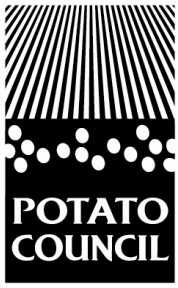

Final Report

Improving decision making for the management of potato diseases using real-time diagnostics

J Brierley & A Lees, *SCRI*
A Hilton & S Wale, *SAC*
J Peters, J Elphinstone & N Boonham, *CSL*

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1. Summary

The overall objective of this project was to improve decision making for the management of potato diseases by using predictive diagnostics, the deployment of relevant sampling techniques and knowledge of the epidemiology of pathogens of potato to establish disease risk assessment criteria. To achieve this, three scientific institutions: Scottish Crop Research Institute, Central Science Laboratory and SAC Aberdeen have been working in partnership with the potato industry (Greenvale AP, Higgins Agriculture Ltd and MBM Produce).

Standardised assays for the detection and quantification of powdery scab, black scurf, black dot and PMTV in both soil and tubers and *Erwinia* in tubers have been developed based on real-time (TaqMan) PCR techniques, and robust soil and seed sampling strategies formulated.

Pathogen inoculum in soil and on seed has been related to levels of progeny disease in three years of monitoring. Results show that for:-

- Black dot: Seed inoculum can cause disease, but at relatively low levels compared to soil inoculum i.e. 20 % incidence compared to 100 %. The level of soil inoculum can be used to predict the risk of progeny disease. Disease risk categories have been set, based on the amount of soil inoculum detected (as determined with real-time PCR). These categories of disease risk can be used to inform growers of the agronomic factors they need to consider to reduce the incidence and severity of black dot on progeny tubers.

- Powdery scab: Both seed and soil-borne inoculum can cause disease. However, it is not the level of inoculum that is important in determining the extent of disease but rather environmental conditions. If any seed or soil inoculum is detected then there is a risk of disease given suitable conditions for its development. In wetter summers when powdery scab is more prevalent compared to drier years, higher levels of soil inoculum do increase the risk of disease occurring.

- Black scurf: Despite a great deal of effort to make the sampling protocols as robust and reliable as possible, we have indications that they have not been adequate in providing a consistent estimate of soil-borne inoculum for *R. solani*. The occurrence of disease when mini-tubers were grown in soil where no inoculum was detected highlights the problem. A new strategy for soil sampling needs to be developed which reflects the patchy and perhaps fluctuating occurrence of *R. solani* mycelium in fields.

- PMTV: There was a good correlation between levels of soil-borne PMTV inoculum, as measured by PCR analysis on tomato bait plant roots, and detection of PMTV in progeny tubers. This correlation was improved by considering the results from both soil- and seed PCR tests. However, there was poor correlation between the detection of seed-borne PMTV and detection of virus in progeny tubers.

- *Erwinia* spp. (*Pectobacterium*): Real-time PCR analysis allowed high throughput detection, quantification and identification of *Pectobacterium* and *Dickeya* populations on potato tuber stocks. Although the incidence of infection (% stocks infected) was high, the average inoculum load of *Pectobacterium atrosepticum* (*Erwinia carotovora* subsp. *atroseptica*) on seed from each stock was generally low in all 3 years studied.

Blackleg incidence was correspondingly low in 2005 and 2006 but was higher in 2007 in response to wetter spring and early summer weather. Further data from a wider variety of seed stocks with a higher range of inoculum levels is needed before the accuracy of blackleg prediction under varying climatic conditions can be assessed. Some correlation was observed between *Pectobacterium atrosepticum* levels on seed and on progeny tubers in two out of three years. No correlation was found in 2007 when bacterial numbers probably increased rapidly under wetter conditions irrespective of the initial inoculum level on the seed.

2. Experimental Section

2.1 Introduction

The requirement of the potato industry for high quality healthy seed and ware is well known. Currently, few scientifically valid testing procedures are available to determine seed health; most fungal disease assessments are based on visual examination. Although diagnostic tests have been developed to detect and quantify a wide range of seed-borne and soil-borne pathogens, these tests have not been taken up by industry for a variety of reasons including difficulties in the interpretation of data. These difficulties in interpretation exist because extensive validation of the tests in relation to the quantitative assessment of disease had not been carried out.

The overall aim of this project was to improve decision making for the management of potato diseases by using predictive diagnostics, the deployment of relevant sampling techniques and knowledge of the epidemiology of pathogens of potato to establish disease risk assessment criteria. To achieve this, three scientific institutions: Scottish Crop Research Institute (SCRI), Central Science Laboratory (CSL) and Scottish Agricultural College (SAC) have been working in partnership with the potato industry (Greenvale AP, Higgins Agriculture Ltd. and MBM Produce).
The work focussed on developing, validating and providing practical risk assessment techniques for black dot (*Colletotrichum coccodes*), black scurf (*Rhizoctonia solani*), powdery scab (*Spongospora subterranea*) and Potato Mop Top Virus (PMTV) in field soils and seed tubers, and for *Erwinia* ssp. on seed tubers. All assays were quantitative and based on real-time (Taqman) PCR, providing a consistent technology base for the future and ensuring that the diagnostic needs of the GB potato industry can be met.

The objectives of this project were to:-

1. Validate bait plant assays and potato pathogen diagnostic assays, based on real-time PCR, ensuring that they are sensitive, specific, operate under standard experimental conditions, are practical and robust and may be used in combination with each other (multiplexed) and can be transferred between laboratories.

2. Investigate methods to improve soil DNA extraction, using increased mixing and grinding of samples and reduction of inhibitory compounds. Soil assays will focus on *C. coccodes, R. solani*, *S. subterranea* and PMTV for risk assessment purposes.

3. To evaluate direct soil extraction and soil baiting methods for the detection of *C. coccodes, R. solani* and *S. subterranea* from soil samples.

4. To evaluate soil bait plant methods for the detection of PMTV, incorporating testing of the plants with the currently available TaqMan assays for PMTV.

5. To validate tuber and soil tests with industry input through the transfer of methodologies to industry and the supply of samples and information from industry to the scientific partners.

6. To develop structured tuber and soil-sampling strategies, based on existing strategies, that will allow individual samples to be tested for a range of pathogens and will ensure the best possible chance of detecting a pathogen if it is present in that sample.

7. Determine the value of diagnostic data in predictive risk assessment of disease development in comparison with actual disease levels that develop in field and store.

8. To produce a risk assessment of disease occurrence through a combination of the detection of a particular pathogen, the environmental and cultural factors and possible control measures, and to give advice on disease risk and prevention.

9. To transfer risk assessment advice to industry.

2.2 Material and methods

Soil and tuber sampling

A review of existing sampling strategies for soil and tubers was carried out (see Appendix 1). On the basis of this review simple sampling guidelines for seed and soil were produced which were used throughout this project.

Sampling tubers from boxes in store

The principle is to sample sufficient representative tubers from as many boxes as possible. When potatoes are stored in boxes access is usually only available to a proportion of the boxes. Access to tubers in the top boxes is easier but sampling from the top only will bias the results (it is where moisture rising from tubers below condenses). Thus samples need to be taken from boxes at all levels of stacking.

Identify the boxes to be sampled (see Figure 1):

Identify where the stock to be sampled is stored. Identify the boxes in the stock which are accessible and from which tubers can be sampled. From these, identify an equal number of boxes at each level of stacking that can be sampled. Divide the number of tubers required (100) by the total number of boxes to be sampled, this will identify how many tubers need to be drawn from each box.

e.g. 10 boxes = 10 tubers from each of 10 boxes = 100
20 boxes = 5 tubers from each of 20 boxes = 100

Stock size: One sample of tubers should be taken for every 25 tonnes of stock.

Taking samples: Only seed sized tubers should be sampled. Tubers should be taken from just under the surface layer. Surface tubers will therefore need to be moved to one side at a number of locations in each box. Care is needed to avoid handling rotting tubers or sampling potatoes adjacent to rots. Once the location for sampling has been determined, as far as possible, the tubers sampled should be taken without bias. It is very important to remember that if a rotted tuber is touched, hands or gloves should be washed before sampling further tubers to avoid cross contamination.

Number of tubers: 100 tubers per stock should be sampled from as many boxes as possible.

Storage and Dispatch: Sampled tubers should be placed in a clean, unused paper sack.
Each bag should be labelled with:

Variety
Stock identifier
Store location
Name of sampler
Date of sampling
Samplers code (if applicable)

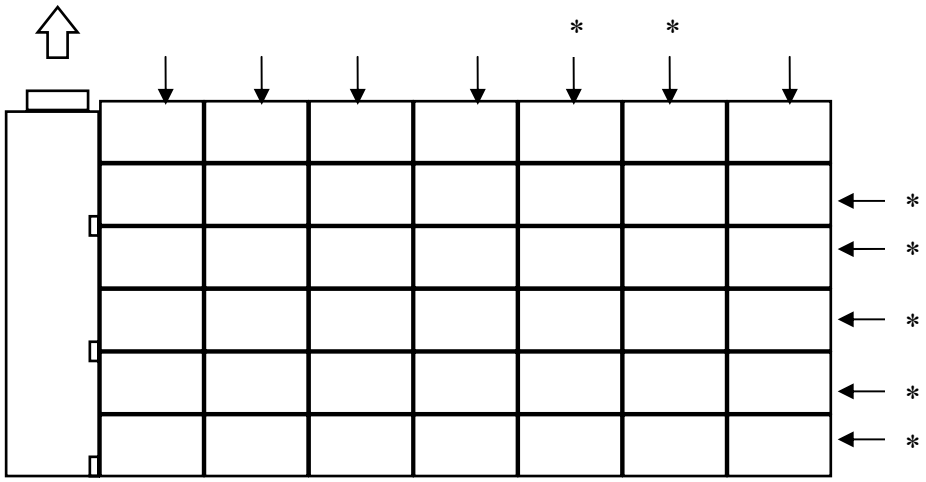


FIGURE 1. ILLUSTRATION INDICATING WHICH BOXES FROM A SINGLE ROW PLACED AGAINST A VENTILATION PLENUM CAN BE SAMPLED FROM. THOSE WITH STARS INDICATE APPROPRIATE BOXES FOR SAMPLING.

Sampling soil for detection of soil-borne potato pathogens

The principle is to sample soil from as many points as practical. There are no specific guidelines or information on distribution that help to determine the most efficient sampling regime for soil-borne potato pathogens. The data that is available has been examined, and it is concluded that clumping and distribution of soil-borne potato diseases are likely to be similar to that of potato cyst nematode (PCN). Thus, it is concluded that sampling for soil-borne pathogens should follow the same procedure as for PCN.

Sampling area: 4ha (10ac) or less. This may mean that fields greater than 4ha in size will need to be divided.

Sampling tool: Soil will be sampled with a mini-auger as used for PCN sampling.

Sample size: For standard testing a total soil sample of 1 kg will be required.

Sampling points: As the mini-augur for PCN samples about 10g each time, samples need to be taken from 100 points. If more than 1 kg is required, two samples should be taken from each sampling point.

Sampling pattern: After consideration of a wide range of patterns, it is concluded that sampling should take the form of a W pattern. In a square 4ha field with boundaries 200m long, approximately 500m will be walked. Thus samples should be taken approximately every 25m. The distance between sampling points will need to be adjusted according to field shape and size (if under 4ha).

Sampling bag: Samples should be placed in a strong plastic bag, sealed to prevent soil leakage and labelled with:
Field name
Section or part of field (if applicable)
Name of sampler
Date of sampling
Samplers code (if applicable)

Monitoring Programme

In each of three years (2005 to 2007), each of the industry partners (Greenvale, Higgins and MBM Produce) identified 15 stocks to be monitored. Within the fields to be planted with these stocks, the industry partners identified a 4 ha (10 acre) area in which soil samples and harvested progeny tuber samples would be taken and records of field disease made. Prior to planting, a 1 kg soil sample was taken by industry partners from the appropriate field area and sent to SCRI. The soil was air dried before being thoroughly mixed. SCRI kept a sub-sample of soil (c.100 g) to determine *C. coccodes*, *R. solani* and *S. subterranea* contamination, and supplied CSL with a sub-sample of soil (c. 600 g) to enable them to assess PMTV contamination, and in 2006 and 2007 SAC were supplied with a sub-sample (c. 300 g) to be tested for *R. solani* contamination using a bioassay.

For each of the seed tuber stocks identified by the industry partners to be monitored, a 100 tuber sample was sent to SAC for a visual disease assessment. In addition, a 100 tuber sample was sent to CSL. CSL processed tuber samples as described below, retaining an aliquot and supplying SCRI with an aliquot of Nucleic acid (NA).

SCRI carried out real-time PCR assessments of *C. coccodes, R. solani* and *S. subterranea* as described below. CSL carried out real-time PCR assessments of PMTV and *S. subterranea* as described below, but in addition, in 2006 and 2007 they also assessed the seed tuber NA for TRV contamination (an assay routinely used at CSL) and the tubers were assessed for spraing. The assessment of tuber stocks for *Erwinia* spp. contamination used the assays described below.

Crops were monitored for disease during the growing season, and records made of blackleg, stem canker and black dot development. This was done at 3 weeks after emergence (stem canker and blackleg), flowering (blackleg) and 1-2 weeks after haulm destruction (black dot).

At harvest, industry partners randomly sampled 100 tubers across the field to send to SAC for visual disease assessment, and a further 100 tubers were sent to CSL for the co-extraction of DNA and RNA as described below. As for the seed stocks, an aliquot of the NA was supplied to SCRI for real-time PCR assessment of *C. coccodes, R. solani* and *S. subterranea,* whilst CSL retained an aliquot to carry out real-time PCR assessments of PMTV, TRV and *Erwinia* spp. and assessed tubers for spraing.

Diagnostic assays

Extraction of soil DNA

A refined method for the direct extraction of soil DNA

The method of soil DNA extraction developed by Cullen et al. (2001) was adapted by substituting the initial processing procedures, sonification and bead beating of a 10g sub-sample with the fine milling of a 60 g sub-sample as described below.

Air dried soil samples (1 kg), sampled as described above, were placed in a large plastic bag and any lumps were flattened out with a roller, the bag was then inflated and rotated to mix the soil. A single 60g sub-sample was taken. Soil sub-samples were placed in a Retsch milling bowl (Planetary Ball Mill PM 400) with 120 ml extraction buffer (SPCB: 120 mM sodium phosphate, 2% CTAB (hexadecyltrimethyl-ammonium bromide), 1.5 M NaCl; pH 8.0) and 12 stainless steel ball bearings and milled at 300 rpm for 5 min until a fine soil suspension was created. Triplicate 1.5 ml aliquots of the soil suspension (equivalent to 0.75 g soil) were then transferred to 2 ml Eppendorf tubes and kept on ice until further processing. Milling bowls were cleaned with 96% Ethanol and 0.2 M NaOH between samples to prevent cross-contamination. The soil suspension was centrifuged (1820 g for 5 min) and the supernatant removed. The subsequent DNA extractions and purification through a Micro Bio-Spin column containing polyvinylpolypyrrolidone (PVPP; Sigma), followed the method of Cullen et al. (2001), except that the pelleted DNA was re-suspended in 100 µl TE buffer (10mM Tris-HCl and 1 mM EDTA, pH 8.0) rather than 75 µl and stored at -20°C prior to use.

Baiting method for detection of PMTV and *S. subterranea* in soil

Approximately 150 g of soil was placed into each of three replicate pots in a tray and 9 ten-day-old tomato seedlings were planted in each pot. The pots containing the seedlings were grown in the glasshouse (17-20ºC) and watered daily. At harvest the plants were carefully removed from each pot and the soil washed off the roots. For each pot a 200 -300 mg segment of root tissue was taken across all roots and placed into a labelled Stomacher bag. These were stored at –80^{o}C prior to RNA extraction.

Each Stomacher bag was placed in liquid nitrogen to freeze the root tissue, which was then quickly transferred to a 2 ml screw cap micro-centrifuge tube containing approx 700 mg of 2.4 mm Zirconia beads (Biospec). The lid was secured and the tube was shaken on a mini bead-beater at speed 4200 for 30 sec. Lysis buffer (1 ML) (Toyobo, Shinko Sangyo co. Japan) was added to the tube. The lysate was transferred to a new 1.5ml tube and spun at 10000 rpm for 3 min. RNA was extracted from the cleared lysate using a robotic magnetic particle processor (Kingfisher mL, Thermo Labsystems) in conjunction with a silica based total RNA extraction kit). The manufacturers' program 'Total_RNA_ML_1' was used. Briefly, in well 1 of the Kingfisher ML 5 tube strips, 800 µl of cleared lysate was mixed with a further 300 µl of the

same lysis buffer and with 50 µl of magnetic silica particles. The other wells were loaded as follows: well 2 – 1000 µl of wash buffer 1, well 3/4 – 1000 µl of wash buffer 2 and well 5 – 100 µl elution buffer. When the particles were deposited into the elution buffer in tube number 5, the 5 tube strips were placed into an oven at 65°C for five minutes to achieve effective elution, before being replaced into the Kingfisher ML to remove the magnetic beads. The resulting RNA in well 5 was transferred to a fresh 1.5 ml tube, diluted (1:10) into 900 µl of nuclease-free water.

Bioassay for *R. solani*

For the *R. solani* bioassay at SAC, the soil sub-sample was air- dried and passed through a 2mm sieve. Beetroot seed were autoclaved three times for 15 min at 121^{o}C prior to use to prevent contamination. Ten seeds were mixed, using a pair of forceps, with approx. 75g of soil in a 9-cm diameter Petri dish. There were four replicate plates for each soil sample. The Petri dish lid was sealed with parafilm and incubated in the dark at 25^{o}C. After 48 hours of incubation in soil at 25^{o}C, seeds from each plate were recovered, washed under running water in a sieve for approx. 1 min, blotted on a sterile filter paper and plated on to modified Ko & Hora (1971) selective medium. Each plate had 10 seeds and all plates were incubated at 25^{o}C. After 48 hours these plates were checked for the appearance of *R. solani* colonies. Plates were examined under a light microscope and assessed for characteristic mycelia. The percentage of the beet seeds considered to be colonised by *Rhizoctonia* hyphae per plate was determined.

Extraction of tuber DNA

A standard method suitable for bacterial, fungal and viral pathogens

In 2005, three separate extractions were carried out for fungal DNA, bacterial DNA and viral RNA. In 2006, a protocol suitable for the co-extraction of DNA and RNA (Nucleic acid – NA) from potato tissue to detect bacterial, fungal and viral targets was developed. In a pilot study, the method proved successful for the detection of *Erwinia* spp., *C. coccodes, S. subterranea*, *R. solani* and PMTV (the method was slightly less sensitive at detecting RNA targets than existing RNA specific extraction methods). Average COX Ct values were 20.9 ± 0.07 standard deviations for seed socks in 2006 and 20.8 ± 0.07 standard deviations in 2007. This indicates that the quality of DNA extracted from seed was excellent and very consistent. Less consistent COX results were obtained in 2005 prior to the refinement of the tuber DNA extraction method (27.4 ± 1.86 standard deviations).

For each stock sampled, 100 potato tubers were subdivided into 10 replicate sub-samples of 10 tubers and 10 separate NA extractions completed. For each NA extraction the tubers were carefully washed to remove soil and a strip of peel was removed from one side of the tuber (from rose end to the stolon-end) with cores from both the stolon- and rose-ends. The sample was placed into a Homex grinding bag and weighed prior to the addition of 15 ml of ice cold phosphate buffer (pH 7.0) containing tetrasodium pyrophosphate antioxidant. The sample was then pulverised to give an oat-meal consistency using a Bioreba grinder. The buffer was removed, centrifuged twice and the Nucleic acid (NA) extracted using a Promega Wizard Magnetic DNA purification for Food in combination with a Kingfisher 96 magnetic particle separator (Thermo Labsystems) following the manufacturer's instructions. The final NA extraction was eluted into 1000 µl of 1 x TE buffer and stored at -20°C prior to use.

Real-time PCR diagnostic assays

Fungal pathogens

The detection and quantification of *R. solani*, *C. coccodes* and *S. subterranea* using the real-time PCR (TaqMan) assays previously developed at SCRI were re-tested to confirm they worked well and in a standard format. The detection of *C.coccodes, R.solani* and *S.subterranea* from soil and tubers by quantitative real-time PCR was carried out according to the methods of Cullen *et al*. 2002, Lees *et al*., 2002 and van de Graaf *et al*., 2003 respectively. These assays have been used throughout this project.

All real-time PCR reactions were performed in 96-well reaction plates using TaqMan Universal PCR MasterMix (Applied Biosystems). For each reaction, 2 µl DNA which had been diluted (1/20 for soils and 1/2 for tubers with HPLC) was added to 23 µl of mastermix in the appropriate well. Forward and reverse primers for all assays were used at a concentration of 0.3 µM per 25 µl. Fluorogenic probes for all assays were used at a concentration of 0.1 µM per 25 µl reaction. Plates were cycled at generic RT PCR system conditions (95ºC for 10 min, and 40 cycles of 60ºC for 1 min plus 95ºC for 15 sec) within the 7700 Sequence Detection System (Applied Biosystems) using real-time data collection.

A range of standards containing known amounts of DNA were included in the real-time PCR assays. In the case of *S. subterranea* the DNA was extracted from a known number of spore balls and diluted. The standards were used to create a standard curve of the critical threshold (Ct) value against the logarithm of the amount of DNA (*C. coccodes* and *R. solani*) or spore balls (*S. subterranea*). Using the standard curves, the amount of DNA in each unknown sample was expressed as pg DNA/assay for *C. coccodes* and *R. solani* and as spore ball equivalents for *S. subterranea* on the basis of their Ct values. Non template controls with 2 µl HPLC instead of DNA were included in every assay and all samples were tested in duplicate and results averaged. For soil DNA assays, results were adjusted for the amount of soil from which the DNA was extracted from and expressed as pg DNA/ g soil (*C. coccodes* and *R. solani*) or spore balls/ g soil (*S. subterranea*). For tuber DNA assays an internal PCR control assay used existing TaqMan primers and probe to amplify a fragment of the potato cytochrome oxidase (COX) gene.

In 2005, the amount of tuber peel from which DNA extractions were made was not recorded, thus target pathogen levels are expressed as pg DNA/ assay. In 2006 and 2007 tuber peel weights were recorded allowing DNA levels to be standardised as pg DNA / g tuber peel. Standardising the data is the best practice, however, the amount of tuber peel within the extractions was not that variable, therefore, to enable comparisons across all three years the non-standardised data is referred to within the report.

PMTV

Prior to the verification of a PCR assay, the 2005 seed stocks were tested for the presence of PMTV by growing-on potato cores for one month, harvesting leaves and testing these by double antibody sandwich (DAS) ELISA. All subsequent testing for the detection of PMTV in seed, soil and progeny tubers was done using real-time PCR primers and probes (Mumford et al., 2000).

PCR assays were performed using either Applied Biosystems 7700 Sequence Detector or Cepheid Smartcycler systems. The PCR mix contained 200 µM d-ntp mix, 300 nM primers 10 nM 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. The whole assay was complete within 2 hours. An internal PCR control assay used existing TaqMan primers and probe to amplify a fragment of the potato cytochrome oxidase (COX) gene.

Spraing Assessments

In 2006 and 2007, seed stocks were assessed for the development of spraing symptoms using a method provided by SASA. Sacks of potatoes were stored at 18°C for 2 weeks and then transferred for a further week at 5°C. Tubers were then sliced three times and all 6 faces assessed for the presence of spraing using a disease key provided by SASA showing slight, moderate and severe disease symptoms. All 100 tubers were assessed in stocks testing positive for either PMTV or TRV. A reduced number of potatoes were assessed for the remaining stocks (20/100).

***Pectobacterium* spp. and *Dickeya* spp. (previously known as *Erwinia* ssp.)**

The soft rot forming bacteria have recently been reclassified, *Erwinia carotovora* subsp. *atroseptica* (Eca) is now known as *Pectobacterium atrosepticum. Erwinia carotovora* subsp. *carotovorum* (Ecc) is now known as *Pectobacterium carotovorum* subsp. *carotovorum. Erwinia chrysanthemi* (Ech) is now known as *Dickeya* spp. Throughout the duration of this project the assays for *P. atrosepticum*, *Dickeya* spp. and total pectolytic populations (*Pectobacterium* + *Dickeya* spp.) will still be referred to as Eca, Ech, and Pec respectively.

Problems were identified with the specificity of the primer and probes originally used in the project and a series of new assays were designed to amplify formate acetyltransferase sequences specific to *Pectobacterium atrosepticum* (De Boer and Ward, 1995). Specificity of primers and probes was initially selected after BLASTn searching for short nearly exact matches amongst the NCBI nucleotide database. The specificity of six selected assays were then investigated using combinations of 3 forward and 4 reverse primers and two probes. Each assay was tested against a panel of bacteria, including 69 isolates of *Pectobacterium* and *Dickeya* spp. and 37 isolates of other related *Enterobacteriaceae*, including a number of isolates from which target sequence was isolated using former Eca primers.

All of the six newly designed assays for *Pectobacterium atrosepticum* (Eca) showed improved specificity over the former assay. Expected target sequence was amplified from all isolates of *Pectobacterium atrosepticum*. No target was amplified from any isolates of *Pectobacterium carotovorum* subsp. *carotovorum*, *P. carotovorum* subsp. *odoriferum*, *P. betavasculorum* or *Dickeya* spp. Two of the six assays amplified expected target from the closely related isolates of *P. wasabiae*. No target sequence was amplified from 37 isolates of related *Enterobacteriaceae*, except in the case of one assay from a single isolate of *Erwinia* sp. One of the assays which demonstrated 100% specificity for *P. atrosepticum* was selected for routine use.

The selected primers and probe for the detection and quantification of *Pectobacterium atrosepticum* (Eca), total pectolytic bacteria (PEC), *Dickeya* spp. (Ech) and COX are listed below.

Primers	
ECA-CSL-1F	5' CGGCATCATAAAAACACGCC 3'
ECA-CSL-89R	5' CCTGTGTAATATCCGAAAGGTGG 3'
PEC-1F	5' GTG CAA GCG TTA ATC GGA ATG 3'
PEC-1R	5' CTC TAC AAG ACT CTA GCC TGT CAG TTT T 3'
ECH-1F	5' GAG TCA AAA GCG TCT TGC GAA 3'
ECH-1R	5' CCC TGT TAC CGC CGT GAA 3'
COX-F	5'- CGT CGC ATT CCA GAT TAT CCA -3'
COX-R	5'- CAA CTA CGG ATA TAT AAG AGC CAA AAC TG -3'
Primers	
ECA-CSL-36T	5' ACATTCAGGCTGATATTCCCCCTGCC 3'
PEC	5' CTG GGC GTA AAG CGC ACG CA 3'
ECH	5' CTG ACA AGT GAT GTC CCC TTC GTC TAG AGG 3'
COX-P	5'-[VIC]- TGC TTA CGC TGG ATG GAA TGC CCT -[TAMRA]-3'

Assays were performed using either Applied Biosystems 7700 Sequence Detector or Cepheid Smartcycler systems. The PCR mix contained 200 µM d-ntp mix, 300 nM primers 10 nM 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. The whole assay was complete within 2 hours. An internal PCR control assay used existing TaqMan primers and probe to amplify a fragment of the potato cytochrome oxidase (COX) gene.

In order to validate procedures for high throughput testing, samples from a large number of additional seed stocks were provided from Higgins Agriculture Ltd. Extracts from these samples were prepared on-site and transferred under refrigeration for DNA extraction and PCR analysis at CSL. In 2004, 303 samples from 2003 seed stocks were supplied by Higgins Agriculture and a further 474 samples from 2004 seed stocks were supplied in 2005. Additional samples of 2003 and 2004 harvested seed stocks were also submitted as tubers from MBM Produce for extraction and testing at CSL. Automated magnetic DNA extraction was performed using the Kingfisher 96 magnetic particle separator (Thermo Labsystems) as described above. Automated real time PCR was performed in 384-well plates using ABI7900 real-time PCR system with robotic liquid handling equipment. Additional stocks were not tested in 2006, as originally planned, since resources were diverted to development and validation of a new real-time PCR assay with improved specificity to *Pectobacterium atrosepticum*.

Trial scale plots of mini-tubers of both resistant and susceptible cultivars

To gain information on the effect of soil-borne inoculum and cultivar resistance on the development of black dot, powdery scab and black scurf, a number of small trials were planted in each of the three years, a total of 26 trials were carried out over three years. At sites where ware crops were planted, mini-tubers of three cultivars, Maris Piper, Saxon and Romano in 2005 and 2006, and Maris Piper, Saxon and Estima in 2007, were provided to Industry partners. In each of two drills 10 mini-tubers of each cultivar were planted and labelled with a marker. Plots were monitored by the commercial partners as for the monitoring crops. At

harvest, daughter tubers were lifted and sent to SAC where a visual disease assessment was performed on 25 tubers from each mini-plot. The results were related to the amount of soil-borne contamination as measured by real-time PCR in the soil sample taken from the total area i.e. that of the commercial crop in which the trial was located.

Data from black dot project field trials

An extension to the black dot project "Developing effective integrated control measures for the control of black dot" (R249) involved the planting of twenty field trials in both England and Scotland. The main objective was to evaluate black dot on progeny tubers at sites with varying levels of soil inoculum and soil types in varieties with a range of black dot resistance ratings. However at all of these sites, not only *C. coccodes* but also *S. subterranea* and *R. solani* soil inoculum levels were determined, and disease symptoms on progeny tubers recorded.

Details of these field trials can be found in the R249 final report. In this current report we describe the relationship between soil inoculum levels and disease in varieties with a range of resistance ratings as found at these nineteen field trial sites (one site was harvested prematurely before tubers could be harvested for disease assessments).

2.3 Results

In each of the next sections we have summarised the results obtained over the three years of monitoring and where relevant results from additional experiments such as mini-tuber trials and work carried out as part of the black dot extension project have been included. A summary of the detection and quantification of soil and seed inoculum levels are given, followed by how inoculum levels relate to disease in the progeny crops.

Black dot

Crop monitoring
Mini-tuber trials
Black dot extension field trial results

Powdery scab

Crop monitoring
Mini-tuber trials
Black dot extension field trial results

Rhizoctonia

Crop monitoring
Mini-tuber trials
Black dot extension field trial results

PMTV

Crop monitoring

Erwinia

Crop monitoring

Black dot

Quantification of *C. coccodes* in soil

Figure 2 illustrates the range of contamination levels found in commercial fields sampled in the three years of monitoring.

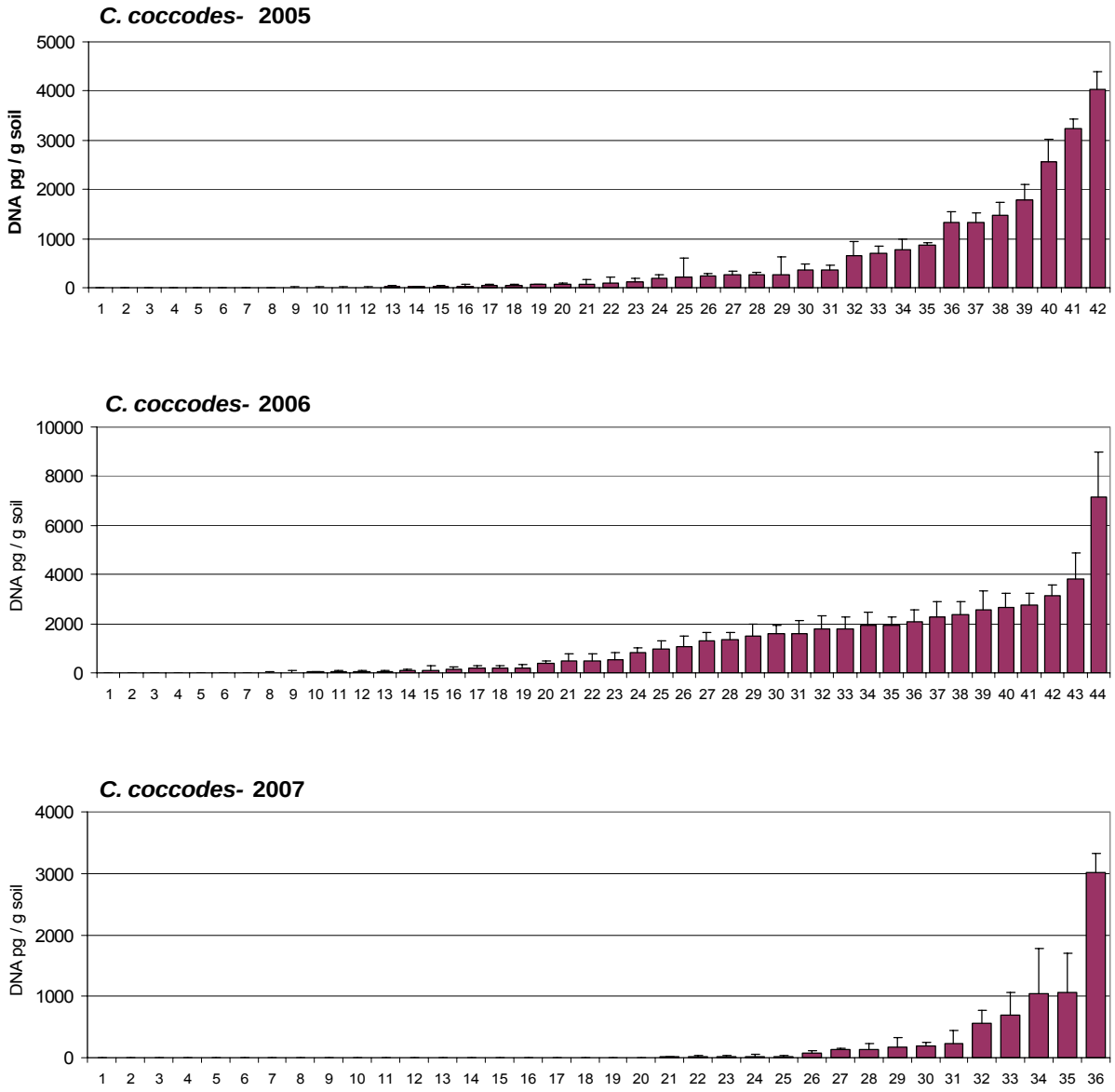


FIGURE 2 . QUANTIFICATION OF *C. COCCODES* DNA IN SOIL SAMPLES IN 2005, 2006 AND 2007. MEAN + ST. DEV OF THREE SUB-SAMPLES TAKEN AFTER MILLING.

Following direct extraction of DNA from soils target DNA was found at levels ranging from zero (un-detectable) to over 7100 pg DNA/ g soil for *C. coccodes* (Figure 2). A high percentage of soils (over 58 %) were contaminated with *C. coccodes* in all three years (Table 1). Degree of soil contamination was lower in 2007 than in the previous two years. This was due to an increased number of seed crops in 2007, as the emphasis of one of the companies switched from ware to seed production.

TABLE 1. THE NUMBER OF SOIL SAMPLES TESTED, THE NUMBER AND PERCENTAGE OF THE TOTAL THAT WERE FOUND TO HAVE DETECTABLE LEVELS OF *C. COCCODES* CONTAMINATION AND THE MAXIMUM LEVEL OF CONTAMINATION FOUND IN SOILS SAMPLED IN 2005, 2006 AND 2007.

Pathogen		Number of soils tested	Number of soils contaminated (percentage)	Max. contamination level (pg DNA/ g soil)
C. coccodes	2005	42	34 (81)	4000
	2006	44	37 (84)	7100
	2007	36	21 (58)	3020

Quantification of *C. coccodes* on seed

In all years, a very high proportion of seed stocks were contaminated with *C. coccodes* (over 75 %) (Table 2). In more than 19 % of the seed stocks tested each year, real-time PCR assays detected *C. coccodes* contamination on seed stocks that did not have visual symptoms of disease (Table 2), indicating the ability of real-time PCR to detect symptomless infection.

TABLE 2. THE NUMBER OF SEED STOCKS TESTED, THE NUMBER AND PERCENTAGE OF THE TOTAL WHICH WERE FOUND TO HAVE DETECTABLE LEVELS OF *C. COCCODES* CONTAMINATION, THE MAXIMUM LEVEL OF CONTAMINATION FOUND ON SEED STOCKS, AND THE NUMBER AND PERCENTAGE OF STOCKS WHICH HAD SYMPTOMLESS INFECTION.

Year	Number of stocks tested	Number of stocks contaminated (percentage)	Max. contamination level (pg DNA/ assay)	Number of stocks with symptomless infection (percentage)
2005	42	32 (76)	2927	8 (19)
2006	43	39 (91)	11309	9 (21)
2007	39	38 (97)	31150	14 (36)

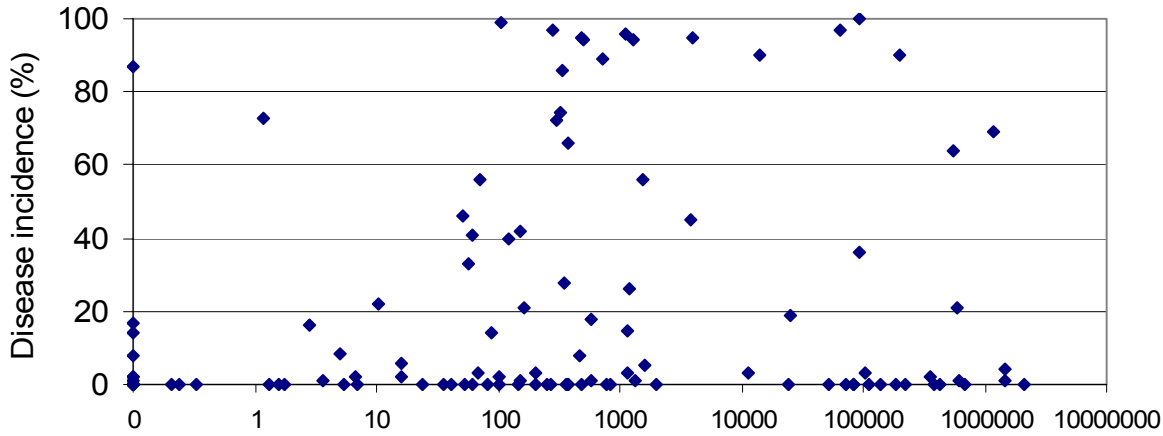
Relating inoculum to disease risk for black dot

Crop Monitoring

Seed inoculum

Overall, there was no relationship between the level of seed inoculum and disease incidence or severity on the progeny tubers as illustrated in Figure 3.

A. Disease incidence



B. Disease severity

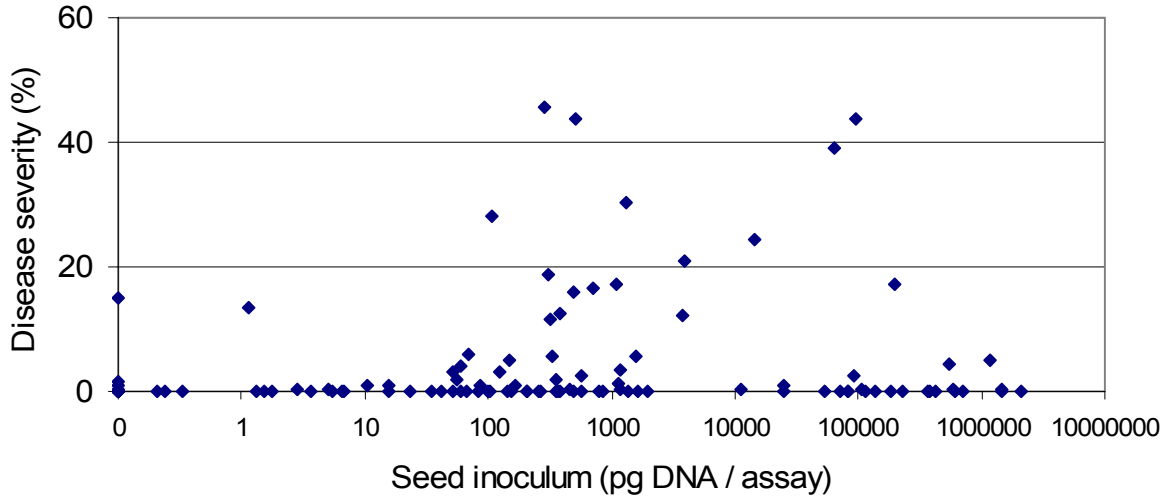
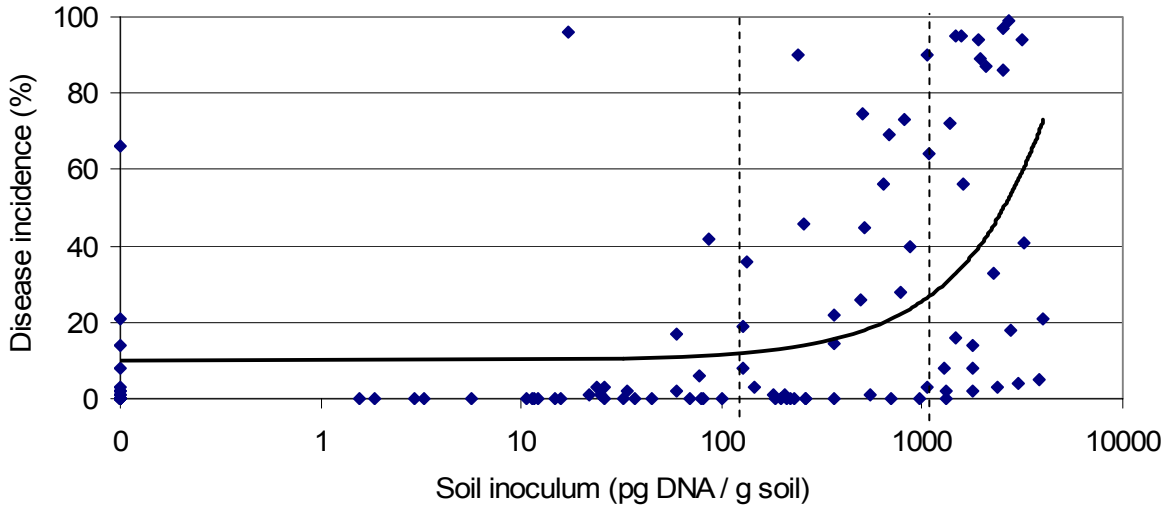


FIGURE 3. THE EFFECT OF SEED INOCULUM (PG DNA / ASSAY) ON A. THE INCIDENCE AND B. THE SEVERITY OF BLACK DOT IN PROGENY CROPS MONITORED IN 2005, 2006 AND 2007.

Soil inoculum

The effect of soil inoculum level (pg DNA / g soil) on the incidence, severity of disease and % unmarketable tubers in the progeny crop is quite marked. At levels of soil inoculum less than 100 pg DNA / g soil, very few crops have an incidence of black dot of more than 20 %. With increasing soil in inoculum the incidence of black dot (determined as the percentage of tubers with black dot symptoms) increases (Figure 4 A). The severity of black dot on progeny tubers, determined as the mean % surface area covered by black dot symptoms, also increased as the level of soil inoculum increased (Figure 4 B).

A. Disease incidence



B. Disease severity

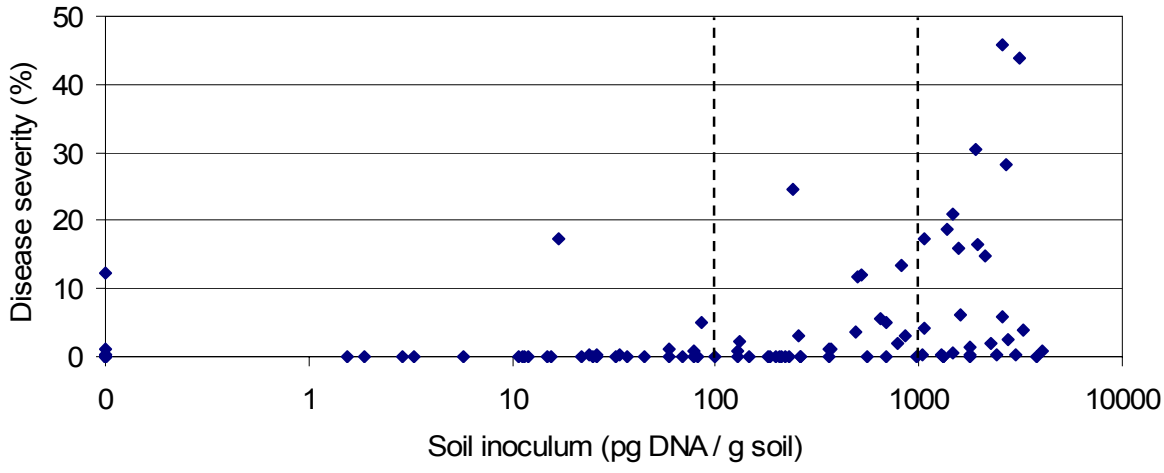


FIGURE 4. EFFECT OF INCREASING SOIL INOCULUM ON A. THE INCIDENCE AND B. THE SEVERITY OF BLACK DOT IN PROGENY CROPS MONITORED IN 2005, 2006 AND 2007.

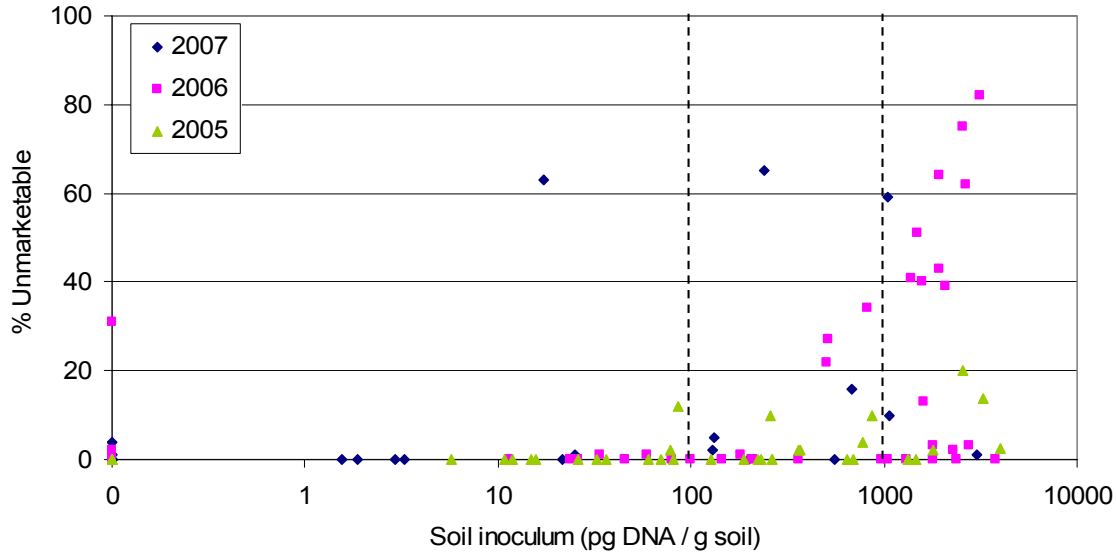


FIGURE 5. EFFECT OF INCREASING SOIL INOCULUM ON THE PERCENTAGE OF UNMARKETABLE TUBERS (> 10 % SEVERITY OF BLACK DOT) IN PROGENY CROPS MONITORED IN 2005, 2006 AND 2007.

With increasing levels of soil inoculum the risk of black dot increased, measured as incidence, severity and % unmarketable tubers (Figures 4 and 5). Figure 5 also illustrates seasonal variations in the extent of black dot development, for example, in general disease levels were higher in 2006 at a given soil inoculum level than in 2005 and 2007, although a few crops in 2007 had unaccountably high levels of disease. Threshold levels have been set which relate the level of soil inoculum to a category of disease risk (Table 4).

TABLE 4. DISEASE RISK CATEGORIES

Soil inoculum level (pg DNA / g soil)	Disease risk category
0	very low
<100	Low
100-1000	Medium
>1000	High

TABLE 5. THE PERCENTAGE OF CROPS WITH BLACK DOT AND THE PERCENTAGE OF CROPS WITH AN INCIDENCE OF BLACK DOT GREATER THAN 20% WHEN GROWN IN SOILS WITH DIFFERENT DISEASE RISK CATEGORIES.

Disease risk category pg DNA / g soil	v. low 0	low <100	medium 100-1000	high >1000
% crops with disease	33	36	63	96
% crops with disease incidence > 20 %	7	7	40	57
Number of crops in category	27	28	30	28

The percentage of crops with disease and with more than a 20 % incidence of disease increased with increasing disease risk category (Table 5). There was however, no difference between the very low and low disease risk categories.

Results from the black dot project (R 249) clearly demonstrated that seed inoculum could cause black dot symptoms on progeny tubers, but the incidence of disease caused by seed inoculum in the absence of soil inoculum was rarely above 20 %. In contrast, soil inoculum could cause disease up to 100 % incidence on the progeny tubers.

Results from monitoring of commercial crops in this study supports experimental evidence from the black dot project. Out of the 27 crops where no soil inoculum was detected, 23 had seed with detectable levels of *C. coccodes* contamination. Disease occurred in approximately a third of these crops (Table 5), however, the majority of these had less than 20% incidence of disease, and seed inoculum could therefore account for the disease found. Of the two crops which had more than 20 % incidence of disease, one had just 21 %, but the other had 66 %, the later was therefore unaccountably high. The soil sample from this site was retested, but no inoculum could be detected.

Within the monitored crops the level of disease at higher inoculum levels is very variable, i.e. at soil inoculum levels above 1000 pg DNA / g soil (high disease risk category), whilst 96 % of crops had black dot the incidence ranged from 2 % to 99 %. These crops represented a wide range of potato crops, consisting of, for example, varieties with different black dot resistance ratings and crops grown with a range of crop durations, irrigation and the use of Amistar.

Varietal resistance to black dot

Figure 6 shows the effect of soil inoculum on disease incidence in forty three crops for which the variety grown and the black dot resistance rating for that variety was known. Crops below the regression have less disease than the average, whilst crops above the regression line have more disease than average. The majority of crops above the regression line are susceptible crops with a resistance rating of 4 or 5, whilst the majority of those below the line are more resistant varieties with ratings of 6 or 7. Varietal resistance cannot explain all the variation as other factors already mentioned will influence the development of black dot.

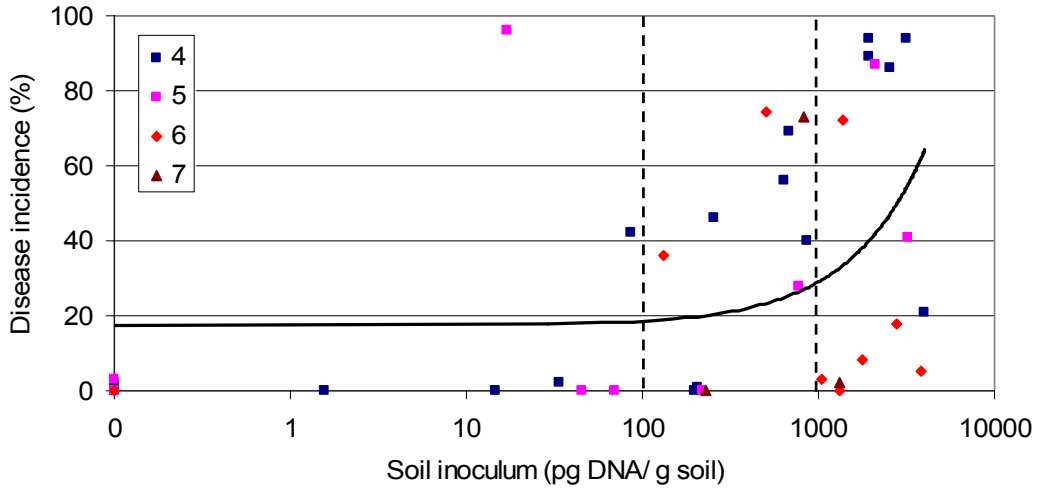


FIGURE 6. EFFECT OF INCREASING SOIL INOCULUM ON THE INCIDENCE OF BLACK DOT IN PROGENY CROPS OF VARIETIES WITH DIFFERENT RESISTANCE RATINGS (4, 5, 6 AND 7) IN MONITORED CROPS IN 2005, 2006 AND 2007. REGRESSION LINE FITTED.

Crop duration

In the black dot field trials carried out as part of the Potato Council-funded black dot project, increasing crop duration increased the incidence and severity of disease for an individual variety. In the monitoring exercise described here, where crop duration is less than 20 weeks the risk of black dot was low (Figure 6). However, when comparing crops with an extended crop duration (>25 weeks) the risk of black dot was not greater than crops with a moderate duration (20-25 weeks). Many of the longer duration crops grown were more resistant varieties such as Cara, King Edward and Saxon and hence the risk of black dot developing in these varieties is less than might be predicted from crop duration alone.

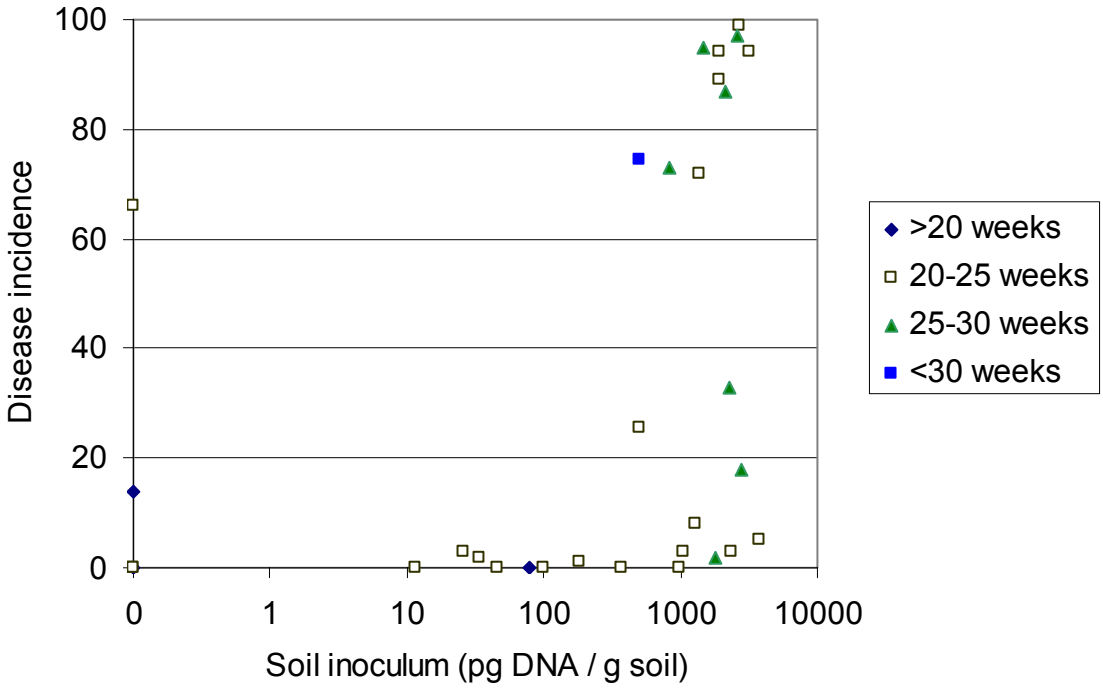


FIGURE 6. EFFECT OF INCREASING SOIL INOCULUM (PG DNA G SOIL) ON THE INCIDENCE OF BLACK DOT IN PROGENY CROPS WITH DIFFERENT CROP DURATIONS (WEEKS FROM PLANTING TO HARVEST) MONITORED CROPS IN 2005, 2006 AND 2007.

Mini-tuber trials

Where no soil inoculum was detected no disease developed in mini-tuber progeny (Figure 7). In both the low (< 100 pg DNA/ g soil) and medium (100-1000 pg DNA / g soil) risk soils only one crop of the susceptible variety Maris Piper (4) developed disease > 20 % incidence. In high risk soils (> 1000 pg DNA / g soil), relatively high levels of disease developed even in varieties with high resistance ratings, indicating that at very high inoculum levels resistance cannot be relied upon to reduce disease risk.

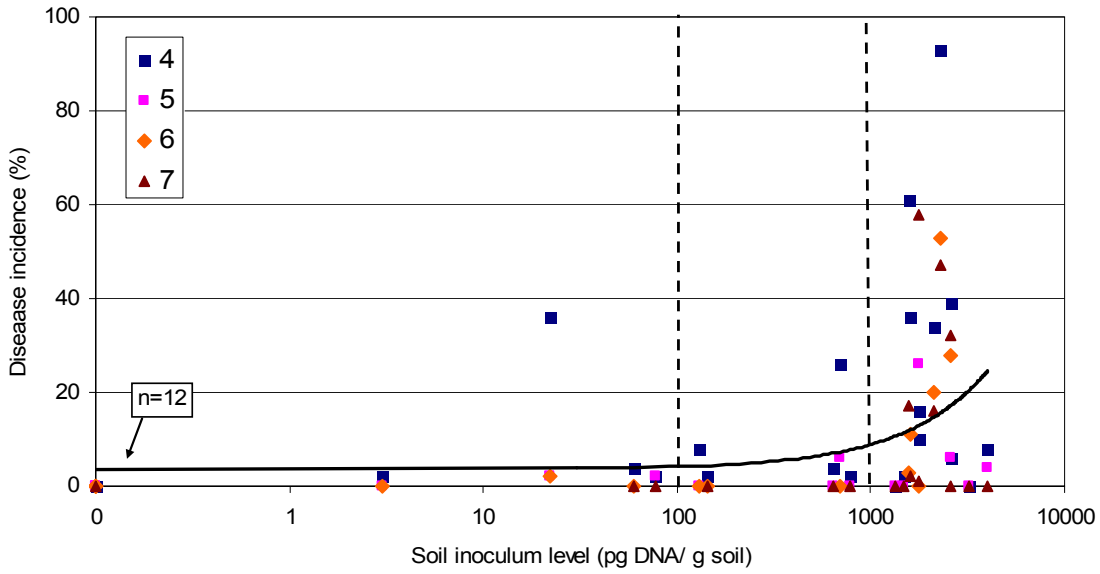


FIGURE 7. EFFECT OF INCREASING SOIL INOCULUM ON THE INCIDENCE OF BLACK DOT ON MINI-TUBER PROGENY OF VARIETIES WITH DIFFERENT RESISTANCE RATINGS GROWN IN 2005, 2006 AND 2007. REGRESSION LINE FITTED.

As with the commercial crops, the likelihood of mini-tuber progeny having black dot and having an incidence of black dot greater than 20 % increased with disease risk category (Table 6). The main difference between the results from commercial crops and the mini-tuber trials, is that in the absence of seed inoculum no disease was found in very low disease risk soils (i.e. those in which no soil inoculum was detected).

TABLE 6. THE PERCENTAGE OF MINI-TUBER PROGENY WITH BLACK DOT AND THE PERCENTAGE OF MINI-TUBER PROGENY WITH AN INCIDENCE OF BLACK DOT GREATER THAN 20% WHEN GROWN IN SOILS OF DIFFERENT DISEASE RISK CATEGORIES.

Disease risk category pg DNA / g soil	v. low 0	low <100	medium 100-1000	high >1000
% crops with disease	0	58	40	69
% crops with disease incidence > 20 %	0	8	7	33
number of crops in category	12	12	15	36

Black dot project field trials

All sites had detectable levels of *C. coccodes* inoculum, and disease was found at all sites in at least some varieties (Figure 8A and B). Considering each variety at each of the 19 sites individually i.e. total of 114 crops (consisting of the six varieties at each of the nineteen field sites), then there was no relationship between disease risk category and the proportion of crops which had black dot. However, the proportion of crops which had more than 20% incidence of disease increased in line with disease risk category (Table 7).

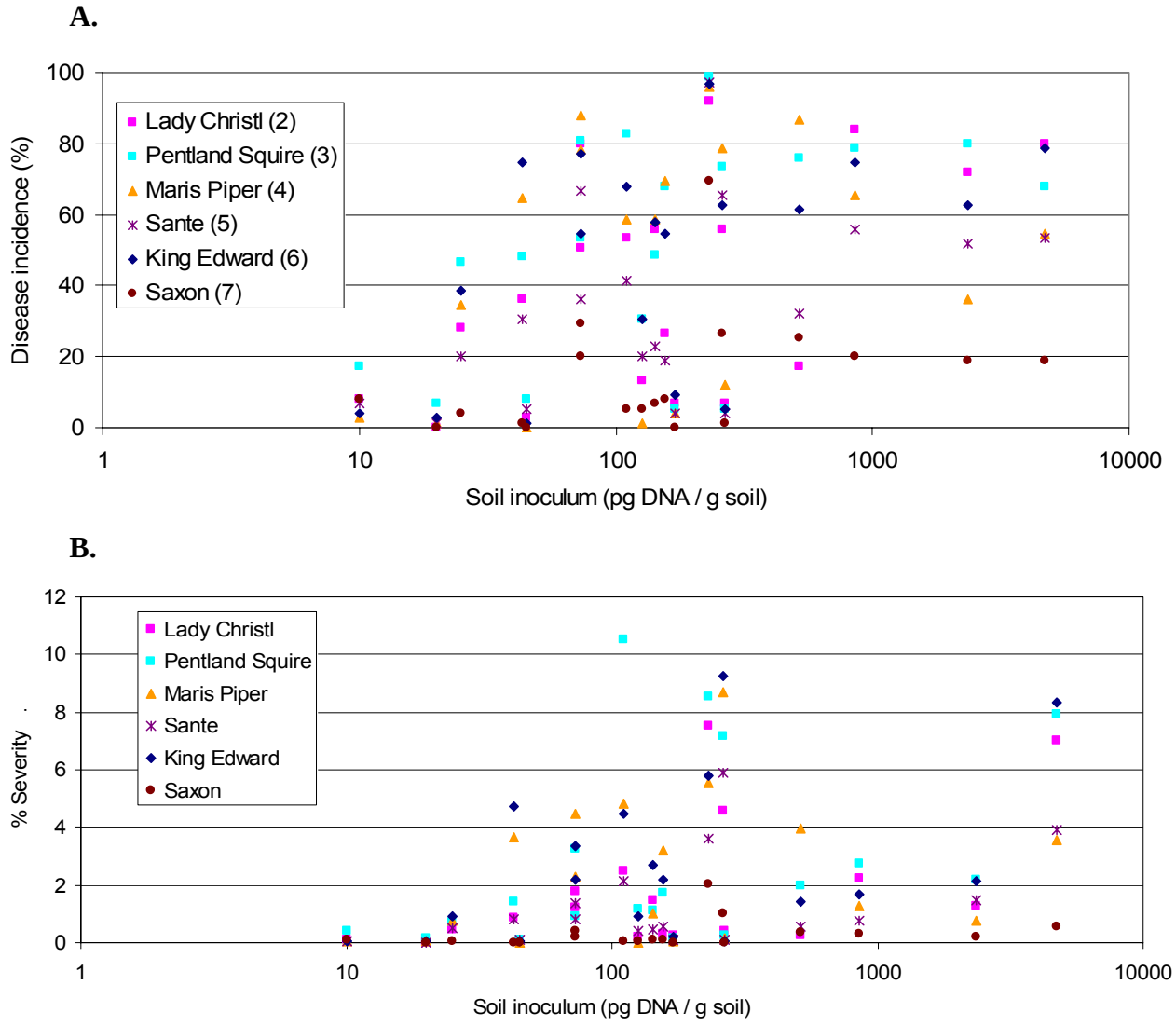


FIGURE 8. THE EFFECT OF INCREASING SOIL INOCULUM (PG DNA / G SOIL) ON A. INCIDENCE AND B. SEVERITY OF BLACK DOT IN PROGENY CROPS. VARIETIES WITH DIFFERENT RESISTANCE RATINGS FOR BLACK DOT (IN BRACKETS) PLANTED IN BLACK DOT FIELD TRIALS IN 2007.

TABLE 7. THE PERCENTAGE OF PROGENY CROPS WITH BLACK DOT AND THE PERCENTAGE OF PROGENY WITH AN INCIDENCE OF BLACK DOT GREATER THAN 20% WHEN GROWN IN SOILS WITH DIFFERENT LEVELS OF INOCULUM.

Disease risk category	v. low	low	medium	high
pg DNA / g soil	0	<100	100-1000	>1000
% crops with disease		90	98	100
% crops with disease incidence > 20 %		48	63	83
number of crops in category	0	42	60	12

At each of the nineteen field sites, the varieties were ranked 1 to 6 depending on the level of progeny disease, one having the least disease and six having the highest incidence of disease on progeny tubers. The rank scores were then summed across all sites (cumulative rank), and across sites with low to medium levels of soil inoculum (<1000 pg DNA / g soil) and sites with high soil inoculum (> 1000 pg DNA / g soil).

TABLE 8. VARIETIES RANKED FOR DISEASE INCIDENCE AT EACH SITE

variety (black dot resistance rating)	L. Christl (2)	P. Squire (3)	M. Piper (4)	Sante (5)	K. Edward (6)	Saxon (7)
Cumulative rank (all sites; n=19)	68	95	77	53	81	26
Cumulative rank (sites < 1000; n=12)	57	85	72	48	72	24
Cumulative rank (sites > 1000; n=2)	11	10	5	5	9	2

Varieties with high resistance ratings should have low cumulative ranks. Across all sites, Saxon which is the most resistant variety within the trials had the least disease. King Edward had more disease than would be expected from its resistance rating and Lady Christl had less.

King Edward had more disease than predicted at sites with either low/medium and high levels of soil inoculum. Lady Chistl performed better than its resistance rating suggests at the low/medium soil inoculum sites, but as expected at high inoculum sites (with the highest cumulative rank at sites with > 1000 pg DNA / g soil).

Factors affecting black dot

Incorporating the information we have from the black dot project into our disease risk assessment, we know that varietal resistance, crop duration, water status (irrigation and seasonal differences), temperature (seasonal differences) and the use of Amistar all significantly affect the development of black dot. Therefore it is not surprising that a range of disease levels are found for any particular soil inoculum level within the monitoring programme. Data from monitored crops was analysed using step wise regression analysis to see if any relationships could be determined using this data set (Table 9). However, due to the patchy records on crop information, only a sub-set of the whole data set can be included, reducing our ability to analyse the data thoroughly and completely.

Using the crops for which we do have the relevant information, we can investigate factors such as varietal resistance to black dot and/or crop duration, and how they affect the extent of black dot development on progeny tubers. The results of this analysis are shown in Table 9.

TABLE 9. SUMMARY OF LINEAR REGRESSION ANALYSIS BETWEEN SEVERITY AND INCIDENCE OF BLACK DOT ON DAUGHTER TUBERS AND A NUMBER OF AGRONOMIC FACTORS ASSOCIATED WITH INDIVIDUAL MONITORING CROPS IN 2005, 2006 AND 2007. DATA IS PRESENTED AS PERCENTAGE OF VARIATION EXPLAINED BY THE LINEAR REGRESSION

Agronomic factors	2005		2006		2007	
	Incidence	Severity	Incidence	Severity	Incidence	Severity
Soil inoculum (pg DNA/ g soil)	27.9***	13.4*	25.5***	28.3***	ns	ns
Seed inoculum (incidence of black dot)	12.9*	9.6*	ns	ns	ns	ns
Years since last potatoes	26.6*	19.4*	ns	ns	ns	ns
Cultivar resistance rating	27.2*	27.0*	ns	ns	ns	ns
Duration of crop (weeks)	16.4*	ns	24.1***	10.0*	ns	ns
Time from burn-off to harvest (weeks)	ns	ns	ns	ns	33.8**	ns
Irrigation	29.7***	18.1*	19.2**	12.5**	60.8***	38.6**

Significance levels *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$

Summary of black dot results

- Results from the monitored crops, where contaminated seed was planted into soils with undetectable levels of soil inoculum support the results of the black dot project (R249). Both found that seed inoculum can cause disease, but the incidence of disease in progeny crops caused by seed inoculum in the absence of soil inoculum rarely exceeds 20%.

- The level of *C. coccodes* soil inoculum does affect the risk of disease occurring in progeny crops. Data from the monitored crops, mini-tuber trials and black dot extension trials all found a positive relationship between the level of soil inoculum and the risk of disease developing.

- Disease risk categories, which reflect the risk of disease occurring, based on the level of soil inoculum (pg DNA / g soil), have been set. In the absence of seed inoculum (in mini-tuber trials), no disease developed on progeny tubers when no soil inoculum was detected. However, as the vast majority of seed stocks tested in this project were contaminated with *C. coccodes,* up to 20 % incidence of disease could be expected to occur even if no soil inoculum is detected. Therefore, for commercial soil tests three disease risk categories are proposed, low (< 100 pg DNA / g soil), medium (100-1000 pg DNA / g soil) and high risk (> 1000 pg DNA / g soil).

- The black dot project (R249) found that under experimental conditions (controlled environment and field trials) a number of factors, including varietal resistance, crop duration, irrigation, temperature and use of Amistar had a significant effect on black dot.

- Varietal resistance is an important factor to consider when managing disease risk. Crops of more resistant cultivars often had less disease than more susceptible crops in the monitoring programme, mini-tuber trials and black dot extension trials. There was some indication that varietal resistance may not be as effective in reducing disease risk at very high levels of soil-borne inoculum compared to relatively low levels.

Powdery Scab

Quantification of *S. subterranea* in soil

Figure 9 illustrates the range of contamination levels found in commercial fields sampled in three years of monitoring.

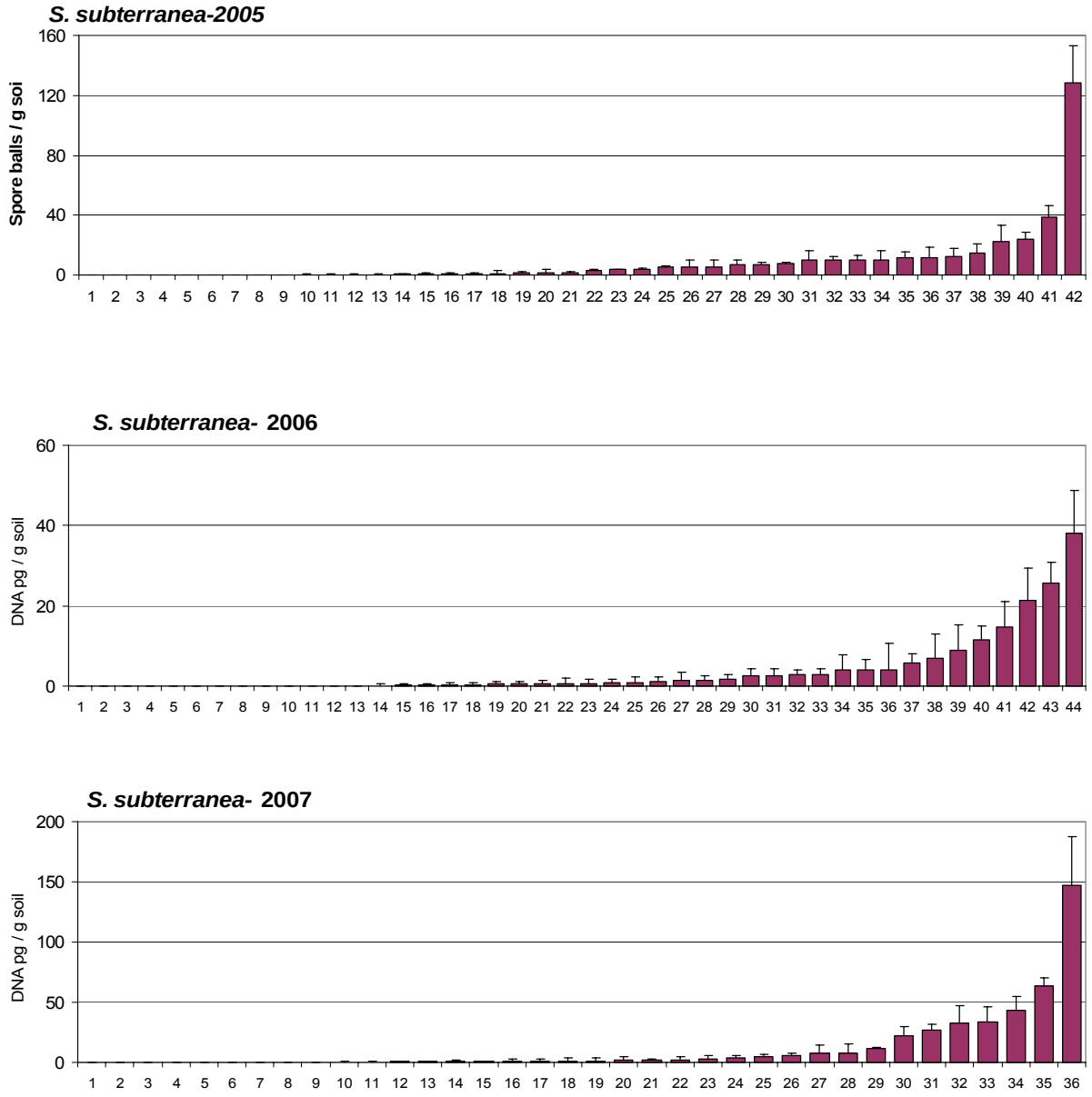


FIGURE 9. QUANTIFICATION OF *S. SUBTERRANEA* DNA IN SOIL SAMPLES IN 2005, 2006 AND 2007. MEAN + ST. DEV OF THREE SUB-SAMPLES TAKEN AFTER MILLING.

Following direct extraction of DNA from soils target DNA was found at levels ranging from zero (un-detectable) to over 148 spore balls per g soil for *S. subterranea* (Figure 9). A high percentage of soils (70 % or more) were contaminated with *S. subterranea* in all three years (Table 10).

TABLE 10. THE NUMBER OF SOIL SAMPLES TESTED, THE NUMBER AND PERCENTAGE OF THE TOTAL WHICH WERE FOUND TO HAVE DETECTABLE LEVELS OF PATHOGEN CONTAMINATION AND THE MAXIMUM LEVEL OF CONTAMINATION FOUND IN SOILS SAMPLED IN 2005, 2006 AND 2007.

Pathogen		Number of soils tested	Number of soils contaminated (percentage)	Max. contamination level (spore balls / g soil)
S. subterranea	2005	42	34 (81)	128
	2006	44	31 (70)	38
	2007	36	27 (75)	148

Quantification of *S. subterranea* on seed

In all years, a high proportion of seed stocks were contaminated with *S. subterranea* (69 % or more) (Table 11). Real-time PCR assays detected *S. subterranea* contamination on seed stocks which did not have visual symptoms of disease in over 21 % of seed stocks in each year, indicating the ability of real-time PCR to detect symptomless infection (Table 11).

TABLE 11. THE NUMBER OF SEED STOCKS TESTED, THE NUMBER AND PERCENTAGE OF THE TOTAL WHICH WERE FOUND TO HAVE DETECTABLE LEVELS OF *S. SUBTERRANEA* CONTAMINATION, THE MAXIMUM LEVEL OF CONTAMINATION FOUND ON SEED STOCKS, AND THE NUMBER AND PERCENTAGE OF STOCKS WHICH HAD SYMPTOMLESS INFECTION.

Year	Number of stocks tested	Number of stocks contaminated (percentage)	Max. contamination level (spore balls / assay)	Number of stocks with symptomless infection (percentage)
2005	42	29 (69)	41	9 (21)
2006	43	31 (72)	63	18 (42)
2007	39	36 (92)	224	29 (74)

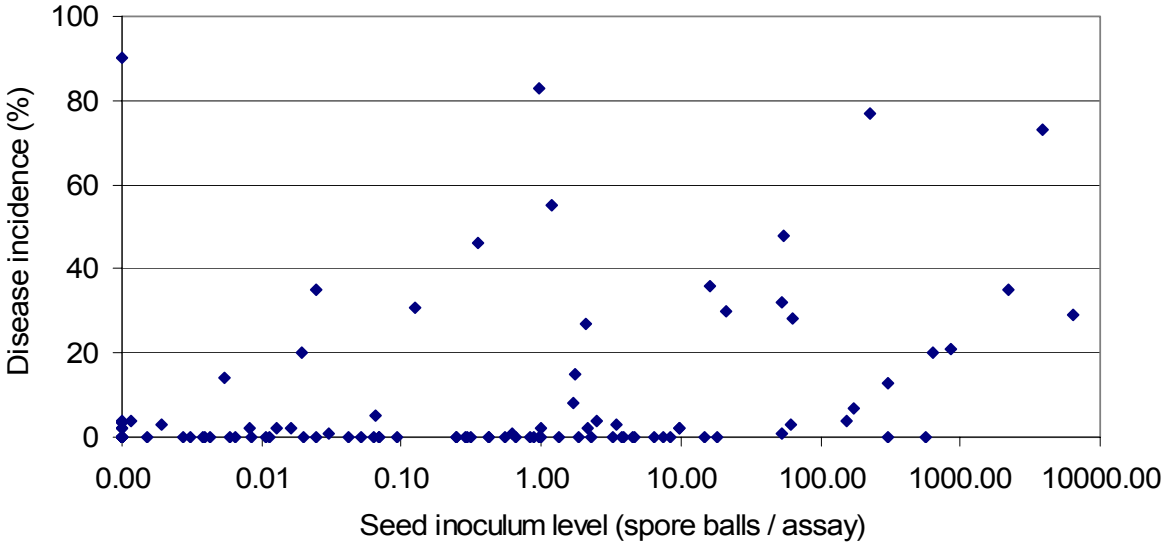
Relating inoculum to disease risk for powdery scab

Crop Monitoring

Seed inoculum

There was no relationship between the level of seed inoculum as determined by real-time PCR (spore balls / assay) and the incidence or severity of powdery scab on progeny tubers (Figure 10).

A. Disease incidence



B. Disease severity

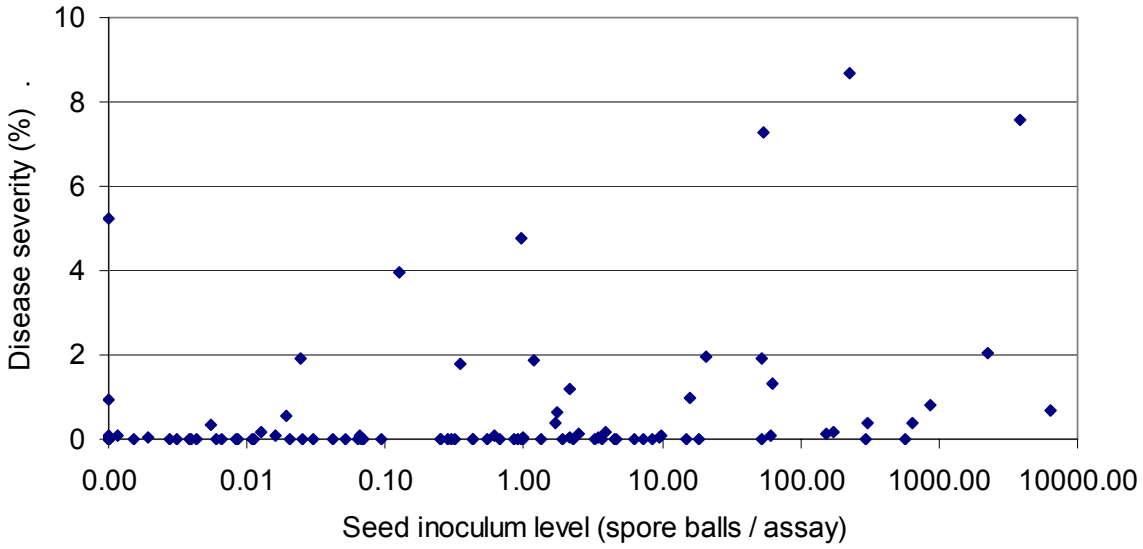
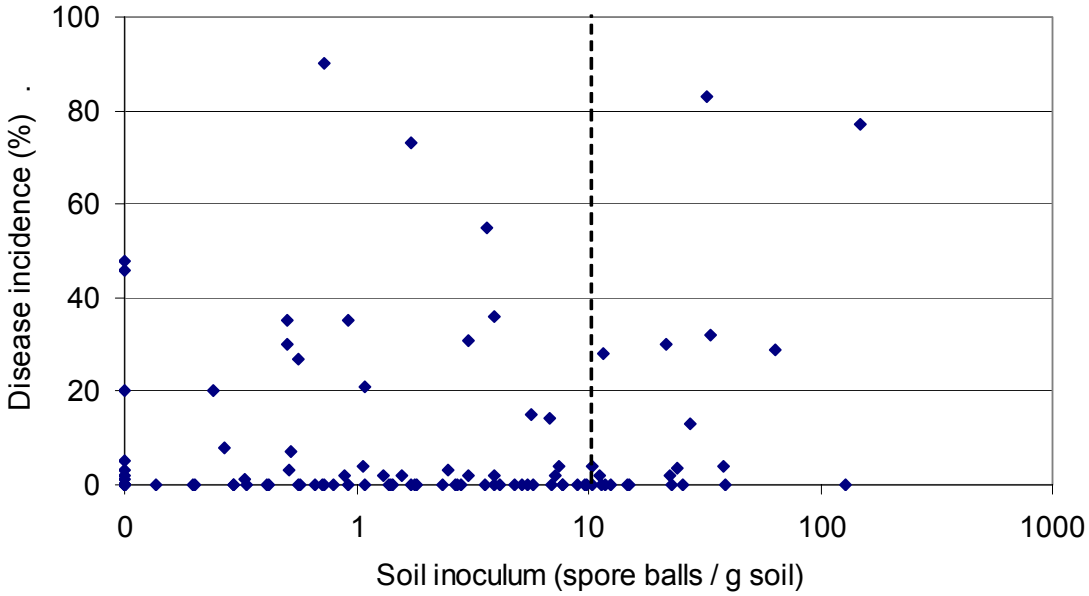


FIGURE 10. THE EFFECT OF SEED INOCULUM ON A. THE INCIDENCE AND B. THE SEVERITY OF POWDERY SCAB IN PROGENY CROPS MONITORED IN 2005, 2006 AND 2007.

Soil inoculum

As with seed inoculum, there was no clear relationship between the level of soil inoculum as determined by real-time PCR (spore balls / g soil) and the incidence and severity of powdery scab on progeny tubers across the three years data (Figure 11). However, there is a small increase in disease risk when soil inoculum levels are > 10 spore balls / g soil (Table 12).

A. Disease incidence



B. Disease severity

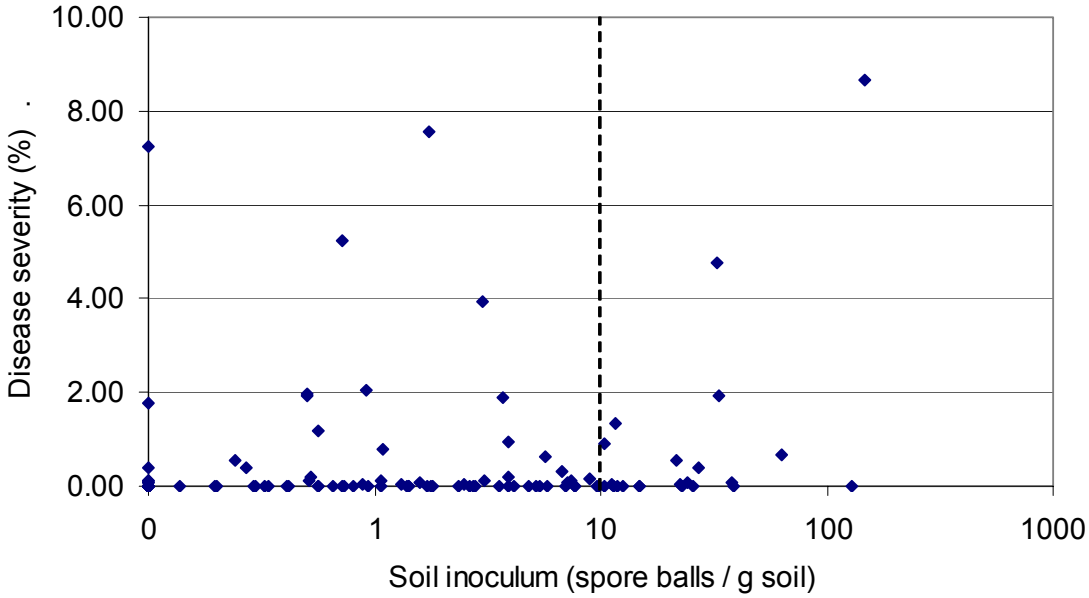


FIGURE 11. THE EFFECT OF INCREASING SOIL INOCULUM ON A. THE INCIDENCE AND B. THE SEVERITY OF POWDERY SCAB IN PROGENY CROPS MONITORED IN 2005, 2006 AND 2007.

TABLE 12. THE PERCENTAGE OF CROPS WITH POWDERY SCAB AND THE PERCENTAGE OF CROPS WITH AN INCIDENCE OF POWDERY SCAB GREATER THAN 20 % WHEN GROWN IN SOILS OF DIFFERENT DISEASE RISK CATEGORIES.

Disease risk category spore balls / g soil	low 0	medium <10	high >10
% crops with disease	27	42	55
% crops with disease incidence > 20 %	12	17	27
Number of crops in category	26	65	22

In contrast to black dot, where soil inoculum appears to be the main factor influencing the incidence of disease on progeny crops, for powdery scab either source of inoculum may cause disease, but the main factor influencing disease development is not the level of inoculum but rather environmental conditions. If conditions suitable for powdery scab are encountered, high levels of disease can arise from very low levels of initial inoculum, conversely, if the conditions are not conducive to disease development, even high levels of inoculum on either seed or in the soil may not result in diseased progeny.

Although disease risk categories have been set for powdery scab soil inoculum levels (Table 12), they must be considered in a wider context, for example:

- Low risk soils where no inoculum has been detected, will only be truly low risk if seed free from *S. subterranea* contamination are planted.
- In seasons when conditions are not generally conducive to disease then powdery scab can still develop on crops with either high inoculum levels or with a microclimate suitable for disease, ie. prone to waterlogging.
- In seasons when conditions are more conducive to disease development, then the level of inoculum becomes more relevant to the incidence of disease.

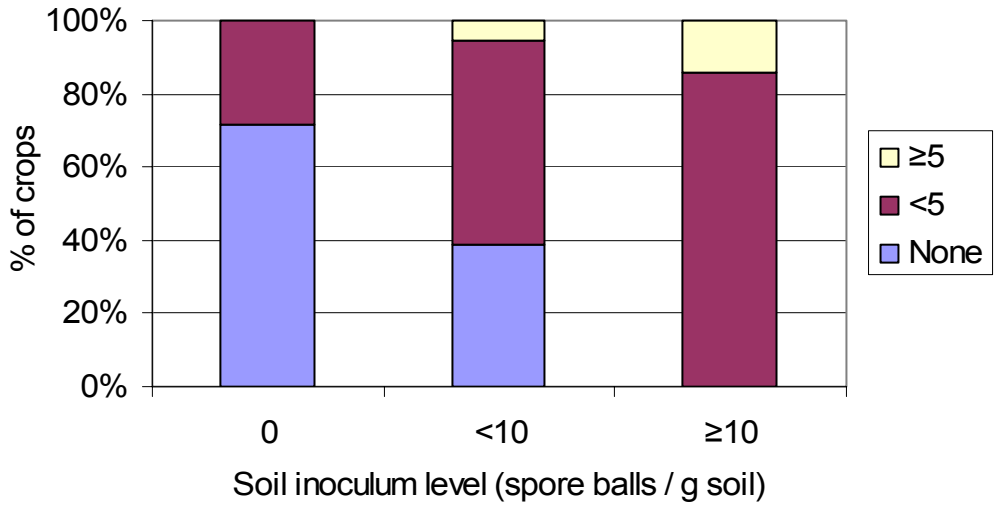
Within the three years during which crops were monitored, two years (2005 and 2006) were relatively dry and little powdery scab was observed in progeny crops. The third year (2007) was much wetter and consequently much higher levels of powdery scab were found. Spring rainfall (March through May) across the UK did not vary much between the three years of this study. However, summer rainfall (June through August) was much higher in 2007 (357 mm) than in 2005 and 2006 (216 and 187 mm respectively). Average UK rainfall in the summer was below the long-term average in 2005 and 2006 and 151 % of the long-term average in 2007 (Met Office UK averages).

TABLE 13. AVERAGE UK RAINFALL DURING SUMMER (JUNE, JULY AND AUGUST) IN THE THREE YEARS OF THIS STUDY.

Year	Summer rainfall (mm)	Relative to long-term average
2005	216	92 %
2006	187	80 %
2007	357	151 %

In 2007, a year when powdery scab was more prevalent than the two previous years, the percentage of crops with higher incidence of powdery scab increased with increasing soil inoculum (Figure 12A.). In 2005 and 2006 (Figure 12B) there is less difference in the incidence levels of powdery scab between the soil inoculum categories.

A. 2007



B. 2005 and 2006

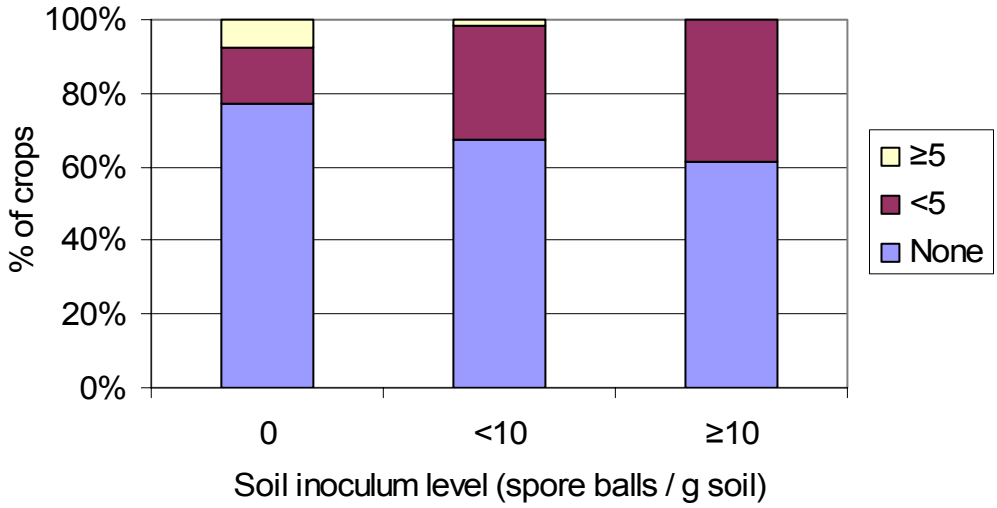


FIGURE 12. THE PERCENTAGE OF MONITORED CROPS IN A. 2007 AND B. COMBINED FOR 2005 AND 2006 WITH DIFFERENT INCIDENCE LEVELS OF POWDERY SCAB (NONE, < 5 AND ≥ 5 %) ON PROGENY TUBERS WHEN GROWN AT SITES WITH VARYING LEVELS OF SOIL INOCULUM (0, <10 AND ≥10 SPORE BALLS/ G SOIL).

Attempting to separate out the effects of soil and seed-borne inoculum in the monitored crops does not yield any useful information (Figure 13). Thirty-two crops had uncontaminated seed (as determined with real-time PCR), but very few of these stocks developed powdery scab, thirty out of the thirty two stocks were planted in 2005 and 2006, the two years with very low levels of powdery scab. Conversely twenty-six stocks were planted into soils with undetectable levels of inoculum. However there was no apparent relationship between seed inoculum level and incidence of disease.

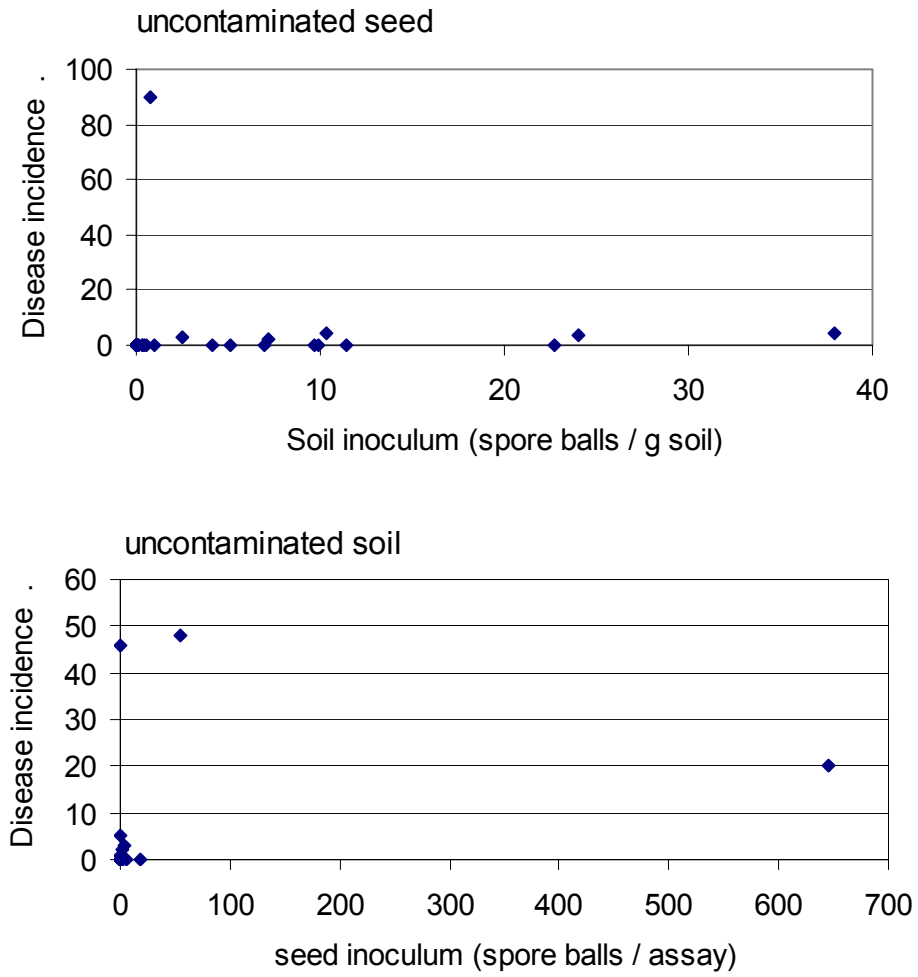


FIGURE 13. A. THE EFFECT OF SOIL INOCULUM (SPORE BALLS / G SOIL) ON THE INCIDENCE OF POWDERY SCAB IN STOCKS WITH UN DETECTABLE LEVELS OF SEED INOCULUM, AND B. THE EFFECT OF SEED INOCULUM (SPORE BALLS / ASSAY) ON THE INCIDENCE OF POWDERY SCAB IN STOCKS WITH UNDETECTABLE LEVELS OF SOIL INOCULUM.

Mini-tuber trials

In total, 25 mini-tuber trials were performed. Little powdery scab developed on the mini-tuber trials in 2005 and 2006. Powdery scab was more common in 2007 particularly on the more susceptible varieties Maris Piper and Nadine. In the trials, powdery scab did develop at 2 trial sites (both in 2007) where no inoculum was detected in the soil, (Figure 14). The was no link between number of spore balls in the soil and incidence of powdery scab (Figure 14), and no difference in the percentage of crops developing disease in the different disease risk categories (Table 14).

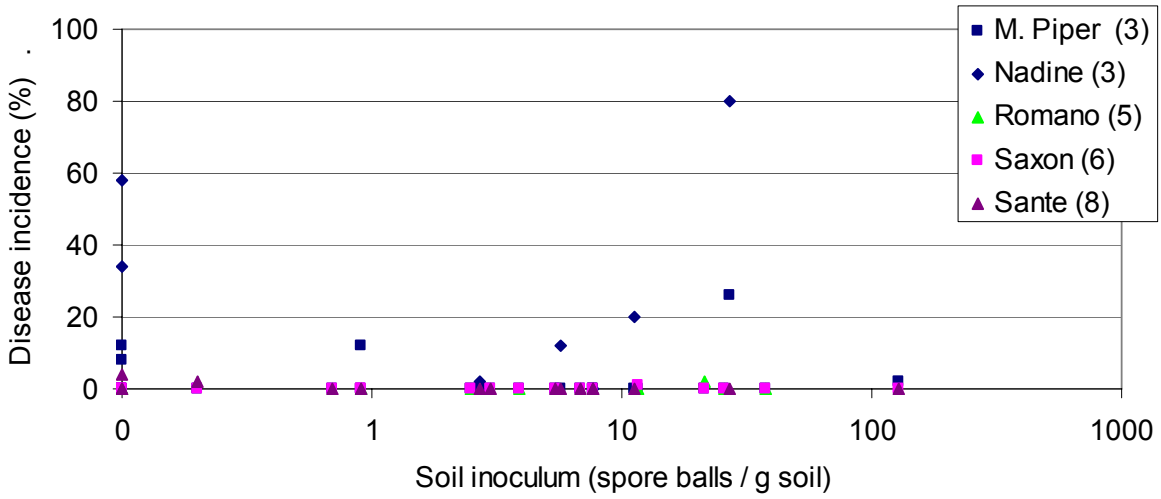


FIGURE 14. THE EFFECT OF INCREASING SOIL INOCULUM ON THE INCIDENCE OF POWDERY SCAB IN MINI-TUBER PROGENY IN 2005, 2006 AND 2007.

TABLE 14. THE PERCENTAGE OF CROPS WITH POWDERY SCAB AND THE PERCENTAGE OF CROPS WITH AN INCIDENCE OF POWDERY SCAB GREATER THAN 20 % WHEN MINI-TUBERS WERE GROWN IN SOILS OF DIFFERENT DISEASE RISK CATEGORIES ACROSS ALL YEARS.

Disease risk category	low	medium	high
spore balls / g soil	0	<10	>10
% crops with disease	33	10	29
% crops with disease incidence > 20 %	13	0	14
Number of crops in category	15	39	21

Powdery scab in black dot project field trials

One site was removed from the analysis as extraction method may have been different from the other trials sites therefore there are eighteen rather than nineteen trial sites (Figure 15). Six varieties were grown at each of the eighteen sites, resulting in a total of 108 crops. Ten of the eighteen field trial sites had no detectable *S. subterranea* soil inoculum. The number of progeny crops at a given soil inoculum level that have greater than 20 % incidence of disease increases as the level of soil inoculum increases (Table 15), i.e. no crops with undetectable levels of *S. subterranea* in the soil had an incidence of disease greater than 20 %, this increased to 19 and 33 % of crops in the medium and high levels of soil inoculum respectively (Table 15).

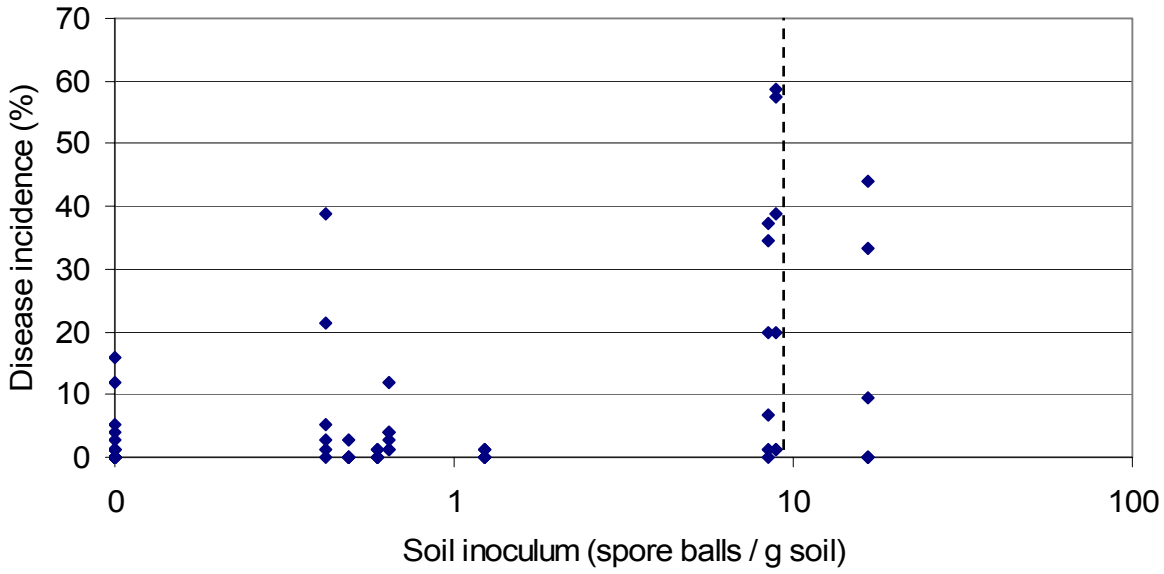


FIGURE 15. THE EFFECT OF INCREASING SOIL INOCULUM (SPORE BALLS / G SOIL) ON THE INCIDENCE OF POWDERY SCAB IN PROGENY TUBERS FROM THE BLACK DOT EXTENSION FIELD TRIALS IN 2007.

TABLE 15. THE PERCENTAGE OF CROPS WITH POWDERY SCAB AND THE PERCENTAGE OF CROPS WITH AN INCIDENCE OF POWDERY SCAB GREATER THAN 20 % WHEN GROWN IN SOILS OF DIFFERENT DISEASE RISK CATEGORIES.

Disease risk category	low	medium	High
spore balls / g soil	0	<10	>10
% crops with disease	18	64	50
% crops with disease incidence > 20 %	0	19	33
Number of crops in category	60	42	6

At each of the eighteen field sites, the varieties were ranked 1 to 6 depending on the level of progeny disease, one having the least disease and six having the highest incidence of disease on progeny tubers. The rank scores were then summed across all sites (cumulative rank). Varieties with high resistance ratings should have low cumulative ranks. Across all sites, Sante which is the most resistant variety within the trials had the least disease, followed by the second most resistant variety, Saxon. The remaining varieties all had resistance ratings of 3 or 4 and had similar cumulative ranks (Table 16).

TABLE 16. VARIETIES RANKED FOR DISEASE INCIDENCE AT NINETEEN FIELD TRIAL SITES RELATIVE TO EACH OTHER

Variety (powdery scab resistance rating)	L. Christl (3)	M. Piper (3)	P. Squire (4)	K. Edward (4)	Saxon (6)	Sante (8)
Cumulative rank (all sites;n =18)	78	62	66	69	54	49

Factors affecting powdery scab

Little disease developed in 2005 and 2006 so no associations between agronomic factors were observed. In 2007, when conditions for powdery scab were favourable, significant associations between level of soil inoculum and incidence and severity of disease on daughter tubers were observed (Table 17). A small but significant association between powdery scab on seed and severity on daughter tubers at harvest was observed.

TABLE 17. SUMMARY OF LINEAR REGRESSION ANALYSIS BETWEEN SEVERITY AND INCIDENCE OF POWDERY SCAB ON DAUGHTER TUBERS AND A NUMBER OF AGRONOMIC FACTORS ASSOCIATED WITH INDIVIDUAL MONITORING CROPS. DATA IS PRESENTED AS PERCENTAGE OF VARIATION EXPLAINED BY THE LINEAR REGRESSION.

	2005		2006		2007	
Agronomic factors	Incidence	Severity	Incidence	Severity	Incidence	Severity
Soil inoculum (pg DNA/ g soil)	ns	ns	ns	ns	28.3**	39.8**
Powdery scab on seed	ns	ns	ns	ns	ns	19.5*
Years since last potatoes	ns	ns	ns	ns	ns	Ns
Cultivar resistance rating	ns	ns	ns	ns	ns	Ns
Duration of crop (weeks)	ns	ns	10.5*	9.3*	ns	Ns
Time from burn-off to harvest (weeks)	ns	ns	ns	ns	ns	Ns
Irrigation	ns	ns	ns	ns	ns	Ns
Volunteers	ns	ns	ns	ns	ns	Ns

Significance levels *** p< 0.001 ; ** p < 0.01 ; * p <0.05

Summary of powdery scab results

- There was no relationship between the level of seed-borne inoculum and powdery scab on progeny tubers. However, in crops where contaminated seed was planted in to soil with undetectable levels of inoculum, disease did develop.

- There was no clear relationship between the level of soil-borne inoculum and powdery scab, although there is a small increase in the risk of disease at soil inoculum levels above 10 pg DNA / spore balls /g soil.

- Environmental conditions, rather than the level of inoculum, are the most important factors influencing disease development. Little powdery scab was found in 2005 and 2006, two years with relatively dry summers. In 2007 which was relatively wet, more disease was observed and there was a relationship between soil inoculum levels and risk of disease. In years when conditions are suitable for powdery scab development, low levels of inoculum can result in high levels of disease. Conversely, if conditions are not suitable for disease then high levels of inoculum may not result in any disease.

- Disease risk categories that reflect the risk of disease based on the level of soil inoculum (pg DNA / g soil) have been set. Low risk (no detectable inoculum), medium (< 10 spore balls / g soil) and high (≥ 10 spore balls / g soil). However it must be stressed that these disease risk categories are only indications of risk and must be considered along with a number of other factors, such as, seed inoculum, seasonal factors and field conditions.

- In the monitoring programme, no progeny crops had powdery scab symptoms in the absence of detectable levels of seed and/or soil inoculum. This indicates that the seed and soil diagnostic tests are adequately detecting inoculum. However, at two of the mini-tuber sites in 2007, powdery scab developed on some progeny. The source of this infection has not been identified.

- Varietal resistance should be considered when managing powdery scab risk. Crops of more resistant cultivars often had less disease than more susceptible crops in the monitoring programme, mini-tuber trials and black dot extension trials.

Black Scurf

Quantification of *R. solani* AG-3 in soil

Following direct extraction of DNA from soils target DNA was found at levels ranging from zero (un-detectable) to over 17000 pg DNA/ g soil for *R. solani* (Figure 16). Compared to the other pathogens (*C. coccodes* and *S. subterranea*) relatively few (21 % or less) soil samples were found to be contaminated with *R. solani*, see Table 18.

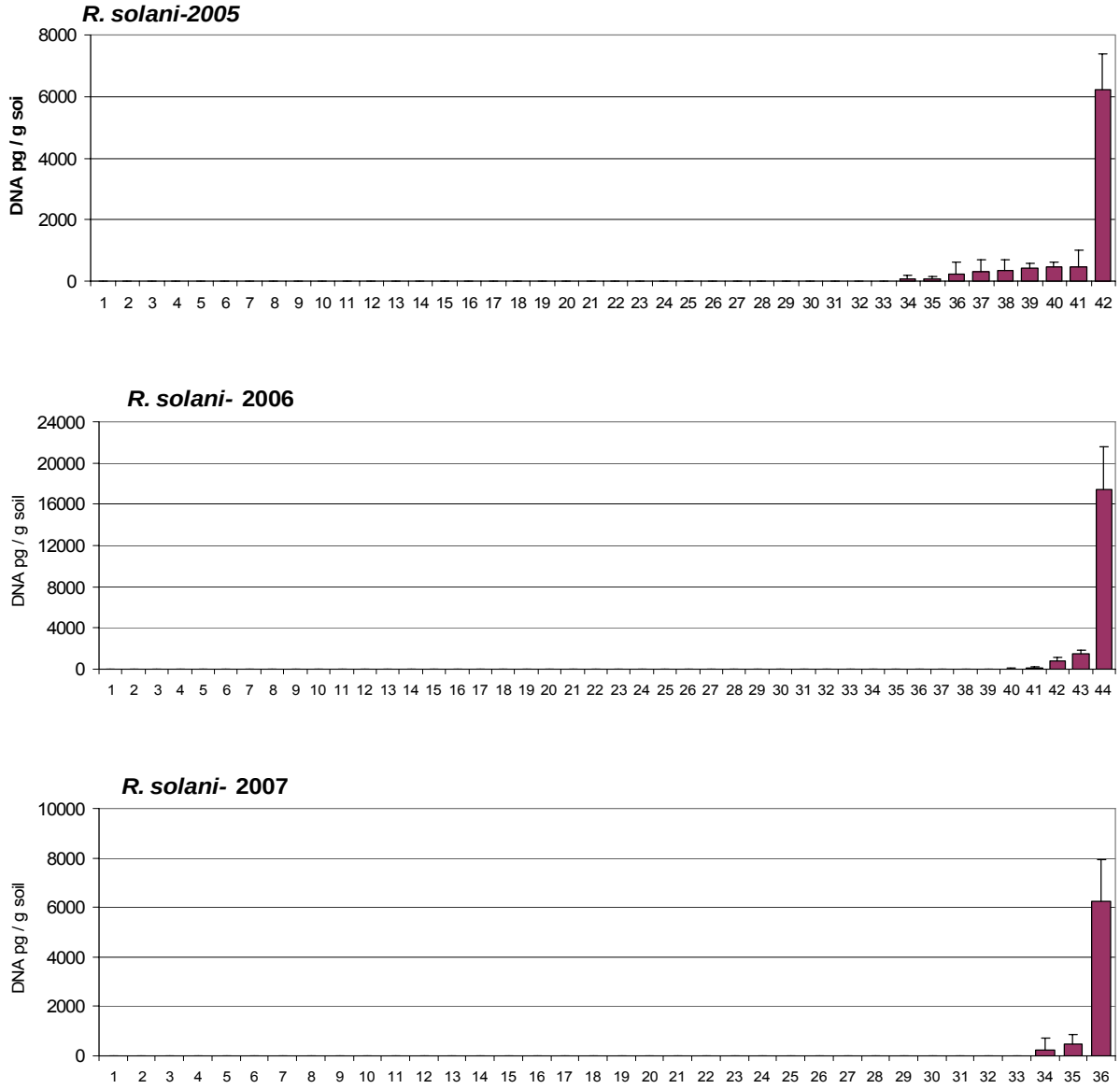


FIGURE 16. QUANTIFICATION OF *R. SOLANI* DNA IN SOIL SAMPLES IN 2005, 2006 AND 2007. MEAN + ST. DEV OF THREE SUB-SAMPLES TAKEN AFTER MILLING.

TABLE 18. THE NUMBER OF SOIL SAMPLES TESTED, THE NUMBER AND PROPORTION OF THE TOTAL WHICH WERE FOUND TO HAVE DETECTABLE LEVELS OF PATHOGEN CONTAMINATION AND THE MAXIMUM LEVEL OF CONTAMINATION FOUND IN SOILS SAMPLED IN 2005, 2006 AND 2007.

Pathogen		Number of soils tested	Number of soils contaminated (percentage)	Max. contamination level (pg DNA/ g soil)
R. solani	2005	42	9 (21)	6200
	2006	44	5 (11)	17000
	2007	36	3 (8)	6300

Quantification of *R. solani* AG-3 on seed

Target DNA was found at levels ranging from zero (un-detectable) to over 20,000 pg DNA / assay for *R. solani*. In all years, 60 % or more of stocks were contaminated with *R. solani* (Table 19). Real-time PCR assays detected *R. solani* contamination on seed stocks that did not have visual symptoms of disease in over 14 % of seed stocks in each year, indicating the ability of real-time PCR to detect symptomless infection (Table 18).

TABLE 19. THE NUMBER OF SEED STOCKS TESTED, THE NUMBER AND PERCENTAGE OF THE TOTAL WHICH WERE FOUND TO HAVE DETECTABLE LEVELS OF *R. SOLANI* CONTAMINATION, THE MAXIMUM LEVEL OF CONTAMINATION FOUND ON SEED STOCKS, AND THE NUMBER AND PERCENTAGE OF STOCKS WHICH HAD SYMPTOMLESS INFECTION.

Year	Number of stocks tested	Number of stocks contaminated (percentage)	Max. contamination level (pg DNA / assay)	Number of stocks with symptomless infection (percentage)
2005	42	25 (60)	3000	11 (26)
2006	43	30 (70)	20460	6 (14)
2007	39	32 (82)	15894	12 (31)

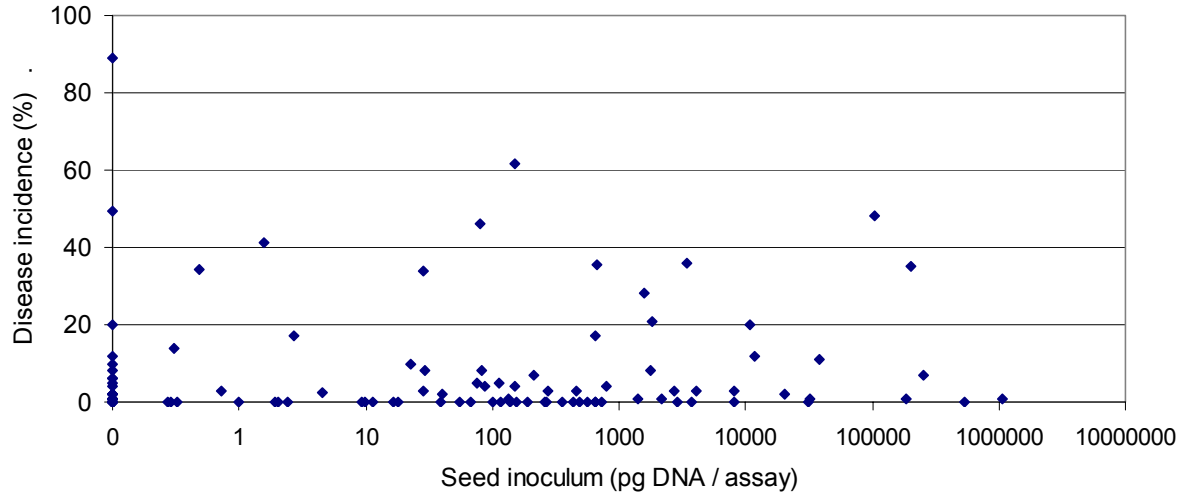
Relating inoculum to disease risk for black scurf

Crop Monitoring

Seed inoculum

There was no relationship between the level of seed inoculum as determined by real-time PCR (pg DNA / assay) and the incidence or severity of black scurf on progeny tubers (Figure 17).

A. Disease incidence



B. Disease severity

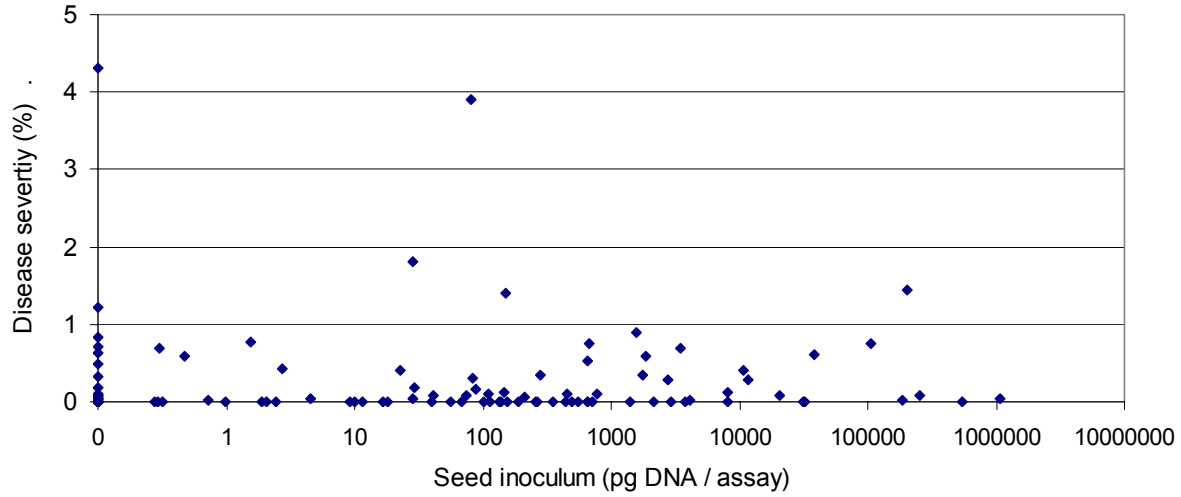


FIGURE 17. THE EFFECT OF SEED INOCULUM (PG DNA / ASSAY) ON A. THE INCIDENCE AND B. THE SEVERITY OF BLACK SCURF IN PROGENY CROPS MONITORED IN 2005, 2006 AND 2007.

No links between the level of soil contamination (pg DNA / g soil) and stem canker or black scurf developing were observed (Figures 18 and 19). In all three years, there were incidences of disease in the progeny crop in the absence of detectable seed and soil-borne inoculum.

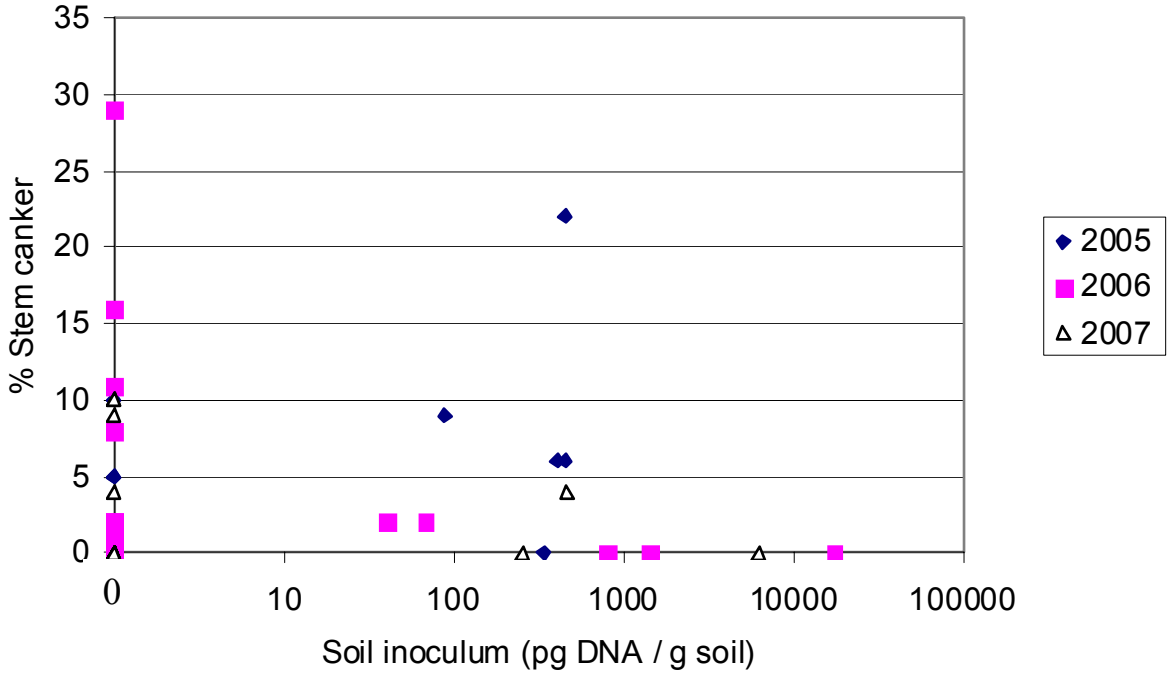


FIGURE 18. THE EFFECT OF SOIL INOCULUM ON THE INCIDENCE OF STEM CANKER IN PROGENY CROPS MONITORED IN 2005, 2006 AND 2007.

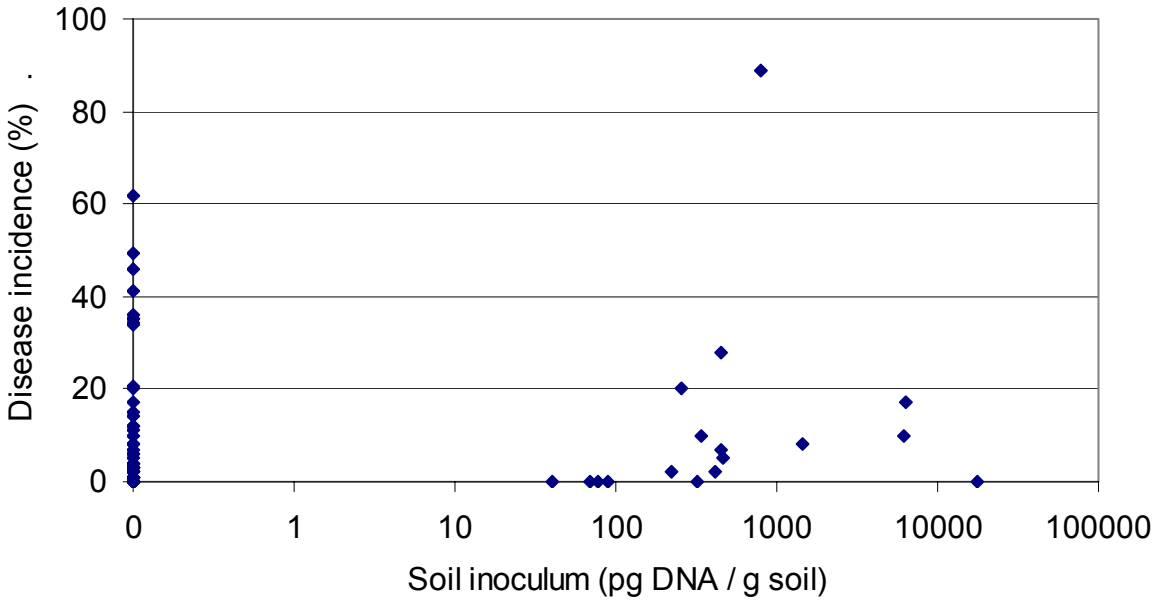


FIGURE 19. THE EFFECT OF SOIL INOCULUM ON THE INCIDENCE OF BLACK SCURF IN PROGENY CROPS MONITORED IN 2005, 2006 AND 2007.

Bioassay for *R. solani* (non AG group specific)

Ongoing work at SAC has identified a threshold of 15 % or more seeds being infected in the bioassay as being indicative of the soil being contaminated with *R. solani*. According to this stipulation, 3 fields were contaminated with *R. solani* in the 2006 season and 3 in the 2007 season. Of these 6 crops, three progeny stocks had black scurf. Without the 15 % threshold, the bioassay identified a higher proportion of fields as being contaminated with *R. solani* than the real-time PCR assay. However it must be highlighted that the bioassay is not AG group specific. There was a weak but significant relationship ($P<0.05$) between soil contamination as determined with the bioassay (without the threshold applied) and incidence of black scurf on progeny tubers in 2006. Although a significant association between the bioassay results and black scurf on daughter tubers were observed in 2006 (Figure 20) there are still are large number of cases where the soil was observed to be un-contaminated according to the bioassay, but disease was observed on the daughter tubers.

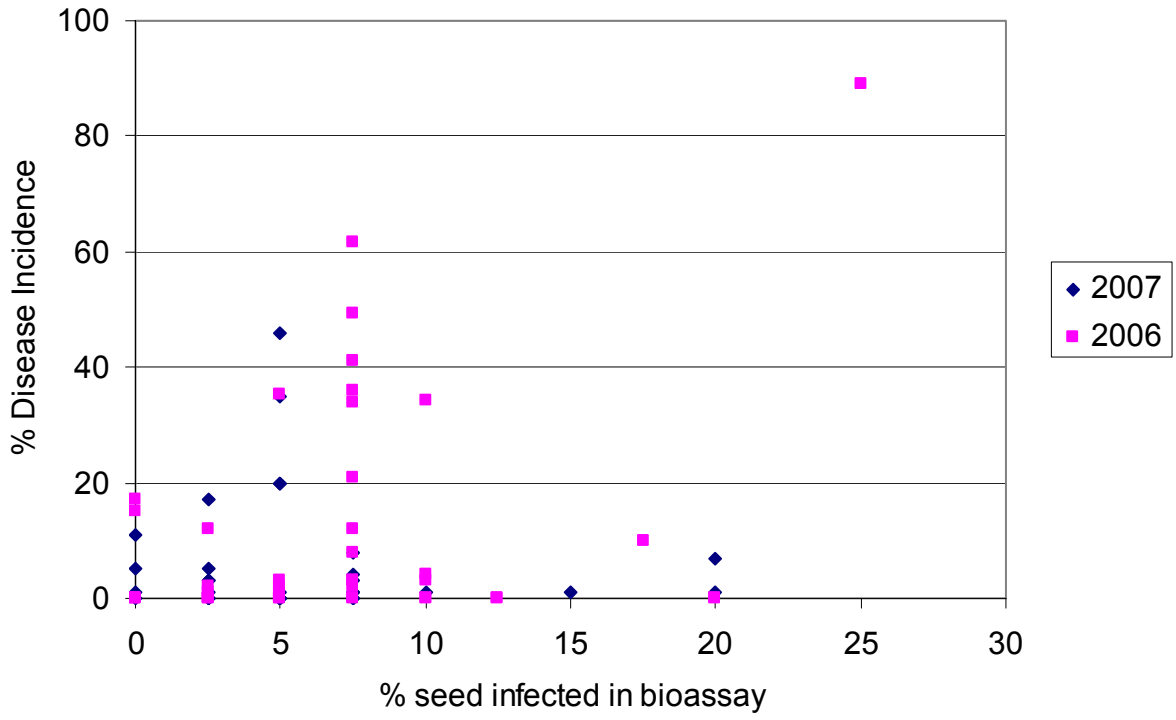


FIGURE 20. THE EFFECT OF SOIL INOCULUM AS DETERMINED IN A BIOASSAY ON THE INCIDENCE OF STEM CANKER IN PROGENY CROPS MONITORED IN 2006 AND 2007.

Both seed and soil-borne inoculum of *R. solani* are capable of causing disease, therefore to aid interpretation of the results from the monitoring study, those crops which had either uncontaminated seed planted in contaminated soil (Figure 21), or conversely contaminated seed planted into soil with undetectable levels of *R. solani* have been looked at (Figure 22).

Over the three years of monitoring, there were only 25 instances in over 120 crops looked at, in which uncontaminated seed (as determined by real-time PCR) was planted in fields with undetectable levels of soil contamination. Of these 25 crops, eleven had black scurf on the progeny tubers (up to 49 % incidence). Only 5 crops had clean seed but planted in to soils with detectable levels of soil contamination (Figure 21) and no relationship between soil inoculum and disease incidence can be drawn from such a small sub-set of data. There were 42 instances where contaminated seed was planted in to soils with undetectable levels of *R. solani* (Figure 22), and no clear relationship between seed inoculum level and incidence of disease is apparent.

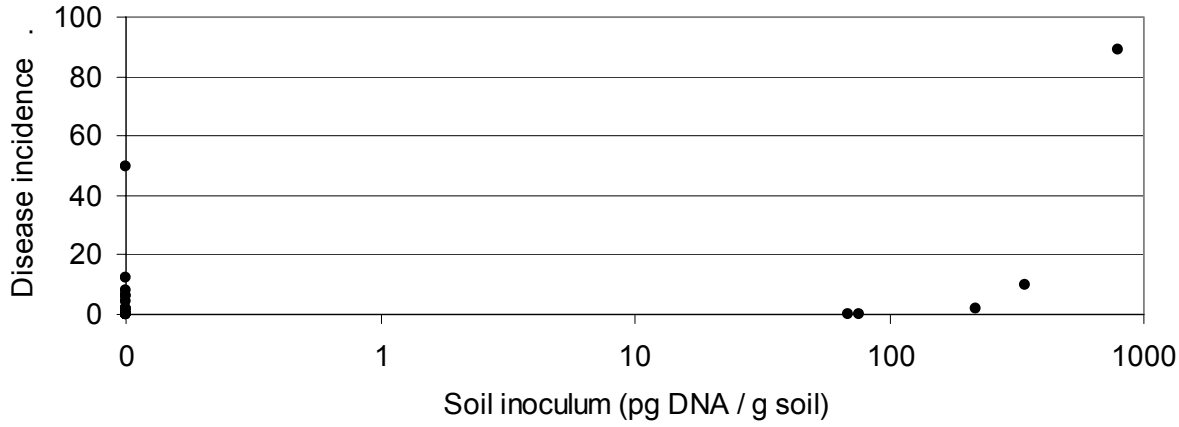


FIGURE 21. THE EFFECT OF SOIL INOCULUM (PG DNA / G SOIL) ON THE INCIDENCE OF BLACK SCURF IN PROGENY CROPS MONITORED IN 2005, 2006 AND 2007 IN THE ABSENCE OF DETECTABLE SEED INOCULUM AS DETERMINED WITH REAL-TIME PCR.

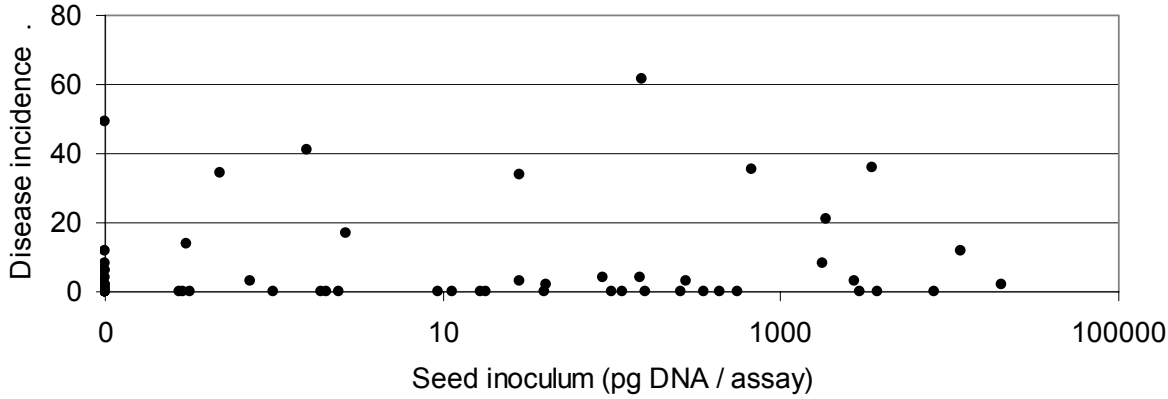


FIGURE 22. THE EFFECT OF SEED INOCULUM (PG DNA / ASSAY) ON THE INCIDENCE OF BLACK SCURF IN PROGENY CROPS MONITORED IN 2005, 2006 AND 2007 IN THE ABSENCE OF DETECTABLE LEVELS OF SOIL INOCULUM AS DETERMINED WITH REAL-TIME PCR.

Mini-tuber trials

In 2005, soil inoculum was detected at 5 out of 9 sites. No disease developed in the four sites with undetectable levels of soil inoculum in this year. However, no inoculum was detected at any of the trial sites in 2006 or 2007, but black scurf was found to be common in many of these trials. Overall there was no link between soil inoculum and incidence of black scurf developing (Figure 23). In general, varietal resistance had little effect on incidence of black scurf, although Nadine with a resistance rating of 7 has less disease than the other varieties grown in the 2007 trials.

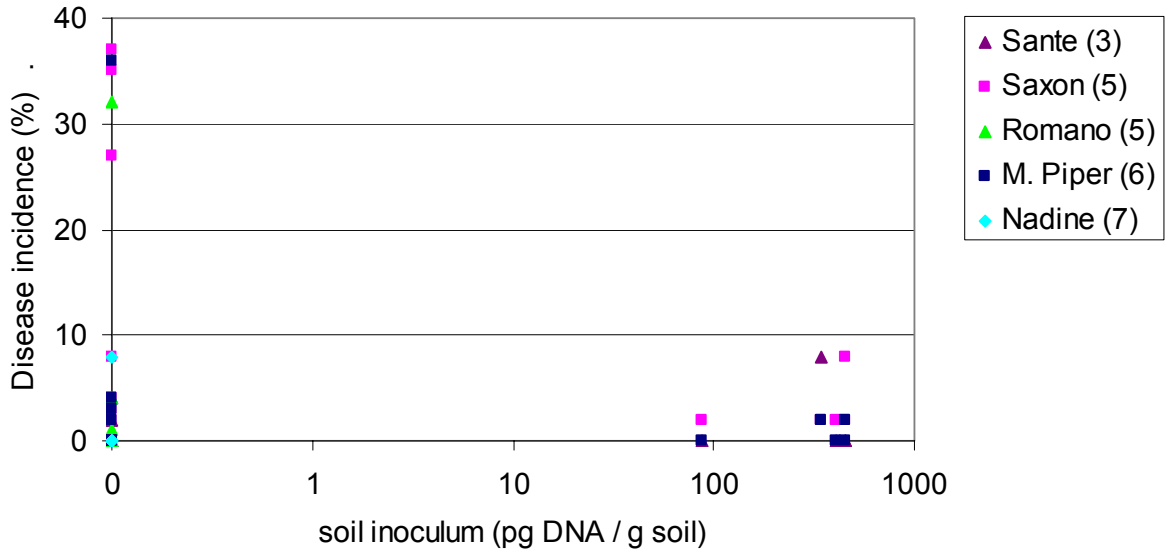


FIGURE 23. THE EFFECT OF SOIL INOCULUM (PG DNA / G SOIL) ON THE INCIDENCE OF BLACK SCURF IN PROGENY CROPS GROWN FROM MINI-TUBERS IN 2005, 2006 AND 2007.

Black scurf in black dot project field trials

None of the soil samples taken pre-planting had detectable levels of *R. solani* inoculum. Low levels of black scurf were detected on progeny tubers at all sites. Seed of two out of the six varieties planted in the field trials had black scurf, however, disease on progeny tubers was not restricted to these two varieties (King Edward and Saxon) at the majority of sites. The possibility that infection can spread from the introduction of contaminated seed through a small field trial area cannot be ruled out as the cause of disease in the uncontaminated varieties, however, its also possible that the disease occurred from undetected soil inoculum.

Factors affecting black scurf

A weak but significant association between the number of years since the last potato crop and the severity of black scurf on daughter tubers was found in 2006. No other significant associations were observed.

TABLE 20. SUMMARY OF LINEAR REGRESSION ANALYSIS BETWEEN SEVERITY AND INCIDENCE OF BLACK SCURF ON DAUGHTER TUBERS AND A NUMBER OF AGRONOMIC FACTORS ASSOCIATED WITH INDIVIDUAL MONITORING CROPS. DATA IS PRESENTED AS PERCENTAGE OF VARIATION EXPLAINED BY THE LINEAR REGRESSION

	2005		2006		2007	
Agronomic factors	Incidence	Severity	Incidence	Severity	Incidence	Severity
Soil inoculum (pg DNA/ g soil)	ns	ns	ns	ns	Ns	ns
Bioassay results	-	-	10.2*	17.3**	Ns	ns
Incidence of disease on seed	ns	ns	ns	ns	Ns	ns
Years since last potatoes	ns	ns	ns	11.6*	Ns	ns
Cultivar resistance rating	ns	ns	ns	ns	Ns	ns
Duration of crop (weeks)	ns	ns	ns	ns	Ns	ns
Time from burn-off to harvest (weeks)	ns	ns	ns	ns	Ns	ns
Irrigation	ns	ns	ns	ns	Ns	ns
Volunteers	ns	ns	16.8**	9.8*	Ns	ns

Significance levels *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$

Problems with *R. solani* detection

When considering the data collected throughout this project, it became evident that there were situations when disease was being found but no source of inoculum had been detected to account for it. Whilst it is recognised that both seed and soil-borne *R. solani* can cause disease, the development of disease in the mini-tuber trials (in the absence of seed inoculum) identifies the problem as being associated with detection of soil-borne inoculum. The ability to detect seed borne inoculum is supported by the finding that over 60 % of seed stocks in each year were found to have *R. solani* contamination, and this is more than the number of stocks with visual black scurf symptoms. However, 21 % or less of soils were found to have detectable levels of inoculum.

Summary of black scurf results

- No relationship was found between either the level of seed or soil-borne inoculum and black scurf on progeny tubers.

- There were a number of crops in which black scurf on the progeny tubers was observed despite no inoculum being detected on either seed or in soil.

- The majority of seed stocks tested had detectable levels of *R. solani* AG-3 contamination (as measured with real-time PCR). More seed stocks had detectable levels of contamination than displayed disease symptoms. It therefore appears unlikely that the assay is not adequately detecting contamination on tubers.

- The development of disease on progeny tubers in the mini-tuber trials, in the absence of soil inoculum, indicates that the detection of soil-borne inoculum may be the limiting factor.

- The real-time PCR assay used in this project reliably detected and quantified the level of *R. solani* in spiked soils. Therefore it is more likely that it is the sampling strategy rather than pathogen detection that is inadequate for the detection of field inoculum.

- Without a method for the reliable detection of soil-inoculum, the diagnostic test cannot be used to predict disease risk.

PMTV and TRV

Detection of PMTV and TRV in soil and seed

Between 12 and 45% of seed stocks tested positive for PMTV in samples collected during 2005 and 2007 (Table 21). Also, during the same period, 0, 20 and 21% of soil samples tested positive for PMTV using a bait test assay (Table 22). The incidence of detection varied markedly (i.e. number of sub-samples from each infected stock or soil sample that tested positive). The vector for PMTV, *S. subterranean*, was detected in 39 to 85% of soils baited using tomato seedlings. In addition, between 12 to 15% of tubers and 5 to 9% of soils tested positive for TRV in samples collected during 2006 and 2007 (Tables 21 and 22).

TABLE 21. THE NUMBER OF SEED TUBER SAMPLES TESTED, THE NUMBER AND PROPORTION OF THE TOTAL WHICH WERE FOUND TO HAVE DETECTABLE LEVELS OF PATHOGEN CONTAMINATION IN SEED TUBER SAMPLES COLLECTED IN 2005, 2006 AND 2007.

		Number of samples tested	Number of samples contaminated (percentage)
PMTV	2005	42	19 (45)[1]
	2006	42	5 (12)
	2007	39	7 (18)
TRV	2005	N/T	N/T
	2006	42	5 (12)
	2007	39	6 (15)

N/T, Not tested
[1] Tested by DAS-ELISA

TABLE 22. THE NUMBER OF SOIL SAMPLES TESTED, THE NUMBER AND PROPORTION OF THE TOTAL WHICH WERE FOUND TO HAVE DETECTABLE LEVELS OF PATHOGEN CONTAMINATION IN SOILS SAMPLED IN 2005, 2006 AND 2007.

Pathogen		Number of soils tested	Number of soils contaminated (percentage)
PMTV	2005	42	0 (0)
	2006	44	9 (20)
	2007	34	7 (21)
TRV	2005	N/T	N/T
	2006	44	2 (5)
	2007	34	3 (9)
S. subterranea	2005	N/T	N/T
	2006	44	17 (39)
	2007	34	29 (85)

N/T, Not tested

Relating inoculum to disease risk for PMTV and TRV

There was an excellent correlation between the detection of PMTV in the combined seed and soil samples, and the detection in progeny tubers where data for the 2006 and 2007 samples were pooled (X^2 =19.69; P<0.001) (Table 23). Within individual years, there was a reasonable correlation between detection of PMTV in the combined seed and soil samples and the detection in progeny tubers in the 2006 (X^2 =17.43, P<0.001) and 2007 crops (X^2 =4.18; P=0.041). The combined data set provided sufficient data points to allow a chi-square test of association between PMTV detection in seed and/or soil, and progeny tubers. However, data from individual years provided insufficient positive values to be analysed by the chi-square test. In those cases where a chi-square test could not be performed, analyses were done using the Fisher's exact test. Detection of PMTV in soil provided less information on the presence of PMTV in progeny crop than the combined seed and soil samples. Nevertheless, association between the detection of PMTV in soil samples and progeny tubers was excellent where data for the 2006 and 2007 samples were combined (X^2 =13.57; P<0.001). However, there was poor correlation between detection in seed only and detection in progeny tubers using the 2006 and 2007 combined data set (X^2 =3.38; P=0.072).

The correlation between the detection of TRV in seed, or seed and soil, and detection in progeny tubers was variable (Table 23). The combined data set for 2006 and 2007 showed that there was a marginal correlation between detection of TRV in seed and detection in progeny (X^2 =8.39; P=0.016); and detection in seed plus soil and detection in progeny (X^2 =6.74; P=0.021). However, care should be taken in interpreting the results as the low number of positive samples means that a chi square test is not possible. Therefore, a Fisher's exact test was used to approximate a chi-square probability.

Despite several stocks testing strongly positive for PMTV and TRV, there was no correlation between detection of virus and spraing symptoms observed in seed or daughter stocks (X^2 = 0.73; P = 0.393; data not shown). Although the second stage of the symptom development (1 week at 5 °C) was outside the optimal range of 6-8°C, it is thought that the change in temperature (18 to 5°C) was sufficient to initiate symptom development in those cultivars likely to show symptoms.

TABLE 23. THE NUMBER OF PROGENY TUBER SAMPLES TESTED, THE NUMBER AND PROPORTION OF THE TOTAL WHICH WERE FOUND TO HAVE DETECTABLE LEVELS OF PATHOGEN CONTAMINATION IN PROGENY TUBER SAMPLES COLLECTED IN 2005, 2006 AND 2007.

Pathogen	Sample year	Number of samples tested	Number of samples contaminated (percentage)	X^2 correlation between virus detection in seed & progeny (probability)	X^2 correlation between virus detection in soil & progeny (probability)	X^2 Correlation between virus detection in seed+soil & progeny (probability)
PMTV	2005	39	7 (18)	-[1]	-[1]	-[1]
	2006	43	13 (30)	2.11 (P=0.147)[2]	12.20 (P=0.001)[2]	17.43 (P<0.001)[2]
	2007	33	9 (27)	1.43 (P=0.232)[2]	2.27 (P=0.161)[2]	4.18 (P=0.041)[2]
	2006-07	76	21 (29)	3.38 (P=0.072)	13.57 (P<0.001)	19.69 (P<0.001)
TRV	2005	N/T	N/T	-	-	-
	2006	43	5 (12)	0.68 (P=0.242)[2]	N/A	2.93 (P=0.148)[2]
	2007	33	5 (15)	9.22 (P=0.017)[2]	N/A	3.41 (P=0.102)[2]
	2006-07	76	10 (13)	8.39 (P=0.016)[2]	N/A	6.74 (P=0.021)[2]

[1] Data unsuitable for analysis because different detection assays used to measure PMTV in seed and progeny tubers.
[2] Fisher's exact test used to determine probability level because of low number of positives.
N/T, Not tested
N/A, Not analysed (insufficient number of positive samples tested)

Erwinia

Three 'erwinia' assays were used: (1) Eca for specific detection of *Pectobacterium atrosepticum* (*Erwinia carotovora* subsp. *atroseptica*) , (2) PEC for total pectolytic bacteria, and (3) Ech for *Dickeya* spp. (*Erwinia chrysanthemi*).

Quantification of '*erwinia*' on seed and progeny tubers

All seed stocks tested in 2006 and 2007 had detectable levels of total pectolytic bacteria and in 2005, 98% of seed stocks tested positive. The incidence of seed stocks with detectable levels of Eca were 71%, 93% and 84% in stocks tested in 2005, 2006 and 2007, respectively (Table 24). In general, the percentage of progeny crops contaminated by Eca was similar or lower than the contamination level in seed. In the 2005 and 2006 crops, the number of progeny tubers contaminated was 63% and 47% respectively (compared with 71% and 93%, respectively, in the seed stocks); in the 2007 progeny tubers, the level of contamination was 82% (seed contamination was 84%). Progeny tuber contamination by total pectolytic bacteria was 98% or 100%.

No seed tuber samples were considered to be infected with *Dickeya* spp. (*Erwinia chrysanthemi*). Suspect results were occasionally observed in 3-9% of progeny tuber samples near the limits of detection (mean CT >38) and in low numbers of subsamples. These results were considered to be false positives and further investigation is required to determine whether improvement to the specificity of the Ech assay is required or whether they could be attributed to laboratory contamination.

TABLE 24. THE NUMBER OF SEED AND PROGENY TUBER SAMPLES TESTED, THE NUMBER AND PROPORTION OF THE TOTAL WHICH WERE FOUND TO HAVE DETECTABLE LEVELS OF 'ERWINIA' CONTAMINATION IN SAMPLES COLLECTED IN 2005, 2006 AND 2007

Year	Number of stocks tested	Number of stocks contaminated with Eca (percentage)	Number of stocks contaminated with PEC (percentage)	Number of stocks contaminated with Ech (percentage)
Seed tubers				
2005	42	30 (71)	41 (98)	0 (0)
2006	42	39 (93)	42 (100)	0 (0)
2007	39	33 (84)	39 (100)	0 (0)
Progeny tubers				
2005	40	25 (63)	40 (100)	1 (3)*
2006	43	20 (47)	42 (98)	4 (9)*
2007	34	28 (82)	34 (100)	3 (9)*

*Results considered to be false positives- see text above.

High throughput testing for '*erwinia*' on multiple stocks

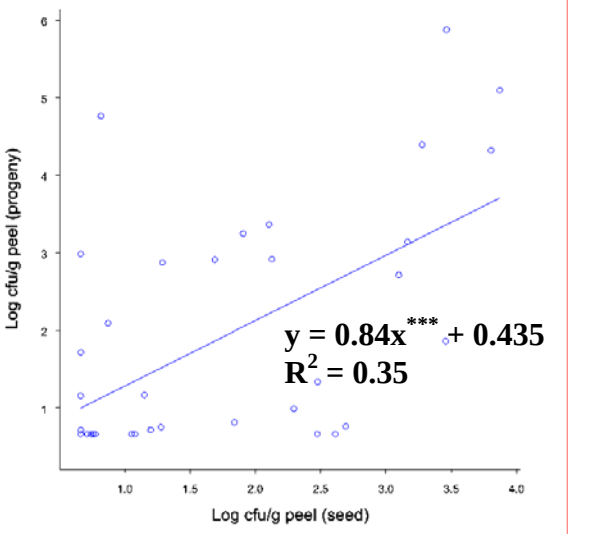
The real-time PCR assays were found to be robust and reproducible and were suitable for high throughput indexing of inoculum load on potato tubers. Over 6000 individual assays were performed on purified DNA extracts using automated DNA extraction and 384-well ABI7900 real-time PCR system with robotic liquid handling. High levels of consistency were observed in quantity and purity of DNA extracted from replicate potato samples. *Dickeya* sp. was not detected in any of the stocks tested. Conversely, *Pectobacterium* sp.were detected in almost all samples tested, although only on very few occasions were high average tuber populations ($>10^5$ cfu per ml) of either *P. atrosepticum* or *P. carotovorum* detected. Results from replicated sub-samples were often but not always similar. The internal PCR control assay indicated reliable and uniform yields of pure DNA from tuber peel extracts, with consistent amplification of the cytochrome oxidase target sequence from co-extracted potato DNA (e.g. mean CT = 19.7 +/- 1.4 $_{n=298}$).

Relating seed-borne inoculum to blackleg risk and levels of contamination in progeny tubers

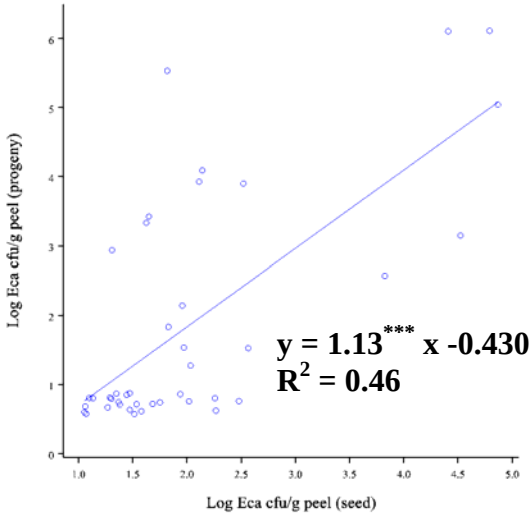
There was good correlation between Eca levels (as measured by PCR cycle thresholds converted to cfu/g peel) in seed and progeny tubers in two out of three years (Fig. 24). In the 2005 and 2006 crops the slopes of the seed Eca levels plotted against progeny Eca levels was 0.84 and 1.13 respectively ($P<0.001$).

The 2007 season was the only year where appreciable levels of blackleg were found in surveyed crops. However, there were only 30 returned blackleg incidence values with which to quantify the correlation between seed-borne contamination by Eca and blackleg. Given this limitation, there was no evidence that blackleg incidence was correlated with numbers of *P. atrosepticum* on seed tubers in 2007 (Fig. 25).

A.



B.



C.

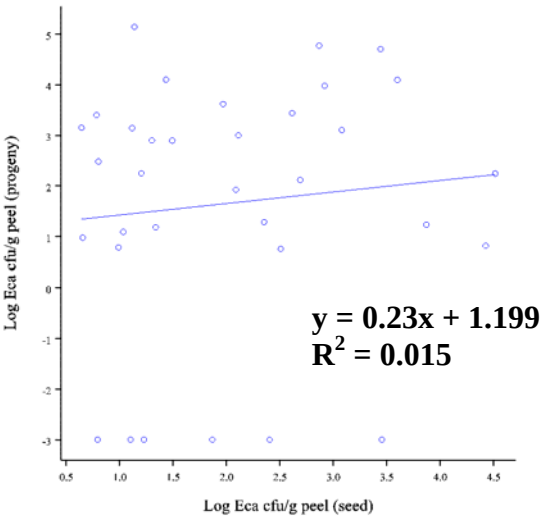


FIGURE 24. THE RELATIONSHIP BETWEEN LOG_{10} COLONY FORMING UNITS (CFU)/G PEEL *PECTOBACTERIUM ATROSEPTICUM* (ECA) ON SEED TUBERS AND CFU/G PEEL ECA ON PROGENY TUBERS DURING A) 2005, B) 2006, AND C) 2007 CROPS. THE SIGNIFICANCE OF THE SLOPE IS DENOTED BY ***, $P \leq 0.001$.

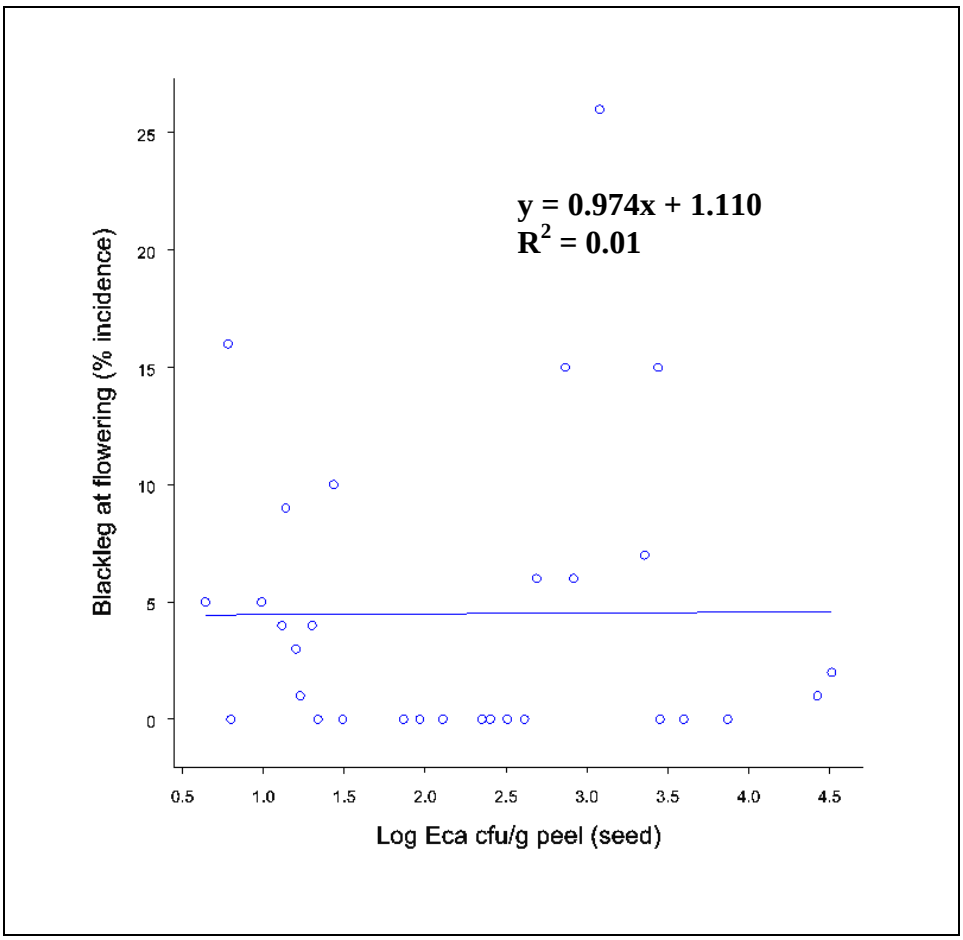


FIGURE 25. THE RELATIONSHIP BETWEEN ECA ON SEED TUBERS AND BLACKLEG INCIDENCE AT FLOWERING IN 2007.

2.4 Discussion

R. solani detection in field soils

When considering the data collected during this project, it became evident that there were situations where black scurf was developing even when no source of inoculum had been detected. Whilst it is recognised that both seed and soil-borne *R. solani* can cause disease, the development of disease in the mini-tuber trials (in the absence of seed inoculum) identifies the problem as being associated with detection of soil-borne inoculum. In contrast to *C. coccodes* and *S. subterranea* which survive in soil as discrete resting bodies (microsclerotia and spore balls respectively), *R. solani* may exist in soils as mycelium, and as such the inoculum source may be very patchy in fields and may also vary in time and space as influenced by various factors, for example, levels of organic matter on which it survives, environmental conditions and the presence of alternative weed or crop hosts.

Another factor that must be taken in to consideration is that the real-time PCR assay used in this project is specific to AG-3. It has been accepted that AG-3 is the *R. solani* anastomosis group predominantly responsible for causing stem canker and black scurf of potato. However, other anastomosis groups can also infect potato. It may be that other AG groups are more important in causing black scurf of potato in the UK than previously thought. The extraction and subsequent storage of DNA from soils in this project provides the opportunity for soil samples to be re-tested for the presence of other AG groups, and in conjunction with the progeny disease data, the relative importance of alternative anastomosis groups could be re-investigated.

Symptomless infections

For all three pathogens investigated in this project, seed stocks with no visual disease symptoms but with detectable levels of pathogen contamination were found. The structure of the monitoring exercise prohibits the investigation of whether such symptomless infections can cause disease, due to the fact that there are very few situations were these seed stocks were planted into soils free of contamination. If however, symptomless infections on seed tubers can lead to disease on progeny tubers or to contamination of previously uncontaminated soil, then this is an important consideration for seed health. Real-time PCR technology could be used to identify such stocks which could be eliminated from high grade seed production.

Commercialisation of diagnostic tests

One aim of the project was to develop real-time PCR protocols for the detection and quantification of seed and soil borne pathogens which could be transferred to either industry "in house" laboratories or commercial service providers. All the protocols developed and used in the project are suitable for transfer. SCRI has successfully transferred the relevant technology to SAC, who already provide the soil diagnostic assays as a testing service. However, it is recognized that if other providers wish to offer the tests as a service and if in future there are modifications to testing procedures, the development of protocols which enable diagnostic testing to be standardized in such a way that would allow assay results to be related back to the disease risk thresholds and interpretation of results obtained in this project would be very beneficial. By providing a basis on which individual service providers can validate there own testing procedures against a standard, it would enable the maximum provision and

therefore hopefully uptake of diagnostic services, which could be beneficial to the potato industry and their ability to manage disease risk.
With this in mind, protocols on the production of standard samples could be developed. The protocols/samples would be used by laboratories to test and understand how their results relate to the 'expected' value. In future if modifications are made to the extraction and detection protocols the protocol/samples would be used to check that the altered methodology/equipment still results in values that can be used in conjunction with the existing risk thresholds e.g. for black dot.

2.5 Interpretation Guidelines

Black dot

A clear relationship between the level of *C. coccodes* soil inoculum, as measured with real-time PCR, and incidence and severity of black dot in field trial sites carried out as part of a Potato Council and SEERAD-funded project, showed that the real-time PCR assay could be used to predict the risk of black dot developing (Figure 26). Differences in black dot development between years showed that environmental conditions also had an impact. In this study, more disease developed in 2004, due to the wetter conditions compared with 2005.

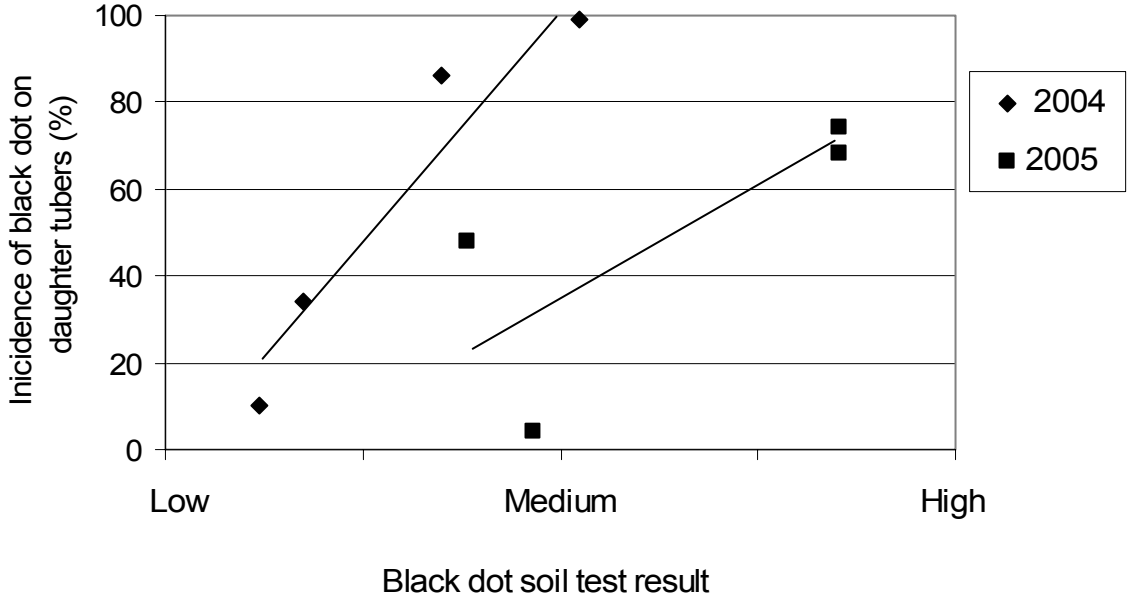


FIGURE 26. RELATIONSHIP BETWEEN SOIL CONTAMINATION, AS MEASURED WITH REAL TIME PCR AND INCIDENCE OF BLACK DOT AT A NUMBER OF FIELD TRIAL SITES USED IN 2004 AND 2005.

The real-time PCR soil test for *C. coccodes* can be used to predict the risk of disease developing

In this study, a clear relationship between soil test results and the resulting disease on daughter tubers assessed over a three year period has been shown (Figure 27). Using the real-time PCR

assay it is possible to quantify amounts of *C. coccodes* DNA in a soil sample and establish thresholds above which the risk of disease development is greater and hence action to control soil-borne black dot must be taken. These include 1) Low risk - below threshold level, 2) Moderate risk - Where control measures are not used or favourable conditions exist disease can be a problem, 3) High risk - High risk of disease developing.

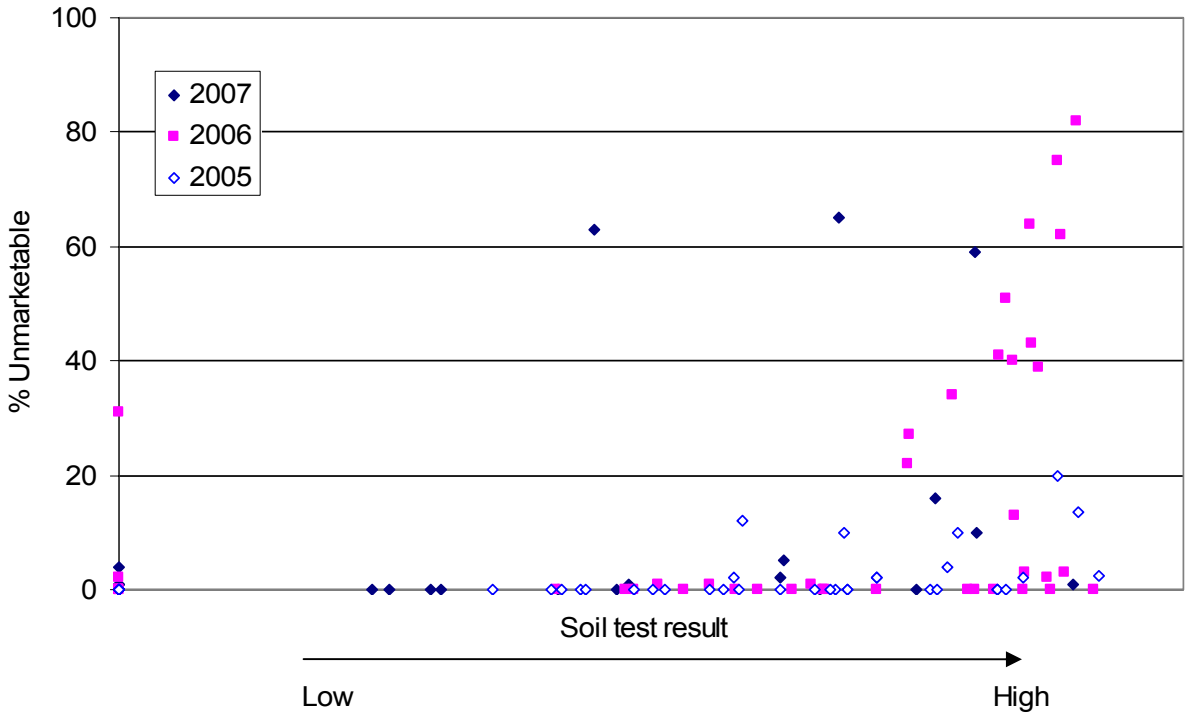


FIGURE 27. RELATIONSHIP BETWEEN SOIL CONTAMINATION, AS MEASURED WITH REAL TIME PCR AND % UNMARKETABLE TUBERS (>10% SURFACE AREA COVERED IN SYMPTOMS) IN CROPS MONITORED IN 2005, 2006 AND 2007.

Threshold levels have been set above which the risk of black dot developing increases

As part of the Potato Council and SEERAD-funded project looking at epidemiology of black dot a number of control options, where soil was found to be contaminated were highlighted. These included: use of more resistant varieties, growing shorter duration crops (late planting and early harvest), application of azoxystrobin and reducing irrigation. Use of a black dot soil test is integral to these control measures and can be used in a number of ways.

- Site selection - Where a number of fields are tested for black dot, those with the highest degree of soil contamination can be avoided. This is particularly useful on rented land where history of cropping may not be known. On land which is not contaminated or where there is a low risk, planting of seed infected with *C. coccodes* should be avoided to prevent further soil contamination.

- Variety choice - Although no variety is completely immune to black dot a number of varieties are moderately resistant. Knowing which fields are contaminated prior to planting means that the most resistant varieties can be used at the most contaminated sites. From the results of 8 field trials replacing Maris Piper (resistance rating 4) with

Sante (resistance rating 5) resulted in just over a 40% disease reduction. Further reductions in disease were observed where Saxon (resistance rating 4) was planted.

- Crop duration - The longer the time between 50% emergence and harvest the greater the risk of black dot developing, particularly where a high level of soil contamination is present. When selecting fields avoid highly contaminated sites for long duration crops. Where planting a crop at a highly contaminated site, ensure this is harvested early to reduce the risk of black dot. In trials, even a delay of 2 weeks in harvest resulted in significant increases in disease.

- Use of azoxystrobin - Amistar has full-approval for control of black dot when applied in-furrow at planting or incorporated prior to planting. In the six trials where it was tested, it resulted in significant reductions overall in incidence and severity. Application of azoxystrobin should only be used where inoculum is present. The results of a soil test can be used to make informed decisions on the use of this product at planting.

- Use of irrigation - In the UK, most ware crops receive irrigation for common scab control and to increase yield. Where applying irrigation at contaminated sites, growers should be aware that the risk of black dot can increase and should pursue other measures to reduce the risk.

Soil test results can be used to make decisions on control measures

Powdery Scab

Powdery scab occurs where the soil is contaminated with spore balls, or in the absence of soil inoculum, where infected seed is planted. In both cases, under conditions favourable for infection, disease can develop even at low levels of inoculum. In this study, where powdery scab occurred on daughter tubers, diagnostic testing found inoculum either on the seed or in soil. Soil-borne inoculum is widespread with 93 out of 113 crops in the monitoring exercise testing positive. In 7 cases, where disease did develop in the absence of soil-borne inoculum *S. subterranea* was found to be present on seed tubers when tested with real-time PCR, however, visual symptoms of disease were not always evident.

A positive soil test result indicates that there is a risk of powdery scab occurring. Where a negative test result is obtained and no inoculum is detected on seed tubers the risk of powdery scab is low.

Relating real-time PCR soil test results to degree of powdery scab development is not possible. It is not the level of spore ball contamination of soil that is the major factor in determining disease development. Rather it is the occurrence of conditions suitable for infection (free water in the soil matrix, temperature range 9-17°C with an optimum at 12°C) during tuber initiation that drives infection.

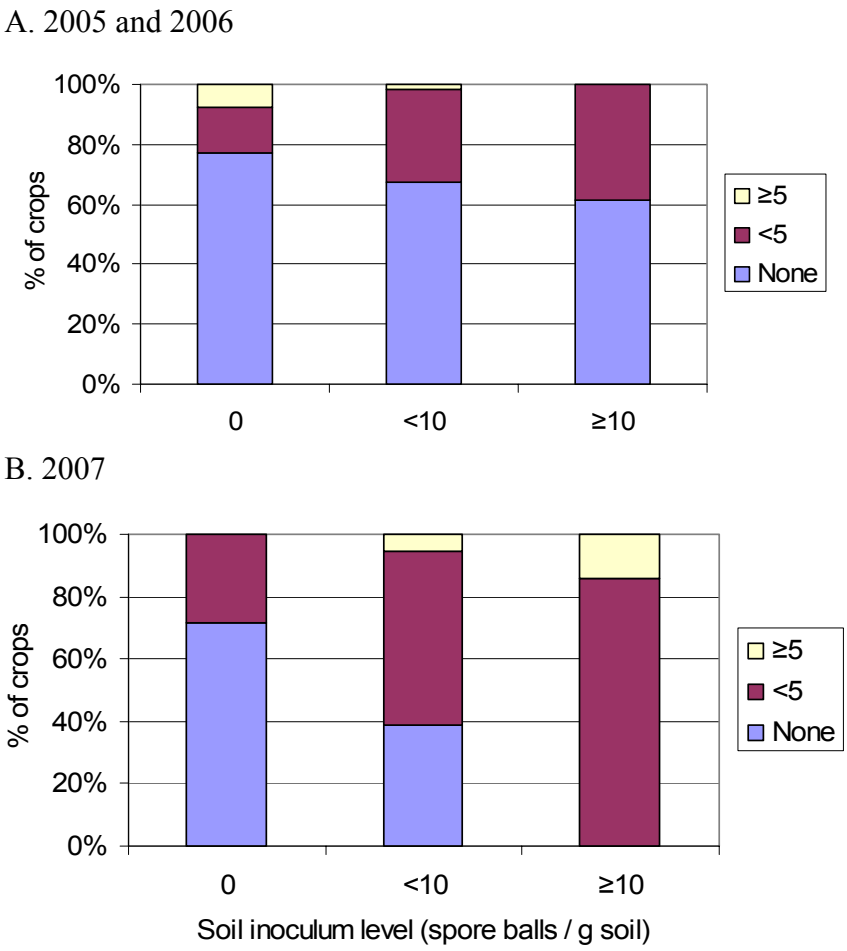


FIGURE 28. THE PERCENTAGE OF MONITORED CROPS IN A. 2005 AND 2006 AND B. 2007 WITH DIFFERENT INCIDENCE LEVELS OF POWDERY SCAB (NONE, < 5 AND ≥ 5 %) ON PROGENY TUBERS WHEN GROWN AT SITES WITH VARYING LEVELS OF SOIL INOCULUM (0, <10 AND ≥10 SPORE BALLS/ G SOIL).

In 2005 and 2006 the number of crops developing powdery scab was low due to the dry soil conditions in these years. There is evidence from these studies (and from commercial experience) that infection can still occur where conditions are sub-optimal. Thus in 2006 when soil conditions were extremely dry throughout tuber initiation in un-irrigated crops in Scotland, powdery scab was still recorded in a few crops. In 2007 the proportion of crops which became diseased was greater than in 2005 and 2006 due to wetter soil conditions, even in non-irrigated crops. In these favourable conditions the risk of powdery scab developing increased in soils contaminated with greater than 10 spore balls / g soil. Powdery scab developed at all sites at which soil contamination was above this level in 2007 (Figure 28B).

Varietal resistance was effective in reducing disease and should be considered for the management of powdery scab.

Environmental conditions determine degree of powdery scab development. When conditions favourable for powdery scab exist, the risk of disease development is greater at levels of soil inoculum of 10 spore balls / g soil or more. Real-time PCR testing of soil can not be used to determine level of powdery scab development when conditions are not conducive to disease development.

Real-time PCR testing of soils can be used in a number of ways:-

- Where a range of fields are tested and a range of varieties with different disease resistance ratings for powdery scab are to be grown, the results of the diagnostic test can be used to assign varieties to specific fields. Thus, a susceptible variety can be considered for a field where powdery scab is not detected. Conversely, a resistant variety can be placed in a field where inoculum levels are higher.

- A field in which high spore ball contamination level is detected (>10 spore balls / g soil) might be avoided for potato production, even where a resistant variety is considered, especially on a farm with history of powdery scab or a field/location where conducive conditions are likely to occur frequently.

In assigning varieties to fields in order to minimise risk of powdery scab, other factors must be borne in mind:-

- The history of powdery scab on a farm. Survey work and experience suggests that occurrence of powdery scab on a farm means that the risk of powdery scab developing is likely.

- Soil type. The soil type must be suitable for the end market, for example, it must be suitable to achieve an acceptable skin finish if pre-packing is the target market. Soil type may affect powdery scab development, disease may be more likely to occur on lighter soils (sands - sandy loams) than heavier soils (silts to clays).

- Drainage. If soil drainage in a field is not 'good', the likelihood of free water persisting in the soil matrix is higher and the risk of powdery scab greater.

- A seed grower may use the result of the diagnostic test to make an informed decision about the use of Shirlan (SOLA) soil treatment. Such a treatment could be beneficial where powdery scab is detected at relatively low levels, < 10 sporeballs/g soil and a

susceptible or moderately susceptible variety (disease resistance ratings of 1-5) is to be grown.

Black scurf

In the real-time PCR diagnostic test, no relationship was detected between the results of the soil test and either stem canker or black scurf on the progeny crops.

Tests using artificially contaminated soils in the laboratory have shown that the diagnostic test can be related to level of soil inoculum. However, the development of black scurf in fields where no soil-borne inoculum was detected and where seed-borne inoculum was not found would suggest that soil sampling is not adequate for detection.

Experience and studies by Kyritis (2005) suggest that the sampling strategy used may be a factor inhibiting the value of the test. Powdery scab and black dot exist in the soil as discrete resting bodies (sporeballs and microsclerotia respectively). Harvesting and soil cultivations between potato crops spread the resting bodies through the soil. Whilst the occurrence of these two pathogens will fluctuate across a field, the sampling strategy adopted in the diagnostic project appears to ensure that both pathogens, if present, will be detected. Furthermore, the level detected (particularly for black dot) is a good guide to what disease may develop. By contrast, soil-borne *Rhizoctonia solani* AG3, whilst it may be present as sclerotia, may also be present as mycelium. As such, the dynamics of *R. solani* survival and distribution in soil are more complicated. In field experience suggests that *R. solani* exists in foci. Within these foci the mycelium of the fungus persists, normally living on organic matter.

Cultivations will spread the pathogen and distort the shape of a focus and may affect its survival where the organic matter upon which it survives is also dispersed. To survive, mycelium of *R. solani* must continue to grow as old mycelium dies naturally. Thus *R. solani* tends to remain as foci that change in shape and extent between potato crops. To incite stem canker a threshold of mycelium must be present within the focus (Kyritis, 2005).

When sampling using the protocol established in the black dot and diagnostics projects, the discrete nature of foci of the pathogen may mean that the fungus is not always included in the sample drawn. PCN also occurs in foci and the sampling strategy adopted in the project followed that for PCN. However, the fact the PCN cysts survive in the soil in the same (inert) way as black dot microsclerotia or powdery scab sporeballs suggest that the sampling strategy for these pathogens is adequate. However, it may be inadequate for the living mycelium of *R. solani*.

Another issue for *R. solani* is that because it survives largely as mycelium, its occurrence may be affected by environmental conditions. *R. solani* is sensitive to a lack of oxygen, and water-logging for example may reduce inoculum levels. Conversely, warm dry conditions may stimulate growth of mycelium. Thus the date of sampling and the weather conditions beforehand may have a significant effect on the extent of *R.solani* mycelium present in soil.

Further studies are required to establish better sampling guidelines for this pathogen.

Potato MopTop Virus

Soil-borne inoculum was shown to be linked to progeny contamination in one out of the two years where PMTV was detected in soil (i.e. 2006 and 2007). The correlation between detecting PMTV in soil and detecting virus in progeny tubers was improved by also testing seed tubers. Thus, seed and soil-borne inoculum was shown to be linked to progeny contamination in both years where PMTV was detected in soil. This illustrates that seed infected with PMTV has the potential to either directly infect progeny tubers or contaminate soils that have *S. subterranea* infestations.

It is not possible to draw any conclusions from the 2005 data. There are two reasons for this. Firstly, seed tubers were tested using the 'growing on' method, whereby leaves produced from germinating seed tubers were tested by DAS ELISA. The soils and progeny tubers were tested by PCR. Therefore, a direct comparison of the results using different detection methods was not possible. Secondly, none of the 42 soils tested in 2005 had detectable levels of PMTV. No follow-up testing was done to confirm whether these were true negative results or false negatives as a result of early assay development.

- The PTMV bioassay allows field soils contaminated with the PMTV virus to be identified.

- Where PTMV and the vector, *S. subterranea*, are detected in soil, PTMV-susceptible varieties should be avoided in the field.

- Where PTMV is detected in a soil test, transmission to the potato crop will depend on conditions being suitable for infection by the vector, *S. subterranea*.

- It is advisable to have seed tubers, as well as soil, tested for the presence of PMTV to provide a more accurate estimate of risk from PMTV infecting progeny tubers.

Erwinia

During the 2005, 2006 and 2007 seasons either 98 or 100% of all seed stocks had detectable levels of total pectolytic bacteria and between 71 and 93% of seed stocks tested positive for Eca. The real time PCR assays permitted rapid and accurate quantitation of *P. atrosepticum* mean inoculum loads per seed stock, from which the mean cfu per g of peel could be calculated. In all 3 years the inoculum loads on seed stocks were found to be uniformly low, rarely exceeding 10^4 cfu per g peel and mostly under 10^3 cfu per g peel. Previous studies (Perombelon, 2000; Toth *et al*., 2003) have indicated the risk of blackleg to be low in crops with such low tuber inoculum loads, especially in non-conducive seasons. This in fact was found to be the case in 2005 and 2006 seasons. In 2007, the higher blackleg incidence recorded in the field was more likely correlated with wetter spring conditions than with tuber inoculum levels. Continued assessment of inoculum loads on seed stocks and resulting blackleg incidence will be required before the accuracy of disease prediction can be determined from seed inoculum. This will allow identification of stocks with a greater range of seed inoculum levels and measurement of resulting disease incidence and severity over several seasons, under extremes of climatic conditions, soil types and potato varieties. Only then will it be possible to increase the accuracy of disease prediction.

There was a good correlation between Eca levels on seed and bacterial numbers on progeny tubers in samples collected during 2005 and 2006. It is likely that during 2005 and 2006, which were considered to be average or below average in terms of rainfall, bacterial numbers on seed tubers were not able to multiply to a large extent and, therefore, this was reflected in the levels found on progeny tubers. During 2007, an extremely wet season during the months of May, June and July, bacterial numbers would have been able to multiply considerably and this is reflected in the poor relationship between the level of Eca contamination on seed and on progeny tubers.

No seed or progeny tuber samples were considered to be contaminated with *Dickeya* spp. (*Erwinia chrysanthemi*). Suspect results were occasionally observed in 3-9% of progeny tuber samples near the limits of detection (mean CT >38) and in low numbers of subsamples. These results were considered to be false positives and further investigation is required to determine whether improvement to the assay specificity is required or whether they could be attributed to laboratory contamination. New research is planned in which the Ech assay will be compared with a second *Dickeya*-specific real-time PCR assay developed independently in the Netherlands (J. van der Wolf, personal communication). In addition, genomic sequencing of isolates of all *Dickeya* species is planned at SCRI. This will permit selection of further appropriate primers and probes if higher specificity is required.

2.6 Conclusions

Assay development

- A new method for co-extracting DNA and RNA (Nucleic acid – NA) from tuber sap has been developed. This enables for the first time a single extraction procedure for fungal, bacterial and viral pathogens to be carried out, substantially reducing the time and cost of carrying out assays for multiple target pathogens.

- Questions over the specificity of the original *Erwinia carotovora* subsp. *atroseptica* (Eca) assay have been addressed with the development of a new set of primers and probes which are specific for *Pectobacterium atroseptica* previously known as *Erwinia carotovora* subsp. *Atroseptica* (Eca).

Soil and stock monitoring

- In all three years of monitoring, the direct extraction and quantification of *C. coccodes*, *S. subterranea* and *R. solani* in soil samples using real-time PCR revealed that soils were commonly contaminated with *C. coccodes* and *S. subterranea,* whilst only a small proportion of soil samples tested were found to have detectable levels of *R. solani*. The proportion of contaminated soils and the range of contamination were generally similar in the three years for each of the three fungal pathogens.

- A bioassay for *R. solani* currently carried out at SAC, does not appear to be more reliable in detecting and quantifying levels of *R. solani* in field soils than the real-time PCR diagnostic test.

- The real-time PCR assay and the bioassay for the detection of *R. solani* in soil do not appear to be reflecting the presence of *R. solani* in field soils reliably. We are confident that inoculum present in the soil sample can be detected by the real-time PCR assay (based on detection and quantification of inoculum in spiked soil samples). However, the current soil sampling strategy does not appear to be adequate in reflecting the field soil as a whole.

- When the detection of *S. subterranea* following direct extraction of DNA from soil was compared with the bait method it was found that the direct extraction method was more sensitive than bait testing, in addition to being a fully quantitative test.

- The proportion of stocks infected with the three soil-borne pathogens was similar in the three years.

- During 2006 and 2007, a bioassay detected PMTV in approximately 20% of soils and TRV in 5 to 9% of soils. Direct tuber testing, using PCR, detected PMTV in 12 to 18% of seed tubers and TRV in 12 to 15% of seed tubers.

- Results from the testing of progeny tuber stocks for 'erwinias' showed that during the 2006 and 2007 seasons, all, or 98%, of seed stocks had detectable levels of total pectolytic bacteria and between 71 and 93% of seed stocks tested positive for Eca (*Pectobacterium atrosepticum*). No seed or progeny tuber samples were considered to

be infected with *Dickeya* spp. (*Erwinia chrysanthemi*) however further investigation is underway to determine whether observed false positive results were due to insufficient assay specificity and/or laboratory contamination.

Investigations into predictive diagnostics and disease risk

- Data from this project supports the results of the associated black dot project (R249), suggesting that the level of soil inoculum is linked to disease risk i.e. soil inoculum levels below 100 pg DNA / g soil are associated with a low risk of disease, whilst soil inoculum levels between 100 and 1000 pg DNA / g soil are associated with an increased (medium) risk of black dot development, whilst levels of soil inoculum greater than 1000 pg DNA / g soil carry a high risk of disease.

- No correlations were found between levels of seed or soil-borne inoculum and powdery scab development in 2005 and 2006. These two years had relatively dry summers and little powdery scab was found in progeny crops. In contrast, 2007 had a relatively wet summer, and consequently powdery scab was more common in this year than the two previous years. In 2007, the level of soil inoculum could be categorised according to level of disease risk. Low risk soils being those with no detectable inoculum, medium risk soils those with detectable levels of inoculum less than 10 spore balls / g soil, and high risk soil being those with 10 spore balls or more / g soil.

- It is believed that the sampling strategy may not be adequate for the detection of *R. solani* in field soils. The occurrence of black scurf on progeny grown from mini-tubers in soils in which no inoculum was detected highlights the problem. New sampling strategies should be considered to investigate whether practical sampling protocols can be formulated which enable the reliable detection of soil-borne *R. solani* inoculum. In addition, the assumption that majority of black scurf is caused by AG-3 in the UK should be re-investigated, to ensure that all relevant anastomosis groups of *R. solani* are being considered.

- Soil-borne PMTV inoculum was shown to be linked to progeny contamination. However, the correlation between detecting PMTV in soil and detecting virus in progeny tubers was improved by also testing seed tubers. This illustrates that seed-borne PMTV has the potential to either directly infect progeny tubers or contaminate soils that have *S. subterranea* infestations.

- Real-time PCR analysis allowed high throughput, robust and accurate detection, quantification and identification of *Pectobacterium* and *Dickeya* populations on potato tuber stocks.

- Although the incidence of infection (% stocks infected) was high, the average inoculum load of *Pectobacterium atrosepticum* (*Erwinia carotovora* subsp. *atroseptica*) on seed from each stock was generally low in all 3 years studied. Blackleg incidence was correspondingly low in 2005 and 2006 but was higher in 2007 in response to wetter spring and early summer weather. Further data from a wider variety of seed stocks with a higher range of inoculum levels is needed before the accuracy of blackleg prediction under varying climatic conditions can be assessed.

- Some correlation was observed between *Pectobacterium atrosepticum* levels on seed and on progeny tubers in two out of three years. No correlation was found in 2007 when bacterial numbers probably increased rapidly under wetter conditions irrespective of the initial inoculum level on the seed.

- A comparison of visual assessments with real-time PCR assays for the fungal pathogens (*C. coccodes, S. subterranean* and *R.* solani) on seed and progeny stocks, showed that for all three pathogens real-time PCR detected contamination on more stocks than visual examination. This demonstrates the existence of symptomless infection of the fungal pathogens. We were unable to ascertain the importance of such infections in causing disease within this study. Ascertaining the frequency of such symptomless infections could be important to high grade seed producers and exporters in identifying potential sources of disease.

Investigations into the effect of various agronomic factors on disease risk

- An analysis of the agronomic factors that affect disease development has been carried out. However, this analysis relied upon having all the crop and agronomic information relevant to each stock which was monitored. This was not always the case and this has influenced the quality of such an analysis. In the Potato Council and SEERAD-funded project "Developing effective integrated control measures for the control of black dot - R249" a combination of controlled environment experiments and field trials was used to investigate the effects of agronomic factors and control measures on the levels of disease. Results from the project have enabled diagnostics results to be interpreted in terms of predicting black dot. Similar experimental work on powdery scab and particularly black scurf would benefit the interpretation of diagnostic results for these pathogens.

2.7 References

Cullen, D. W., Lees, A. K., Toth, I. K. and Duncan, J. M. (2001). Conventional PCR and real-time quantitative PCR detection of *Helminthosporium solani* in soil and on potato tubers. *European Journal of Plant Pathology*. **107**: 387-398.

Cullen, D. W., Lees, A. K., Toth, I. K. and Duncan, J. M. (2002). Detection of *Colletotrichum coccodes* from soil and potato tubers by conventional and quantitative real-time PCR. *Plant Pathology*. **51**: 281-292.

De Boer, S. H., and Ward, L. J. 1995. PCR detection of *Erwinia carotovora* subsp. *atroseptica* associated with potato tissue. *Phytopathology* **85**:854-858.

Ko, W.H. and Hora, F.K. 1971. A selective medium for the quantitative determination of Rhizoctonia solani in soil, *Phytopathology* **61**, 707-710.

Kyritis, P (2003) Epidemiology and pathogenesis of mycelial soil-borne *Rhizoctonia solani* AG3 on potatoes (*Solanum tuberosum).* PhD Thesis. University of Aberdeen.

Lees, A. K., Cullen, D. W., Sullivan, L. and Nicholson, M. J. (2002). Development of conventional and quantitative real-time PCR assays for the detection and identification of *Rhizoctonia solani* AG-3 in potato and soil. *Plant Pathology* **51**:293-301

Mumford, R.A., K. Walsh, I. Barker, N. Boonham (2000): Detection of *Potato mop top virus* and *Tobacco rattle virus* using multiplex real-time fluorescent reverse-transcription polymerase chain reaction assay. *Phytopathology*. **90**: 448-453.

Perombelon, M.C.M. 2000. Blackleg risk potential of seed potatoes determined by quantification of tuber contamination by the causal agent and *Erwinia carotovora* subsp. *atroseptica*: a critical review. *Bulletin OEPP/EPPO Bulletin 30; 413-420*.

Toth, I. K. Sullivan, L. Brierley, J. L. Avrova, A. O. Hyman, L. J. Holeva, M. Broadfoot, L. Perombelon, M. C. M. McNicol, J. 2003. Relationship between potato seed tuber contamination by *Erwinia carotovora* ssp. *atroseptica*, blackleg disease development and progeny tuber contamination. *Plant Pathology. 52: 2, 119-126.*

van de Graaf, P., Lees, A. K., Cullen, D. W. and Duncan, J. M. (2003). Detection and quantification of *Spongospora subterranea* in soil, water and plant tissue samples using real-time PCR. *European Journal of Plant Pathology* **109**: 589-597

Appendix 1

Review of sampling strategies

Jacqueline M. Potts
Biomathematics and Statistics Scotland

There is very little literature on optimal sampling strategies for potato diseases but there is an extensive literature on sampling for nematodes in potatoes and other crops and many of the same general principles apply. However, without knowledge of the anticipated mean levels and degree of aggregation of the particular diseases being studied it is impossible to determine the accuracy of any given sampling scheme and thus the sample size required.

The usual procedure in sampling for nematodes is to take many cores at random or regularly spaced locations over the study area and bulk these to form a sample. Nematodes along with plant diseases are not usually distributed randomly but have an aggregated or contagious distribution. This may be described using a clumped distribution, such as a negative binomial or Neyman Type A distribution, or by Taylor's Power Law. An alternative approach is to use geostatistical techniques. However, this relies on a large number of samples being taken at different locations and analysed separately rather than being bulked. It is therefore too costly to be used on a routine basis.

If the standard method for sampling for potato cyst nematodes is to be adopted for soil-borne plant pathogens there are then three decisions to be taken: the collecting pattern, the number of samples to be taken (or the maximum area from which a single sample should be taken before the field is subdivided) and the number of cores to be taken. Statutory sampling for potato cysts nematodes in Scotland currently collects samples randomly in a W-shaped configuration, uses a maximum area of 4ha and enough cores to provide about 1kg of soil.

A W-shaped path or similar pattern has the advantage that samples are easier to collect than if random sampling or stratified random sampling is used. Basu *et al*. (1977) compared three methods of sampling alfalfa foliage diseases: sites located on a W-shaped path covering the entire field, the same number of sites along a W-path in a smaller area of the field chosen at random and sites along the diagonal of this area. They concluded that it is important to take samples from the entire field because samples from a small portion of the field may not be representative. They also found that a W-shaped path is better than a diagonal one because it gives a wider spread of sampling sites. Lin *et al*. (1979) used simulated data again to compare sampling methods that differed with respect to area covered (whole field vs. one-eighth of field) and shape of path (X, W or diagonal). For clustered disease distributions the X and W paths over the whole field gave the smallest variability, the diagonal path the next largest and the X and W paths over a portion of the field the largest. Even a W-shaped collection pattern still leaves large areas of the field unsampled. For nematode sampling in a uniform crop Barker and Campbell (1981) recommended a regular pattern collected while traversing a zig-zag, but this could give biased results if features which influence density run in parallel lines between the sampling points and it may therefore be less suitable for crops like potatoes which are grown in rows. Francl (1986) found that a systematic zig-zag pattern with samples taken only from plant rows produced biased estimates of mean density over the whole plot, although

this could be avoided by taking samples between the rows as well as in the rows. Francl (1986) found that a ziz-zag over the entire study area had a percent average deviation that is comparable with random sampling. Another way of overcoming the problem of bias is to use stratified unaligned sampling. One variation of this uses a zig-zag pattern but the location on each leg where the core is taken is chosen at random (Boag *et al*., 1992).

For populations that can be described by the negative binomial model the number of cores required to provide an estimate within a specified range of the true mean with a specified confidence level may be determined from the following formula (Ferris, 1984)

$$n = (Z_{\alpha/2})^2 \frac{(1/\bar{x} + 1/k)}{D^2}$$

where n is the number of samples, $Z_{\alpha/2}$ is the upper $\alpha/2$ point of the standard normal distribution, $\bar{x}$ is the population mean, k is the dispersion parameter of the negative binomial distribution and D is half the length of the acceptable confidence interval as a proportion of the mean. Taylor's power law states that in aggregated populations the variance is an exponential function of the mean, that is

$$s^2 = a\bar{x}^b$$

The corresponding formula for the number of cores required (Ferris, 1984) is then

$$n = (t_{\alpha/2})^2 \frac{a\bar{x}^{(b-2)}}{D^2}$$

where $t_{\alpha/2}$ is the upper $\alpha/2$ point of Student's t distribution with the appropriate degrees of freedom depending on the number of samples. However, in order to apply these formulae some knowledge of the likely values of $\bar{x}$ and the parameter k or the parameters a and b is required. Approaches based on the analysis of large numbers of separate samples from a field are too costly for general use. However, some limited experiments of this type on potato diseases are needed in order to determine the likely values of these parameters and so design appropriate sampling schemes.

Barker et al. (1985) state that sampling areas of approximately 2ha or less are usually recommended for advisory purposes and that in sampling larger fields 2-ha sampling units should be selected randomly from areas that have a uniform cropping history and soil type. McSorley and Parrado (1982) suggest that populations in fields of more than 1ha in size are difficult to estimate because of the large number of cores that must be taken. They found that k values for fitted negative binomial distributions normally show a gradual increase as field size decreases. Thus fewer cores are required to estimate populations in smaller fields. If k is very low the field may have to be subdivided into smaller units for individual sampling. They also noted that there are cases where a single composite sample of multiple cores may be impractical even in a small field unit and that in these cases more efficient sampling schemes must be developed by comparing combinations of several composite samples each containing multiple cores.

Another approach to the detection of potato cyst nematodes has been developed in the Netherlands by Been and Schomaker (1996). Data from about 40 infested fields that had been sampled intensively using a sequential approach were used to develop a model which was then used to determine the combination of grid spacing and sample size required to detect a standard focus (a focus with a central population density of 50 cysts/kg soil) in a field of size 0.33ha with 90% probability. They found that the wider the grid, the larger the soil sample needed to detect the standard focus with 90% probability. Foci of infestation often have an oval shape since dispersion depends largely on the activities of farmers and an increase in grid size in the direction of cultivation is therefore less sensitive than the same increase perpendicular to that

direction. When sample sizes are adjusted to give the same average detection probability the variance of the detection probability is greater the wider the grid. The statutory soil sampling method in the Netherlands is a 7.5 x 7.5 m grid and a core size of 3.3g (sample size 200g/0.33 ha). This has less than 10% probability of being able to detect a standard focus. For the detection probability to be 90% under this statutory method the density at the centre would have to be around 5000 cysts/kg. Instead Been and Schomaker recommend a 5 x 5 m sampling grid and a core size of 52g (sample size 6.9 kg/0.33 ha) as the best compromise between minimizing the sample size and the variance of the detection probability on the one hand and the time needed to collect and process the samples on the other.

Composite bulk samples are sometimes mixed and subsampled (Francl, 1986). Subsampling contributes to the uncertainty of mean estimation. Nevertheless if the bulk sample can be well-mixed so that the distribution is close to random and the cost of processing a large volume of soil is high relative to the cost of collecting additional cores then there are advantages to collecting a larger number of cores and subsampling from the bulk sample rather than collecting a smaller number of cores and processing the entire sample. Alternatively in this situation, instead of subsampling, a larger number of cores could be taken with a reduced core size.

If the aim is to map the distribution of infestation using geostatistical techniques samples must be taken at each point on a regular grid and kept separate. Evans et al. (2003) found that spatial independence in potato cyst nematode counts occurred at about 60m, although there was also evidence of spatial independence at a range of 10-20m in intensively sampled areas of the field. Evans and Barker (2004) conclude that since geostatistical analyses of several fields showed that population densities were correlated over a range of separations of 37-80m, samples must be taken at less than this range for the generation of reliable maps and they therefore opted for a sample separation of 20m on a regular grid. By drawing maps from the same dataset ignoring some of the points they showed that the longer range variation in the 20m map could be seen in the 40m map, but an increasingly different pattern is observed with more widely spaced sampling grids. A treatment map derived from the 100m sampling grid would provide a poor estimation of the true distribution.

Analysis of previously collected data on the spatial distribution of *Rhizoctonia solani*

Gilligan, Simons and Hide (1996) examined the incidence and severity of stem canker on individual plants in a row from plots with different cropping history. The degree of spatial correlation varied with the cropping history. Levels were similar over short distances (1-5 plants, 0.4-1.9 m). Beyond this distance there was evidence of larger clusters in certain transects of the order of 12-20 plants (4.5-7.5 m) and occasionally >30 plants (>11.25m). There was also some evidence of short-scale patchiness within larger patches.

In a previous project (Wale, van de Graaf and Lees, 2004) two fields were intensively sampled at 25 points on a grid to determine levels of contamination with soil-borne *Rhizoctonia solani* (AG-3). PCR detection methods only detected any contamination at a very small number of sites but higher levels of contamination were detected using a visually assessed beetroot seed bioassay. However, results were not consistent between replicates. Using Moran's I to test the visually assessed data one of the four replicates for the Moray field showed significant autocorrelation as did one of the six replicates for the Aberdeenshire field. Taking the replicates together, incidence was significantly autocorrelated for the visually assessed data in the Moray field and for the bait seed DNA method in the Aberdeenshire field. Thus we found no evidence of different degrees of spatial autocorrelation in the two field despite the wider

grid spacing (25m) in the Moray field than in the Aberdeenshire field (5m). However, there are insufficient data available to enable us to reach any conclusions about the spatial distribution of *Rhizoctonia solani*.

References

Barker K.R. and Campbell C.L. 1981. Sampling nematode populations. In: B.M. Zuckerman and R.A. Rohde (Editors), Plant Parasitic Nematodes. Vol. III. Academic Press, New York, pp.451-474.

Barker K.R., Schmitt D.P. and Noe J.P. 1985. Role of sampling for crop-loss assessment and nematode management. *Agriculture, Ecosystems and Environment*, **12**: 355-369.

Basu P.K., Lin C.S. and Binns M.R. 1977. A comparison of sampling methods for surveying alfalfa foliage diseases. *Canadian Journal of Plant Science* **57**: 1091-1097.

Been T.H. and Schomaker C.H. 1996. A new sampling method for the detection of low population densities of potato cyst nematodes (*Globodera pallida* and *G. rostochiensis*). *Crop Protection* **15**: 375-382.

Boag B., Neilson R. and Brown D.J.F. 1992. Nematode sampling and prediction. *Nematologica* **38**: 459-465.

Evans K. and Barker A.D.P. 2004. Economies in nematode management from precision agriculture – limitations and possibilities. *Nematology Monographs & Perspectives* **2**: 23-32.

Evans K., Webster R., Barker A., Halford P., Russell M., Stafford J. and Griffin S. 2003. Mapping infestations of potato cyst nematodes and the potential for spatially varying applications of nematicides. *Precision Agriculture* **4**: 149-162.

Ferris H. 1984. Probability range in damage predictions as related to sampling decisions. *Journal of Nematology* **16**: 246-251.

Francl L.J. 1986. Improving the accuracy of sampling field plots for plant-parasitic nematodes. *Journal of Nematology* **18**: 190-195.

Gilligan C.A., Simons S.A. and Hide G.A. 1996. Inoculum density and spatial pattern of *Rhizoctonia solani* in field plots of *Solanum tuberosum*: effects of cropping frequency. *Plant Pathology* **45**: 232-244.

Lin C.S., Poushinsky G. and Mauer M. 1979. An examination of five sampling methods under random and clustered disease distributions using simulation. *Canadian Journal of Plant Science* **59**: 121-130.

McSorley R. and Parrado J.L. 1982. Estimating relative error in nematode numbers from single soil samples composed of multiple cores. *Journal of Nematology* **14**: 522-529.

Wale S.J., van de Graaf P. and Lees A.K. 2004 Detection and distribution of soil-borne *Rhizoctonia solani*. Unpublished project report.