAGE.

1.4.5. Skin spot development (2009-10)

At the end of a 20-week storage period, 100% of the tuber samples developed visible symptoms resembling skin spot and 100% had detectable levels of *P. pustulans* DNA. The incidence of skin spot varied from 2 to 70%. The percentage surface area (or severity) of sample tubers affected by the disease was low: all samples had less than 1% severity, except for one sample which had a mean severity of 2.7%. As the severity of skin spot was low, all analyses were carried out on incidence data.

1.4.6. PCR quantification: the relationship between DNA levels in tuber peel at harvest and skin spot development (2009-10)

At harvest, the amount of detectable pathogen DNA increased with increasing incidence of skin spot detected at the end of a 20-week storage period for varieties Desiree and Maris Piper (*P*<0.001, Fig. 4). As previously found, the relationship between pathogen DNA levels and skin spot incidence was consistent with an exponential model or 'asymptotic regression' The goodness of fit for the exponential regressions, R^2, at 0.63, was reasonable and similar to that found in 2008-09 samples.

The relationship between pathogen DNA and skin spot incidence was poor for four samples of the varieties Cultra and King Edward. For this reason, an exponential model for samples of those varieties were fitted separately from samples of Desiree and Maris Piper. Whereas for cvs Desiree and Maris Piper the slope of the curve decreased with increasing skin spot (Figure 4), for cvs Cultra and King Edward it increased with increasing skin spot incidence (not shown). This was because one data point disproportionately influenced the overall regression. Therefore, it was decided that the data could not support the fitting of any model.

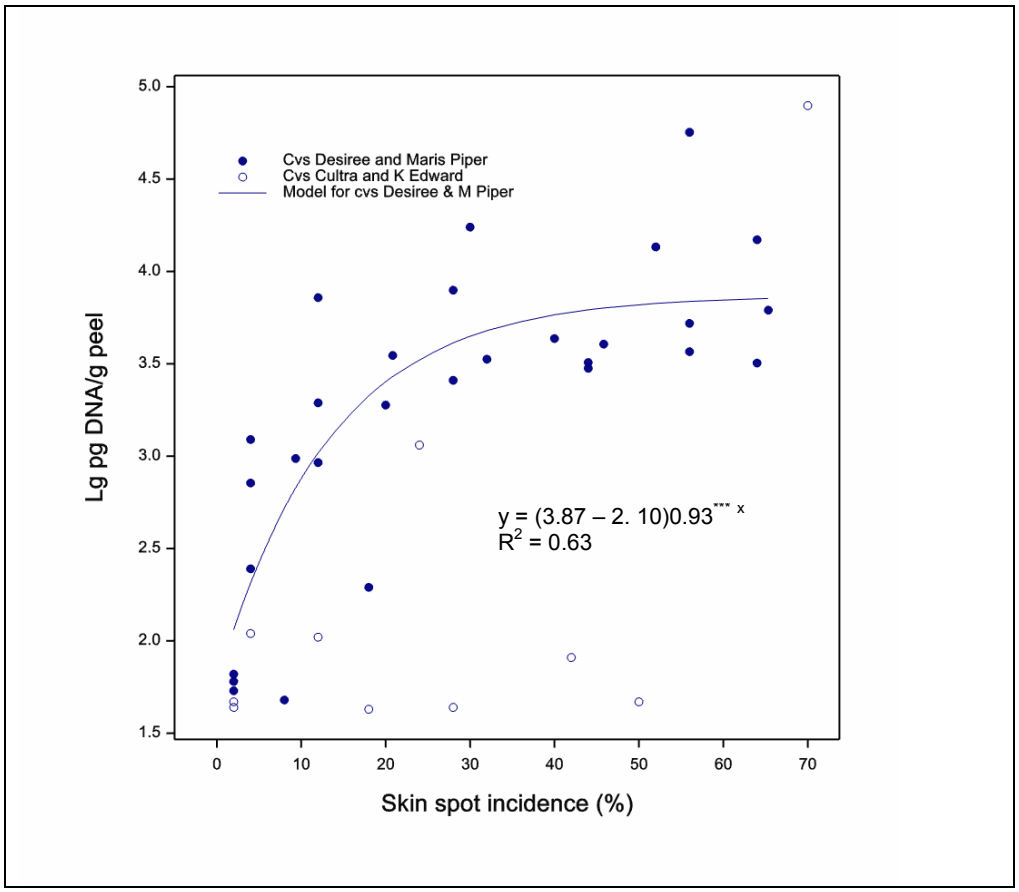

FIGURE 4. SEASON 2009/10. THE EFFECT OF VARIETY ON THE RELATIONSHIP BETWEEN *POLYSCYTALUM PUSTULANS* DNA LEVEL (LOG_{10} PG DNA/G PEEL) DETECTED ON PROGENY TUBERS AT HARVEST AND SKIN SPOT INCIDENCE ON PROGENY TUBERS AFTER 20-WEEKS STORAGE AT 6°C.

The significance of the rate of exponential increase (r) for varieties Desiree and Maris Piper is denoted by ***, P ≤ 0.001.

1.5. Discussion

As was found in the 2007 season (project R294, Peters *et al*, 2008), the amount of *P. pustulans* DNA detected in tuber peel of samples tested during the 2008-09 and 2009-10 seasons generally increased with increasing risk of skin spot development. The relationship between DNA levels in tuber samples at harvest and at the end of the subsequent storage period, and skin spot incidence on progeny tubers at the end of storage was best described by an exponential model or 'asymptotic regression'. In 2007, there were only two sets of tuber samples with skin spot incidences above 20%. Therefore, it is difficult to correlate the relationship between inoculum levels in peel with disease risk across all levels of disease. For this reason, only the 2008 and 2009 samples are considered further in this section. Prior to fitting the regression, four datapoints were removed from the 2009 dataset as these had a high and disproportionate influence on the model. These data will be discussed below. A small difference was observed between the asymptote of the curve in 2008 and 2008 (P=0.025; Fig 5). The effect of season on the correlation between pathogen DNA and disease was small compared with the effect of skin spot incidence on the regression. For this reason, it was decided that a single regression would be fitted. This gave an overall goodness of fit of R^2 = 0.69.

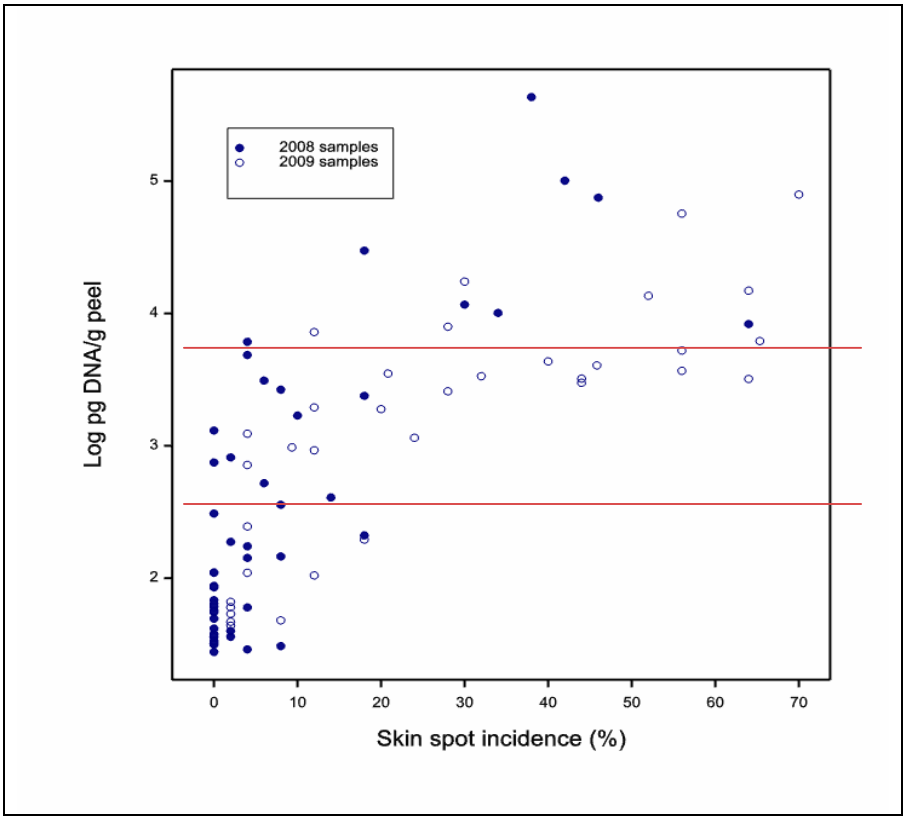

FIGURE 5. THE RELATIONSHIP BETWEEN *POLYSCYTALUM PUSTULANS* DNA LEVEL (LOG PG DNA/G PEEL) DETECTED ON PROGENY TUBERS AT HARVEST PLUS 3 WEEKS IN 2007 AND AT HARVEST IN 2008, AND SKIN SPOT INCIDENCE ON PROGENY TUBERS AFTER STORAGE.

The horizontal lines represent the suggested low risk threshold (< log 2) and high risk threshold (>log 4).

Work carried out during 2008-09 and 2009-10 showed that cultivar had an effect on the relationship between pathogen DNA and skin spot incidence (P=0.021 and P<0.001 in 2008-09 and 2009-10, respectively). This could be due to a real effect of cultivar on the relationship between pathogen inoculum and disease. Alternatively, it could have been due to not being able to adequately sample the peel of such cultivars that have deep eyes and/or inaccessible stolon bases. However, the differences appear to be independent of tuber shape or eye depth (see Table 5).

Variety	Log DNA *P. pustulans*/g peel	Tuber uniformity (1-9, 9 = uniform)	Eye depth (1-9, 9 = shallow)
2008-09			
Lady Rosetta	4.340	7	6
Kerrs Pink	5.199	4	5
King Edward	5.296	5	7
Maris Piper	5.378	7	8
Saturna	5.757	4	4
Uncommon	5.530	-	-
2009-10			
King Edward	4.300	5	7
Maris Piper	4.913†	7	8
Desiree	5.309	5	5
Cultra	NC	-	-

Not calculated because too few samples. † Based on three samples

TABLE 5. THE EFFECT OF CULTIVAR ON THE RELATIONSHIP BETWEEN PATHOGEN DNA AND SKIN SPOT INCIDENCE. VALUES ARE ASYMPTOTE OF THE EXPONENTIAL MODEL CALCULATED USING PARALLEL REGRESSIONS.

Despite differences between cultivars, when testing the 2009-10 tubers, four samples of cultivars Cultra and King Edward showed a poor relationship between DNA levels in peel at harvest and the amount of skin spot that developed after a 20-week storage period. The possibility that this was due to failed extractions was discounted because the plant DNA internal controls were acceptable in all cases. It was noticeable when examining tubers after storage that much of the skin spot pustules were present around the eyes and stolons. It is possible that pathogen inoculum was not adequately sampled where the peel is difficult to reach around the eyes and stolon. Also, when sampling for PCR testing, only a strip of approximately 20mm around the circumference of each tuber is taken. It is possible that this sampling strategy misses areas affected with skin spot. It is recommended that future sampling should involve the removal and testing of peel from the entire surface of the tuber, or as a minimum include cores taken from around the stolon and eyes.

Over the two years trials work reported here, 88 out of 92 samples gave a reasonable correlation between pathogen DNA and skin spot incidence. These data support the use of a quantitative PCR test in predicting which stocks are at risk of skin spot. However, factors such as cultivar and crop duration (or harvest date) also need to be considered. It is suggested that for samples tested at or around harvest, *P. pustulans* DNA levels of $<10^3$ pg DNA/g peel represents low skin spot risk; 10^3 to 10^4 pg DNA/g peel represents moderate skin spot risk; and $> 10^4$ pg DNA/g peel represents high skin spot risk.

1.6. Conclusions

- Over two years of trials involving the testing of 106 sets of tuber samples, there was a good relationship between *P. pustulans* DNA levels in tuber peel at harvest and skin spot incidence on tubers after storage. This relationship promises to enable skin spot risk assessment and management strategies to be made prior to storage.
- It is suggested that for samples tested at or around harvest, *P. pustulans* DNA levels of $<10^3$ pg DNA/g peel represents low skin spot risk; 10^3 to 10^4 pg DNA/g peel represents moderate skin spot risk; and $> 10^4$ pg DNA/g peel represents high skin spot risk.
- There was broad agreement between results presented in this report and those reported in a previous PCL-funded project (R294) for samples collected at or around harvest
- The discrimination between low and high risk samples, in terms of skin spot development, was good in progeny tubers collected at harvest. However, this disagrees with the findings of R294 where better discrimination was achieved when testing tubers at the end of a 20-week storage period. A likely reason for this is that tubers investigated in R294 developed higher skin spot severity than was found on tubers in this report.
- This work indicates that crop duration is a significant component of the disease epidemiology. This, as a factor, has been under-investigated.
- Future sampling should involve the removal and testing of peel from the entire surface of the tuber, or as a minimum include cores taken from around the stolon and eyes.
- It is recommended that further work is done to refine the relationship between pathogen DNA levels in peel and skin spot levels by considering factors such as cultivar, crop duration and geographical location of crop.

1.7. Project deliverables

Building on the delivery of conventional and real-time assays for the detection of *P. pustulans* DNA, a set of thresholds have been developed to enable the assessment of a crop for risk from skin spot. The real-time assay has the potential to enable the early prediction of skin spot levels during storage.

1.8. References

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2. WORK UNDERTAKEN BETWEEN JUNE 2007 AND JUNE 2008

2.1. Summary

A real-time molecular assay for the detection and quantification of *P. pustulans* DNA was developed in a previous Potato Council funded project (R285). The aim of the project reported here was to establish whether this assay can detect and quantify *P. pustulans* in tuber peel before symptoms develop. The real-time assay was shown to enable the early prediction of skin spot levels during storage. This has huge benefit to the industry as it would provide an early warning of skin spot risk at the time of harvest/store loading before any symptoms are visible.

The main conclusions are:

- There was a good relationship between *P. pustulans* DNA levels in tuber peel, and skin spot incidence on tubers after storage. This relationship was found in samples collected from seed prior to planting, progeny at harvest, and on progeny collected three and twenty weeks after harvest.
- The discrimination between low and high risk samples, in terms of skin spot development, was better in progeny tubers collected at the end of storage or when testing seed prior to planting.
- The accuracy of the assay in predicting the risk of skin spot developing on tubers (including the risk of seed transmitting the skin spot pathogen to progeny crop) needs to be validated. Few tuber samples were available during 2007/08 with sufficiently high levels of skin spot to accurately model the relationship between latent infections (measured by DNA levels in peel) and development of skin spot during storage across a wide range of incidence levels.

2.2. Introduction

The purpose of the work presented here was to validate real-time PCR assay (developed during R285) for the detection and quantification of *P. pustulans* and determine the ability of this novel assay to detect latent infections at harvest, three weeks after harvest and at the end of a c. 20-week storage period.

2.3. Material and methods

2.3.1. Field trial: production of tubers with different levels of skin spot inoculum

In March 2006, two stocks of seed with visible skin spot were treated with imazalil, imazalil plus TBZ, fludioxonil, and water (control) as part of Potato Council funded project R251 (Alternative seed treatments for controlling skin spot). The seed stocks were planted at Potatoes in Practice 2006 (PIP 2006), Auchincruive and the ADAS Terrington site in Lincolnshire. Seed for planting at PIP 2006 were sent to SAC, Aberdeen on 19 April 2006 prior to planting on 16 May 2006. Seed for planting at ADAS Terrington were warmed up to 7.5°C on 28 April 2006 and planted on 2 May 2006. These treatments provided seed with varying degrees of skin spot infection. Sub-samples of 50 seed tubers per plot were sent to CSL for DNA extraction and analysis.

Plots were harvested on 30 October 2006 and delivered immediately to SBEU where crop from each plot was weighed and material was prepared for storage. Before going into store, tubers were hand-graded to remove <45 mm and >85 mm sized tubers, and then weighed into labelled plastic trays (to approximately 5 kg per tray). Trays were loaded into 3-tonne experimental stores at 12.0°C within 12 hours of receipt. The temperature was immediately dropped by 0.5°C/day to holding temperature (2.5ºC and 4.0ºC). At the end of the 2006/07 storage season, eight stocks with varying levels of skin spot were assessed for visible signs of infection (table 6). The stocks were planted at ADAS Terrington on 26 April 2007. The experimental design consisted of a complete randomised block design with each treatment replicated three times. Plots were harvested on 10 October 2007 and placed in a 3 tonne experimental store at SBEU with minimal curing to encourage skin spot development. Upon loading, the store temperature was set to 12°C and crop temperature immediately dropped by 0.5°C/day until the holding temperature (c.4°C) is reached. Upon reaching holding temperature, the store RH was set to 95%.

Treatment label (+ relative skin spot level)	Mean skin spot incidence (%)
Stock 1 (inoculum x 16)	29.0
Stock 2 (inoculum x 8)	13.0
Stock 3 (inoculum x 4)	7.7
Stock 4 (inoculum x 2)	3.7
Stock 5 (inoculum x 1)	1.7
Stock 6 (inoculum x 0. 5)	0.7
Stock 7 (inoculum x 0.25)	0.3
Stock 8 (inoculum x 0)	0.0

TABLE 6. MEAN SKIN SPOT INCIDENCE, BASED ON VISUAL ASSESSMENTS ON SEED PRIOR TO PLANTING IN APRIL 2007, AND DESIGNATED TREATMENT LABELS WITH NOTIONAL RELATIVE SKIN SPOT LEVELS.

2.3.2. Sample preparation and assessments

The harvested progeny were delivered immediately to SBEU and placed in labelled plastic trays (to approximately 5 kg per tray). Sub-samples of 50 tubers from each plot were immediately sent to CSL for DNA extraction and analysis. Further samples were removed from store and delivered to CSL for DNA analysis 3-weeks after harvest and 20-weeks after harvest (i.e. at end of storage).

2.3.3. DNA extraction

DNA was extracted at CSL 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 1.0 ml TE buffer 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.

2.3.4. PCR quantification

All real-time PCR reactions were performed in 96-well reaction plates using TaqMan Universal PCR MasterMix (Applied Biosystems). For each reaction, 1 μl DNA (which had been diluted 1/5 for tubers with TE buffer) was added to 24 μl of mastermix in the appropriate well. Assays were performed using an Applied Biosystems 7900HT Sequence Detector system. The PCR mix contained 200 μM dNTP mix, 0.3 μM primers, 0.1 μM fluorogenic probe and 0.125 U AmpliTaq Gold DNA polymerase in a 25 μl reaction volume. Reaction conditions were an initial 95°C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. These conditions are generic to other Potato Council-funded disease diagnostic assays. An internal PCR control assay used existing TaqMan primers and probe to amplify a fragment of the potato cytochrome oxidase (COX) gene. This provides a check that the quality of DNA following the extraction process is suitable for analysis.

2.3.5. Development of conventional PCR primers to P. pustulans

The 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 min 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.

2.3.6. 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 PP03PCRF1 and PP03PCRR1 were selected to amplify a *P. pustulans* specific fragment within the 18S ribosomal RNA (AF087480) using Primer Express software (Applied Biosystems, Branchburg, New Jersey, USA).

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 sequences were checked for specificity against all published sequences on the EMBL database using the blast search algorithm.

2.4. Results

2.4.1. The correlation between skin spot incidence on seed and progeny tubers

There was a good relationship between the amount of visual skin spot symptoms on seed prior to planting and levels of skin spot on progeny tubers after storage ($P \leq 0.001$, $R^2 = 0.61$, Figure 6). The range of incidence levels of skin spot varied from 0 to 59% on a per plot basis. However, the mean surface area affected by skin spot pustules remained low throughout, at around 1 to 2% tuber surface area.

The relationship between skin spot incidence on progeny tubers and on seed was close to 1 (the slope was 1.1, Figure 6).

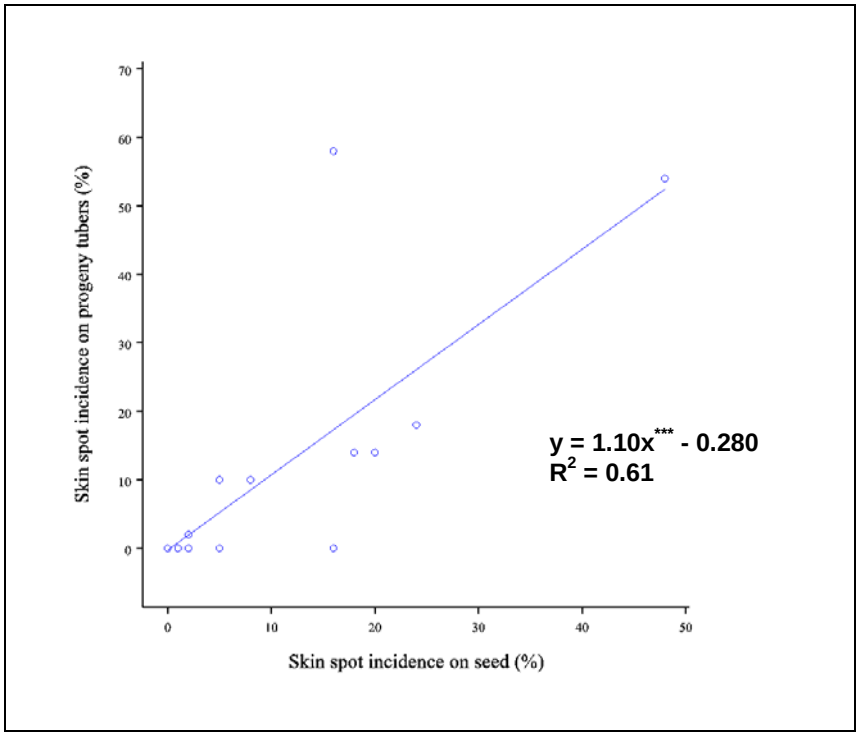


FIGURE 6. SEASON 2008/09. THE RELATIONSHIP BETWEEN SKIN SPOT INCIDENCE ON SEED AT PLANTING AND PROGENY TUBERS AT HARVEST. THE SIGNIFICANCE OF THE SLOPE IS DENOTED BY ***, P ≤ 0.001.

2.4.2. PCR quantification: the relationship between DNA levels in tuber peel and skin spot development

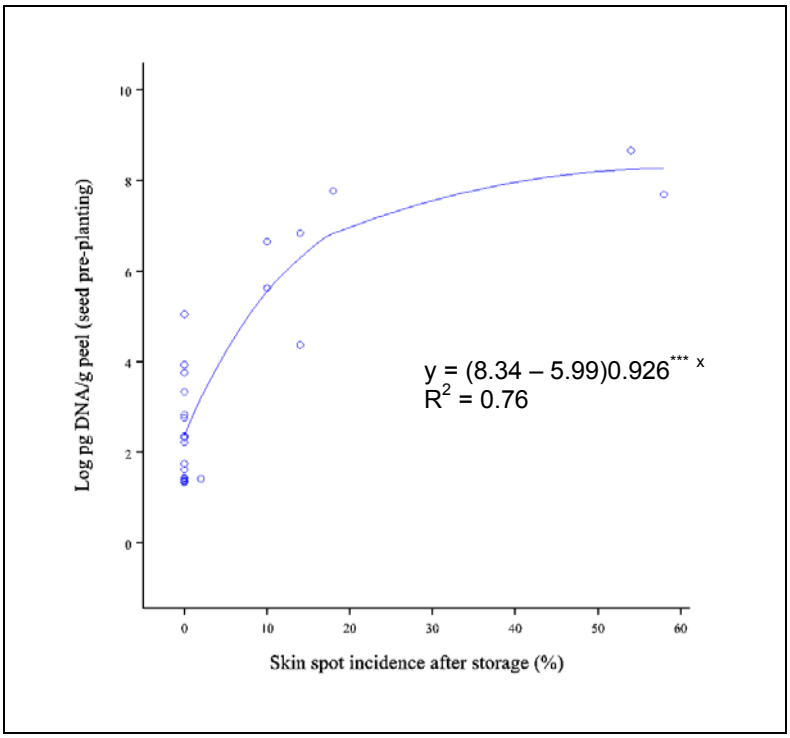
At all four sampling occasions (seed, pre-planting; progeny at harvest; progeny at harvest plus 3 weeks; and progeny after storage) the amount of detectable pathogen DNA increased with increasing incidence of skin spot detected at the end of a 20-week storage period (P<0.001, Figs 7 to 10).

The relationship between pathogen DNA levels and skin spot incidence was consistent with an exponential or 'asymptotic regression' model (Equation 1).

$$y = \alpha + \beta r^{x} \quad (1)$$

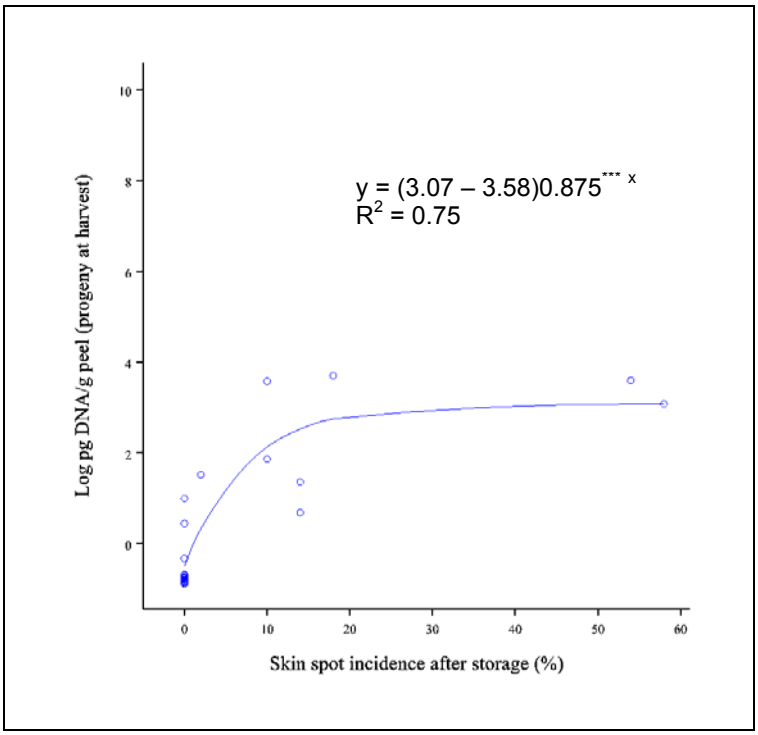
Where α, is the upper asymptote; β, is the range of curve between the value x=0 and the asymptote; and r, is the rate of exponential increase.

The goodness of fit for the exponential regressions, R^2, was reasonable, ranging from 0.64 to 0.78. The asymptotes were higher in tubers sampled at the end of storage (i.e. on seed at pre-planting and progeny tubers after storage).



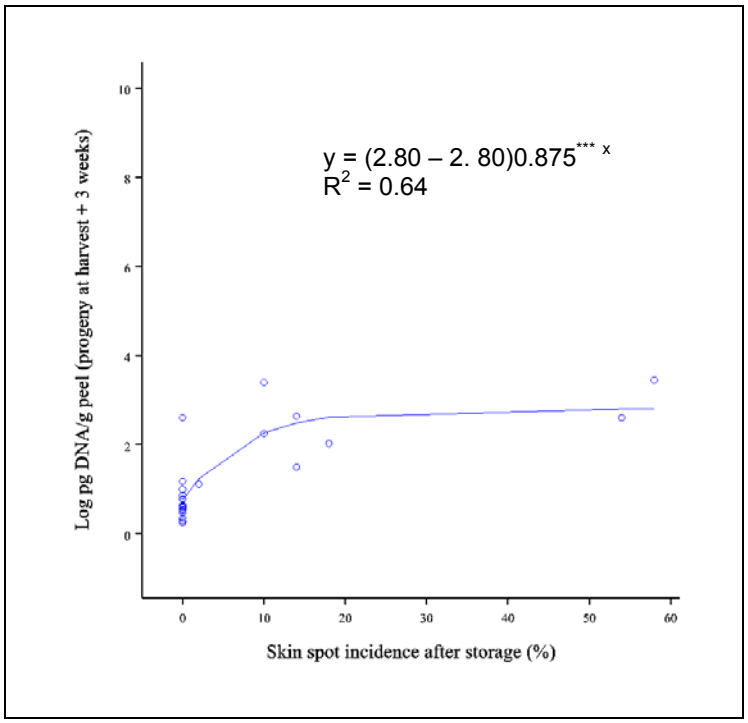
The significance of the rate of exponential increase (r) is denoted by ***, P ≤ 0.001.

FIGURE 7. SEASON 2008/09. THE RELATIONSHIP BETWEEN *POLYSCYTALUM PUSTULANS* DNA LEVEL (PG DNA/G PEEL) DETECTED ON SEED TUBERS PRIOR TO PLANTING AND SKIN SPOT INCIDENCE ON PROGENY TUBERS AFTER STORAGE.



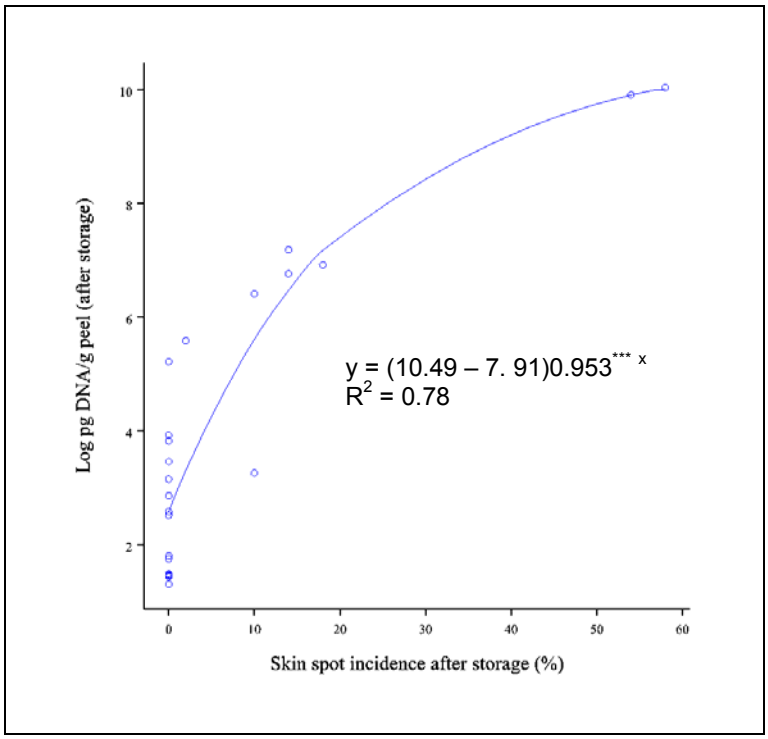
The significance of the rate of exponential increase (r) is denoted by ***, P ≤ 0.001.

FIGURE 8. SEASON 2008/09. THE RELATIONSHIP BETWEEN *POLYSCYTALUM PUSTULANS* DNA LEVEL (PG DNA/G PEEL) DETECTED ON PROGENY TUBERS AT HARVEST AND SKIN SPOT INCIDENCE ON PROGENY TUBERS AFTER STORAGE.



The significance of the rate of exponential increase (r) is denoted by ***, P ≤ 0.001.

FIGURE 9. SEASON 2008/09. THE RELATIONSHIP BETWEEN *POLYSCYTALUM PUSTULANS* DNA LEVEL (PG DNA/G PEEL) DETECTED ON PROGENY TUBERS AT HARVEST PLUS 3 WEEKS AND SKIN SPOT INCIDENCE ON PROGENY TUBERS AFTER STORAGE.



The significance of the rate of exponential increase (r) is denoted by ***, P ≤ 0.001.

FIGURE 10. SEASON 2008/09. THE RELATIONSHIP BETWEEN *POLYSCYTALUM PUSTULANS* DNA LEVEL (PG DNA/G PEEL) DETECTED ON PROGENY TUBERS AFTER STORAGE AND SKIN SPOT INCIDENCE ON PROGENY TUBERS AFTER STORAGE.

2.4.3. Validation of conventional PCR primers

A single PCR product of 556 bp was obtained from the two test isolates of *P. pustulans*. A search of the EMBL database using the blastn algorithm revealed the most closely related sequences were from *Cadophora* and *Rhexocercosporidium* spp.. DNA extracts from *Cadophora* sp. did not produce a PCR product when tested using the *P. pustulans* primer assay. In addition, extracts from *Helminthosporium solani* and *Colletotrichum coccodes* isolates also tested negative with the assay. No *Rhexocercosporidium* spp. cultures were available during this reporting period. However, *Rhexocercosporidium,* a common soil inhabitant, is not thought to cause disease of potato.

At SBEU, tubers with symptoms of skin spot produced a single product of 556 bp when tested using the PCR primers. Tubers with no skin spot symptoms did not produce a product when tested with the skin spot PCR assay.

2.5. Discussion

The amount of *P. pustulans* DNA detected in tuber peel increased with increasing risk of skin spot development. The relationship between DNA levels in samples processed from seed before planting through to progeny tubers at the end of the subsequent storage period, and skin spot incidence on progeny tubers at the end of storage, was best described by a negative exponential model or 'asymptotic regression'. However, care is needed in interpreting the data as the extreme high values displayed a high influence on the model. Further data, particularly at the middle to high range of skin spot incidences, would help to validate the relationship between pathogen DNA and disease development.

Timing of sample collection influenced the relationship between levels of pathogen DNA and skin spot incidence after storage. The upper asymptote of the exponential curves was the same for tubers sampled at harvest and for those sampled three weeks later. However, the asymptote of the curve was considerably higher for samples collected 20 weeks after harvest than for those collected close to harvest. There was only an approximate log 3 difference in DNA (i.e. β) between low- and high risk samples when tubers were collected within three weeks of harvest whereas there was an approximate log 8 difference in DNA levels between low- and high risk samples when tubers were collected 20 weeks after harvest. This suggests that the predictive nature of the assay is best done as close to the point of assessment as possible (i.e. at end of storage). However, this renders the test unsuitable as a predictor of skin spot risk. An alternative, given that the relationship between disease symptoms on seed and progeny were close to unity, is that DNA levels on seed are used to predict risk of disease developing on progeny tubers. However, this relationship has only been investigated over one year. It is imperative that another seasons' data is investigated to test if this relationship is subject to seasonal variation.

2.6. Conclusions

- There was a good relationship between *P. pustulans* DNA levels in tuber peel and skin spot incidence on tubers after storage. This relationship was found in samples collected from seed prior to planting, progeny at harvest, and on progeny collected three and twenty weeks after harvest.
- The discrimination between low and high risk samples, in terms of skin spot development, was better in progeny tubers collected at the end of storage or when testing seed prior to planting.
- The accuracy of the real-time PCR assay in predicting the risk of skin spot developing on tubers (including the risk of seed transmitting the skin spot pathogen to progeny crop) needs to be validated. Few tuber samples were available during 2007/08 with sufficiently high levels of skin spot to accurately model the relationship between latent infections (measured by DNA levels in peel) and development of skin spot during storage across a wide range of incidence levels.
- In addition to the quantitative real-time PCR assay, conventional PCR primers have been tested at CSL and SBEU and have been found to be useful in detecting *P. pustulans* in tuber peel.

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2.8. Project deliverables

Conventional and real-time assays for the detection of *P. pustulans* DNA have been developed and delivered.

The real-time assay has the potential to enable the early prediction of skin spot levels during storage. This has huge benefit to the industry as it would provide a quantification of risk of skin spot development at the time of harvest/store loading before any symptoms are visible.

However, the current data sets are inadequate to validate the assay with sufficient confidence for it to be used predictively. Nevertheless, if this research group were provided with enough seed samples with moderate to high levels of skin spot, this would provide the information needed to establish the correlation between pathogen DNA prior to planting, and in harvested progeny and skin spot levels after storage with reasonable confidence.