Final Report

Extending the Scope of Modern Potato Diagnostics and their Interpretation

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1. SUMMARY

This project investigated four aspects of potato disease diagnostics that remained unclear from two previous diagnostic projects.

Whilst a successful soil sampling protocol has been established for detection and quantification of black dot and powdery scab, it was clear from testing commercial fields that it was inadequate for *Rhizoctonia solani*. For example, on occasions, disease developed where the pathogen was not detected in a soil test. The first part of this project examined improved soil sampling options for *Rhizoctonia*. An initial 15 fields were sampled using the standard protocol for black dot and powdery scab and tested for the presence of *R. solani* AG3 (the main pathogen causing stem canker and black scurf). From the results two fields were chosen for intensive sampling to ascertain the pattern of distribution of the pathogen. From an analysis of the distribution and using data from elsewhere, four new sampling protocols were tested along with the existing protocol in three fields. At the same time optimum sampling depth was examined.

There were inconsistencies between laboratories in the comparison of sampling protocols although there was a trend to improved detection with more intensive sampling. However, the inconsistencies of detection suggest that, currently, further understanding is required about the distribution and epidemiology of the pathogen before a sampling procedure can be recommended and commercial testing and interpretation is possible. It is suggested that low levels of inoculum that may escape detection may be sufficient to cause disease, if environmental conditions favour development of the pathogen. Despite the inconsistencies, future sampling will continue to sample from a 4 ha block but adopt increased sampling points (200) and sample only from the top 5cm of soil.

The same samples in the intensive sampling and comparison of sampling procedures were also tested for presence of the powdery scab and black dot pathogens. This additional testing has provided data on the distribution of these two pathogens in soil but also revealed that no sampling method showed improved consistency for detecting them over the current standard. The relative level of contamination in each of the three soils was consistent irrespective of sampling method, especially for powdery scab.

It was proposed that real-time PCR assays developed at Fera for different *R. solani* anastomosis groups (AG 2.1, 4 and 5) would be used to test DNA extracted from soil samples in previous projects by SCRI. However, preliminary testing revealed that only high levels of DNA could be detected (200 pg DNA or more) and thus effective quantification of inoculum in soils was difficult. Soils collected during the Potato Council-funded diagnostics project (R253) were re-tested for AG 2.1, and 7 out of 108 had detectable levels of AG 2.1. However there was no relationship between the detection of AG 2.1 in soils and the occurrence of disease. Of 35 soils tested for AG 4 and AG 5, all were negative.

It is the intention that soil-borne disease diagnostics developed as a result of Potato Council funding can be adopted by other laboratories. To ensure consistency of testing an inter-laboratory comparison was carried out on four pathogens, *Colletotrichum coccodes*, *Spongospora subterranea*, *Rhizoctonia solani* AG3 and *Pectobacterium atrosepticum*. Standard spiked samples of soil or potato peel were constructed and dispersed to the three laboratories of participating organisations for testing blind. Some limited variation in testing can be expected and on the whole there was consistency in testing between laboratories. However, for some tests there was also inconsistency.

Variations that were substantially different from expected were ascribed to various factors such as construction of a standard curve and the way samples were handled after receipt. Experience from this inter-laboratory testing has allowed a protocol to be written on the preparation of samples for an inter-laboratory comparison trial. This will form the basis for inter-laboratory comparison in an international diagnostics project (Potato Council project R422).

Whilst the relationship between a test result for soil-borne *C. coccodes* and risk of black dot has been established, the relationship between a soil test result for *S. subterranea* and risk of subsequent disease remains crude because environmental conditions drive disease development with this pathogen. What is lacking is a clear understanding of how environmental conditions relate to infection and disease development. Two trials (at SAC & SCRI) were carried out as part of a three year European-wide project to examine this relationship. Full interpretation cannot be made until the European project is complete but there were several useful findings in the first year.

Two varieties were grown in each trial, one very susceptible (Agria or Estima) and one moderately susceptible (Nicola). Root infection (assessed visually and using PCR DNA testing) was evident in both varieties from soon after emergence and was present at high levels throughout the period of monitoring. Root galling was observed around 4 weeks after tuber initiation and at least 5 weeks after root infection, Agria showed significantly more gall symptoms than Nicola, and Estima the least. This is in agreement with previous EU trials where root galling was monitored across several sites.

Powdery scab symptoms were first observed in week 8 of sampling at both sites, around 6 weeks after tuber initiation. Overall, Estima was the most susceptible cultivar followed by Agria and then Nicola. This is in accordance with previous results of EU trials. DNA of *S. subterranea* was detectable in symptomless tubers from the time of tuber formation at both sites, showing that infection takes place at a very early stage of development. Significantly more DNA was detected in symptomless tubers of cultivar Agria (which also had more severe disease) compared with Nicola at the SCRI site. This may be because symptomless infections went on to cause disease in Agria. The dates at which infection occurred and symptoms developed were remarkably consistent between sites.

At both sites conditions were conducive to disease development. The mean daily temperature was approximately 15℃ at both sites. The SAC site was marginally cooler and wetter than the SCRI site, which may account for the differences in powdery scab severity, particularly where Nicola was shown to have more disease at SAC than SCRI. Results from international trials will add to the interpretation with regard to weather conditions.

Currently, after extraction of DNA from soil, soil-borne disease diagnostic tests have to be carried out separately for each pathogen. The potential to carry out multiple tests simultaneously (multiplexing) was investigated. Depending upon the target pathogens of interest, multiplex assays are now possible for *C. coccodes* and *S. subterranea* (Applied Biosystems TaqMan Universal PCR Master Mix) and *S. subterranea* and *R. solani* (QuantiTect Multiplex PCR Kit). However more work would be required to find a suitable multiplex assay for *R. solani* in conjunction with *C. coccodes*.

2. INTRODUCTION

2.1. General Introduction

This project followed on from two previous Potato Council-funded diagnostic projects - R249 and R253. In these earlier projects, significant progress had been made developing and validating PCR soil tests for *Colletotrichum coccodes* and *Spongospora subterranea*. Furthermore, through studying the epidemiology and control of *C. coccodes* and monitoring commercial crops over four years, interpretation criteria for soil tests for *C. coccodes* and *S. subterranea* have been developed. By contrast, whilst a robust soil test for *Rhizoctonia solani* AG3 had been developed, ascertaining a relationship between soil test results and disease development had not been possible. Whilst soil-borne *R. solani* inoculum can be quantified accurately in artificially inoculated soils using real-time PCR assays, it was believed that sampling issues were a major factor affecting accurate quantification of contamination of field soils.

2.2. *Rhizoctonia solani*

This project focuses on sampling for detection of *R. solani*. This is an important starting point for developing future research and control studies. It is possible that knowledge of how *R. solani* survives, is transmitted and causes disease will be required in addition to effective sampling methodology to confidently predict or make a risk assessment of future stem canker or black scurf development or to make disease control recommendations.

Both soil-borne inoculum as mycelium and sclerotia have been studied in recent years by PhD students (Kyritsis, 2003; Ritchie, 2006). These projects did not attempt to quantify inoculum using diagnostic tests but they demonstrated that both forms of inoculum could incite disease. The relative frequency of these two types of inoculum and their relative impact on disease development needs to be determined. This is important because the type of propagule may have implications for PCR quantification.

The risk associated with seed inoculum has yet to be adequately described. Simons and Gilligan (1997 a, b) found that the density of tuber-borne inoculum had a significant effect on the incidence and severity of disease. Based on this finding, it would be expected that a relationship between seed inoculum and disease would be found in crops where infected seed was planted into fields in which no soil inoculum was detected. This occurred in 44 out of 122 crops within an earlier project (R253). However, no relationship between level of seed inoculum (as determined by either visual assessment or real time PCR) and disease was found. It is possible that disease development from seed inoculum was prevented by use of seed treatments (which are widely applied). Indeed seed treatments may effectively limit seed inoculum as for disease development within a crop. Thus soil inoculum may take on greater importance. However, due to the confounding problems associated with soil inoculum quantification, it has not been possible so far to determine whether the absence of a relationship between seed inoculum and disease was because of the use of seed treatments, an interaction between disease and soil borne inoculum that was undetected, or whether the relationship between seed-borne inoculum and disease is not straightforward.

As inconsistencies in AG3 detection and disease development have been found, and there is evidence from Australia that AG2-1 may be important in their system, this project

will retest existing soil DNA samples for the presence of other AG groups to eliminate the possibility that other AG groups confounded the relationship between detection and disease development. If other AG groups are found to be more important in causing disease in GB than previously thought future experimental work would have to take this into account.

2.3. Sampling and testing

There is little published information on sampling strategies for *Rhizoctonia solani*. Potts (2005) reviewed sampling strategies for soil borne fungal pathogens as part of the Potato Council-funded diagnostics project (R253) and concluded that without data on mean pathogen levels and degree of aggregation of particular pathogens, it would not be possible to determine the accuracy of any sampling strategy or the sample size required. However, there has been a lot of research into sampling for potato cyst nematodes (Turner, 1993; Schomaker & Been, 1999; Anon, 2000).

As a preliminary study before embarking on an international diagnostics project, it was decided to compare laboratory testing for potato pathogens. The rationale behind a comparison of laboratories was to ensure that results for diagnostic tests could be compared wherever they were carried out.

2.4. Other aspects of diagnostics

Two of the project team had attended the 2nd European Powdery Scab workshop in Switzerland. At this workshop an international project was proposed to evaluate the interaction of environmental conditions, infection and disease development of *S. subterranea*. As part of this project, two trial sites were proposed following the European protocol established at the workshop.

Finally, diagnostic tests for soil-borne pathogens are currently carried out individually. It was decided that a final component of the project would be to evaluate the potential for multiple testing for soil-borne pathogens

Thus this project comprised four parts:
1. Evaluation of sampling strategies for detection of *Rhizoctonia solani* AG3
2. An inter-laboratory comparison of testing methodology
3. Effect of environmental conditions on powdery scab disease development
4. Evaluation of the potential for multiplexing of potato pathogens

2.5. Evaluation of sampling strategies for the detection of *Rhizoctonia solani* AG3

In a monitoring exercise conducted within project R253, the sampling procedure (sample in a W shape across 4 ha taking samples from 100 locations at equally spaced points along the W) used to take a soil sample for the detection of soil-borne *C. coccodes* also proved effective in quantifying *S. subterranea,* but not *R. solani.* In a number of cases black scurf developed on mini-tuber progeny grown at sites where no soil inoculum had been detected. It is thought that the sampling procedure was effective for *C. coccodes* and *S. subterranea* because the resting bodies of these pathogens were dispersed throughout soil in fields - cultivations having distributed them. In contrast, *R. solani* may also be present in soil as mycelium living on organic matter that may occur in discrete patches. These patches will change shape and form as a result of cultivations, through

the exhaustion of organic matter to live on and possibly as a result of changes in environmental conditions. Whilst there is some scientific literature on distribution and survival of *R. solani* in soil, (Gilligan *et al*., 1996), patterns of distribution have not been established on a large scale. Crop pests, notably PCN, also exist in a patchy distribution and sampling procedures for this pest have been firmly established. In order for soil-borne diagnostic tests, that permit levels of soil-borne *R. solani* to be confidently determined, to be offered commercially, soil sampling strategies for this pathogen (that also suit other soil-borne pathogens) need to be established. This part of the project was designed to develop such a robust soil-sampling strategy.

Based on the findings of Woodhall (2004) that over 92 % of UK *Rhizoctonia solani* isolates on potato were AG 3, with only 7 % being AG 2-1 and less than 1 % being AG 5, diagnostic work on detecting *R. solani* on tubers and in soils in project R253 focussed on AG 3. However, no relationship between inoculum level on tubers and/or in soil with disease was observed. In addition, the development of disease in a crop when no inoculum source had previously been detected was observed on a number of occasions. In 11 out of a total of 25 crops for which both the seed and soil tested negative using real-time PCR for *R. solani* AG 3, black scurf symptoms were recorded on progeny tubers. Failure to adequately detect soil inoculum was identified as the likely source of this anomaly, as disease developed even when mini-tubers were planted (eliminating seed inoculum as a factor). Whilst developing the soil sampling strategy has been the main focus of improving detection of *R. solani* AG 3 in field soils, an investigation in to the detection of other AG groups and their association with disease (black scurf) on potatoes was also deemed worthwhile.

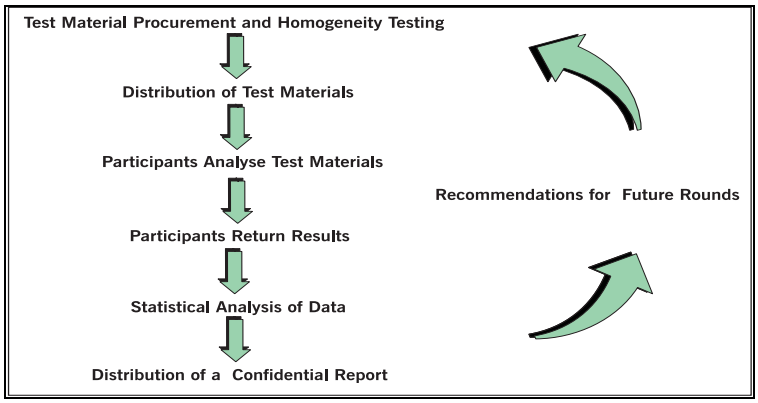
2.6. An inter-laboratory comparison of testing methodology

As part of the Potato Council's diagnostics project (R253), molecular tools for detecting and quantifying a range of potato pathogens (*Pectobacterium atrosepticum*, *Rhizoctonia solani*, *Spongospora subterannea*, and *Colletotrichum coccodes*) have been developed and refined. Soil-borne diagnostic testing for *C. coccodes* has already been offered as a routine test to determine and reduce disease risk in potato crops. Multiple testing laboratories in the UK may wish to offer the services developed from this R&D. To ensure that the laboratories offering the services, as well as future service providers, are producing accurate and critically comparable results, a system to compare results from standard test materials is required.

The inter-laboratory comparison trial (ICT) was managed as a form of proficiency test (PT). Proficiency testing comprises an inter-laboratory analytical quality assurance scheme for assessing the accuracy that participant laboratories can achieve. In other words, the quality of the result is checked against criteria that are set independently of the laboratory carrying out the analyses. Normally, participants in a PT receive only one sample from an independent organising body (such as FAPAS and PhytoPAS) and are free to use whatever method they wish for the analysis. The collated results are used to derive the best estimate of the 'true' level of the analyte and then each result is assessed against a predetermined allowable variation around this 'true' level. In the language of PT, the participants' results are used to derive the assigned value. Then, the difference between each result and the assigned value is compared to the target standard deviation. The end product of the performance assessment is a standardised statistic known as a z-score. The difference between the ICT run here and a PT is that in the trial described here, the samples were prepared by one of the participating labs but analysed by Fera's Proficiency Testing Group (which administers the FAPAS and PhytoPAS schemes). Therefore, the ICT was designed as a trial to establish methods for

developing a scheme to enable the later establishment of an analytical quality assurance scheme for soil-borne pathogen testing.

All the schemes administered by FAPAS (food chemistry testing) and PhytoPAS (plant pathogen testing) follow a similar pattern as shown in the figure below. Suitable test materials are selected and tested to ensure sufficient homogeneity and then distributed to requesting participants. Laboratories usually have 6-8 weeks to analyse their samples and return their results. The statistical analysis is carried out on the submitted results and a report compiled for issue to laboratories.



2.7. Effect of environmental conditions on powdery scab disease development

Previous research, including that conducted by the authors, has helped to describe conditions favourable for powdery scab development, but some basic knowledge of the etiology and epidemiology of the disease is still lacking. For example, we lack sufficient knowledge of the factors that govern survival, infection and disease development, which is vital in predicting disease risk and recommending management strategies. It was generally recognised, at the 1st European powdery scab meeting hosted by SAC in 2000, that the effective control of powdery scab would require an integrated approach. At that meeting the development of techniques with which to detect and quantify inoculum in plant and soil samples was also deemed essential. Through subsequent Potato Council and RERAD funding, robust techniques for the detection and quantification of *Spongospora* now exist and work has been carried out to establish relationships between inoculum and disease. However, the major factors affecting powdery scab development in relation to inoculum concentration are environmental and in order to progress risk assessment and decision support for powdery scab control, work is needed to determine the conditions required for infection (e.g. temperature, water and window of infection). This work was identified as the next important research task by international researchers at the 2nd powdery scab workshop held in Switzerland in 2007. This type of data cannot be gathered in isolation and it was therefore proposed that similar trials were conducted by experienced powdery scab researchers in as many countries and as many sites as possible that were pre-disposed to powdery scab epidemics (Scotland being amongst the most important of these). This study, carried out at 2 sites in Scotland, comprised another component of this project. The EU and other countries in this study are shown below. Standard trials protocols were followed and in addition, diagnostic tools developed at SCRI were used as an additional tool by SAC and SCRI in determining the time and extent of infection, thus bringing added value to the data.

2008	2009	2010 (provisional)
Switzerland x 2 sites	Switzerland x 2 sites	Switzerland x 2 sites
France	France	France
Netherlands	Netherlands	Netherlands
Norway	Australia x 2 sites	Australia
UK x 2 sites	UK x 2 sites	UK x 2 sites

Results of the 2008 Potato Council-funded powdery scab trials are presented in this project. Details and descriptions of the results obtained from the 2008/2009 trials in UK and Australia will be presented in the first annual report of Potato Council project R422. Additional trials will be completed in 2010 and reported on in the 2nd annual report of R422. A final analysis and pulling together of all the results from UK and Australian trials will be completed for the 2012 report. This will be led by SCRI. The other EU trials are coordinated by E. Merz (Switzerland) and will be disseminated to participating countries when they become available, but it is anticipated that they will be included in the 2012 report.

2.8. Evaluation of the potential for multiplexing of potato pathogens

SCRI has primers for almost all soil-borne pests and pathogens of potato and these were assessed for suitability for multiplexing (to allow multiple pathogens to be detected in a single DNA sample). The advantages of multiplex assays are savings in time and costs. The costs of consumables and reagents when performing real-time PCR assays are considerable. The exact cost will depend upon the specific assay being performed and reagents used, but as a rough estimate, a single plate can cost in the region of £100. If samples are to be tested for more than one target pathogen, then by multiplexing assays you halve the number of plates required, thus reducing the overall cost considerably. Multiplexing of real-time PCR assays is in theory possible, by choosing probes for the different targets labelled with dyes which fluoresce at different wavelengths. However, in practice few routine assays are carried out as multiplexed reactions. All the assays currently used at SCRI to quantify potato pathogens are in a format suitable for multiplexing, but have as yet not been tested. To ensure comparability of results, assays used in a multiplex must be shown to be as sensitive and accurate as the assays carried out alone. Potato Council suggested possible combinations of tests for soil pathogens as follows: black dot, powdery scab, *Rhizoctonia*. In the last part of this project a short piece of work was carried out to assess the possibility of multiplexing combinations for 2 or 3 of these pathogen tests.

3. MATERIALS AND METHODS

3.1. Evaluation of sampling strategies for detection of *Rhizoctonia solani* AG3

3.1.1. Intensive sampling of fields to establish distribution pattern of *R. solani* AG3

Fifteen fields across north and east Scotland in which potatoes were grown in 2007 and where progeny exhibited black scurf (Table 1) were identified. Within each field, a 4ha (10 acre) area was identified and its location carefully recorded. The area selected in each field was sampled in a W pattern (100 sample points) - as used for the commercial black dot soil test. However, instead of using PCN sampling spears, samples were taken to 15cm depth with a narrow blade trowel and the top 5cm of the sample discarded. Samples were bulked into a plastic bag and carefully labelled with farm name, date of sampling and sampler name. The total quantity of soil sampled was c. 3 kg. After thorough mixing, the samples were divided into three. One sample was retained at SAC and the other samples dispatched to SCRI and CSL (now Fera). Sampling took place on 22 & 24 April 2008 and the three sub-samples were dispatched on the 28 April.

On receipt of the soils at SCRI (29 April 2008), they were put at 4℃ overnight. The following day the soils were divided in two and half milled immediately, whilst the remaining soil was air dried at room temperature for 5 days before being milled. This comparison was carried out to ascertain if it had a detrimental effect on *R. solani* detection. At SAC, the samples were stored in a cold store at 4^{o}C until drying and extraction 2 days later. A similar approach was adopted at Fera. This pattern of handling by each organisation was repeated for all subsequent samples.

Each Institute tested the 15 soil samples for contamination by *Rhizoctonia solani* AG3 as described in the PCL diagnostics project (R253). Results are shown in Table 2. From the results, two fields, one in Tayside (Field 3) and one in Aberdeenshire (Field 7), were selected for intensive sampling. However, the field in Tayside (Field 3) was growing winter cereals and the grower would not allow sampling and only the Aberdeenshire field (Field 7) was intensively sampled. This was carried out on 3 & 4 June 2008. Subsequently, another field in Morayshire (Field 16 - Keem) was identified following a 2008 potato crop where black scurf was frequent on tubers. This was intensively sampled on 11 & 12 December 2008.

In each field of these 2 selected fields, the 4ha area sampled in the initial sampling was identified. The corners of the square representing the 4 ha area were marked with canes. GPS locations of the corner markers were determined for further sampling. Within the 4 ha, 100 points for sampling were identified on a 10 x 10 grid. These were marked temporarily by canes. These points are indicated at the intersections in Figure 1.

Field no.	Location	Variety
1	Laurencekirk, Aberdeenshire	Lady Rosetta
2	Perth, Perthshire	Lady Rosetta
3	Forfar, Tayside*	Unknown
4	Elgin, Morayshire	Atlantic
5	Banff, Aberdeenshire	Hermes
6	Portsoy, Aberdeenshire	K Edward
7	Portsoy, Aberdeenshire*	K Edward
8	Montrose, Tayside	Saxon
9	Montrose, Tayside	Premier
10	Forres, Morayshire	Maris Peer
11	Perth, Perthshire	Maris Piper
12	Perth, Perthshire	Maris Piper
13	Skene, Aberdeenshire	Cabaret
14	Cromarty, Black Isle	Hermes SE1
15	Cromarty, Black Isle	Hermes PB3

TABLE 1. LOCATION OF FIELDS WHERE BLACK SCURF WAS IDENTIFIED ON POTATOES HARVESTED IN 2007 AND VARIETY GROWN

Site	SAC (pg DNA/g soil)	SCRI (pg DNA/ g soil)		Fera (pg DNA/ g soil)	
		Undried	Dried	Small sample	Bulk sample
1	12919	0	105	0	0
2	0	0	0	0	0
3*	0	123	1553	0	0
4	3678	32	29	0	0
5	0	2.9	0	0	0
6	0	0	19.3	0	0
7*	1073	0	0	85.6	0
8	2723	0	50.1	0	0
9	0	0	40	0	0
10	0	0	0	0	0
11	0	0	0	0	0
12	0	0	0	0	0
13	0	70.1	0	0	35.8
14	0	0	0	0	0
15	0	0	0	0	0

* Fields targeted for intensive sampling

TABLE 2. RESULTS OF SOIL TESTING 15 FIELDS IN NORTH AND EAST SCOTLAND WHERE THE PREVIOUS POTATO CROP HAD EXHIBITED BLACK SCURF FOR CONTAMINATION BY *RHIZOCTONIA SOLANI* AG3

At each intersection, 1 kg of soil was sampled across a 2m x 2m area around the intersection point (i.e. where the cane was placed). Sampling methodology was exactly as described for initial sampling above i.e. samples were taken to 15 cm with a narrow blade trowel and the top 5 cm of the sample discarded. At least 20 cores were taken around each intersection point. Samples from each intersection were bulked into a plastic bag and carefully labelled with Farm name, date of sampling, intersection number and sampler name. A map was drawn and retained to indicate layout of plot and sampling point numbers.

Samples were thoroughly mixed. One half of the samples from each field (either odd or even numbered samples) were dispatched to SCRI and the other half retained by SAC Aberdeen for soil testing. If there was any delay in handling the samples, they were stored in a cold room (4^{o}C). Every attempt was made to make DNA extractions within 2-3 weeks of receipt. If DNA extraction extended over 2 weeks, alternate samples were tested in week 1 and the rest in week 2. In addition to *R. solani* AG3, SCRI also tested their samples for *Colletotrichum coccodes* and *Spongospora subterranea* in fields 7 & 16.

A)

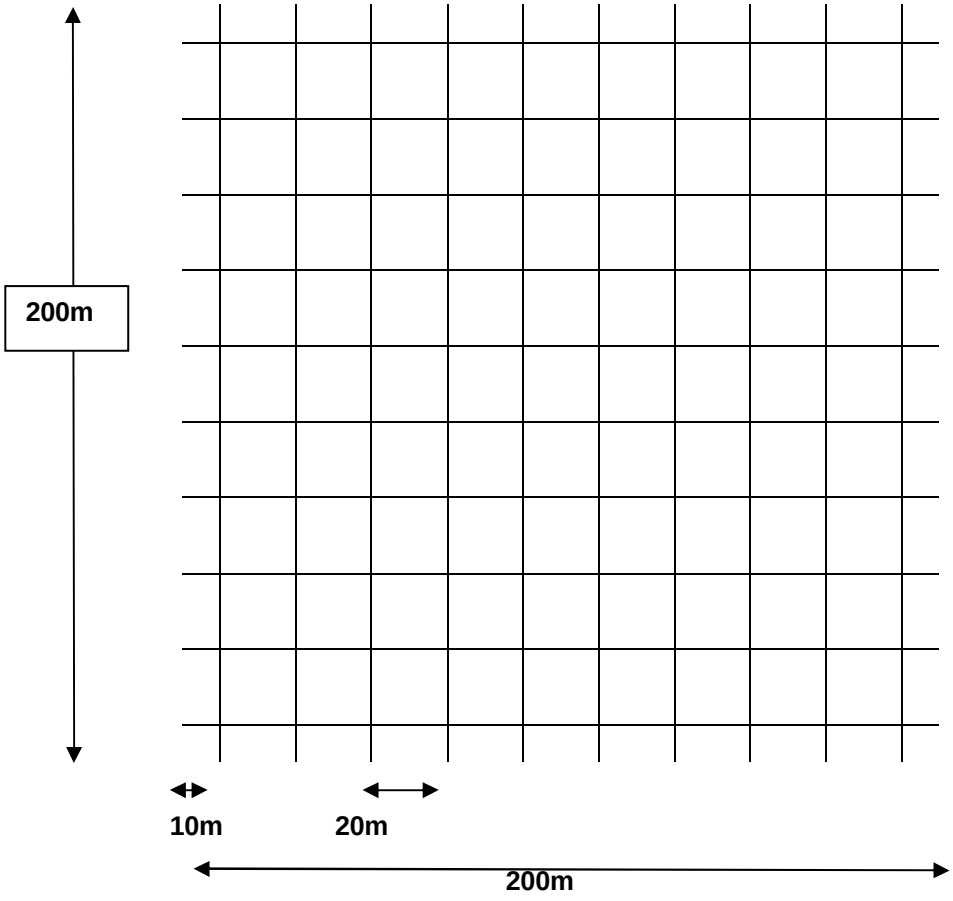


FIGURE 1(A). SAMPLING GRID FOR INTENSIVELY SAMPLED FIELDS

B)

91	92	93	94	95	96	97	98	99	100
81	82	83	84	85	86	87	88	89	90
71	72	73	74	75	76	77	78	79	80
61	62	63	64	65	66	67	68	69	70
51	52	53	54	55	56	57	58	59	60
41	42	43	44	45	46	47	48	49	50
31	32	33	34	35	36	37	38	39	40
21	22	23	24	25	26	27	28	29	30
11	12	13	14	15	16	17	18	19	20
1	2	3	4	5	6	7	8	9	10

FIGURE 1(B). NUMBERING OF SAMPLES FROM INTERSECTION POINTS

3.1.2. Evaluation of sampling methodologies

In order to compare potential sampling methodologies, results of soil testing from the intensive monitoring in Aberdeenshire, along with all other data on distribution of *R. solani* in soil from the UK and Australasia was sent to Dr Jackie Potts, statistician for BioSS, for analysis. The data available from Australia was two intensively monitored sites, one in South Australia where AG 2.1 and AG 2.2 were present and a site in Victoria where AG 2.1 was detected. Specifically, she was asked to examine the data sets and suggest improved sampling strategies appropriate for this pathogen which would improve detection. In Dr Potts opinion the data provided was insufficient to determine consistent patterns of distribution that could be confidently used to advise on sampling approaches. However, from a previous review of sampling for a range of pests and diseases (Potts, 2005) two further procedures were proposed. These two methods (4 & 5 see below) increased the sampling frequency and were proposed to increase the chance of detection of *R. solani*.

Thus, in addition to the standard sampling procedure, based on the original PCN sampling protocol, two procedures proposed by Dr Potts and a further two were added, one from Australian research and another representing a proposed new EU sampling strategy for PCN. These five sampling methodologies were:

- **Standard**. Sample in a W shape across 4 ha taking samples from 100 locations at equally spaced points along the W. This is the standard soil sampling method used for commercial testing of black dot and powdery scab soil contamination.
- **Australian**. Sample in a W shape across 4 ha taking 3 cores at each of 33 locations equally spaced along the W, thus making a composite sample of 99 cores.
- **New PCN sampling method**. Samples taken with at least 100 cores/ha preferably in a rectangular grid of not less than 5 metres in width and not more than 20 metres in

length between sampling points covering the entire field. That is, for a 4ha field 400 cores, systematically sampled.

- **Experimental 1**. Sample in a W shape across 4 ha taking samples from 200 locations at equally spaced points along the W
- **Experimental 2**. Divide 4ha area into 4 sections of approximately 1ha each. Take separate samples from 100 cores arranged in a W-shape in each section. Samples from the 4 sections will be analysed separately. Each bag was marked with the section sampled.

All five sampling methodologies were compared in the intensively sampled field in Aberdeenshire (Field 7 on 14 January 2009), the intensively sampled field in Morayshire (Field 16 on 14 January 2009) and a second field in Morayshire tested as part of the first sampling (Table 1: Field 4 on 14 January 2009). Fields were re-sampled from the same 4ha area identified originally. Samples were taken to 15cm depth with a narrow blade trowel and the top 5 cm of the sample discarded (except for sampling method 1 – see below). Soil from each sampling method (4 samples in method 5) were bulked into a plastic bag and carefully labelled with Farm name, date of sampling and sampler name (and section number in method 5). Mixing, dispatch, and testing of the samples were exactly as described above with half of each sample tested at SAC and half at SCRI.

3.1.2.1. Evaluation of depth of sampling

At the same time as the sampling was being carried out on sampling methodologies, when the Standard sampling was being carried out, the soil in the top 5cm was retained in a separate sampling bag to test the effect of sampling depth on test result. The soil from the top 5cm was handled and tested in exactly the same way as those from the 5 sampling methods above.

3.1.2.2. Repeat evaluation of the most effective methods

In order to confirm effectiveness of sampling, the most promising methods were used to sample the intensively sampled Aberdeenshire field and a field in Morayshire on 28 & 29 March 2009 (Fields 7 & 4). This sampling and subsequent testing was undertaken to investigate changes in soil population and to confirm the effectiveness of sampling. The methods used were the Australian method and the Standard method but taking the top 5cm of soil.

3.1.2.3. Method for testing past DNA soil extracts for **R. solani** *AG groups – SCRI*

DNA previously extracted from approximately 80 field soils as part of the Potato Council-funded diagnostic project (R253) were to be tested for the presence of other AG groups of *R.solani*, particularly AG's 2-1, 4 and 5. These soil DNA samples have corresponding crop disease data and therefore would allow disease to be compared to the level of other AG groups in the soil.

A number of isolates were chosen from the SCRI collection on which to test the assays previously developed at FERA. A pure culture was grown from each of the isolates listed in Table 1, DNA extracted and a standard dilution series made. All the isolates were tested with all three assays.

3.1.2.4. Spatial distribution of Rhizoctonia solani AG3

A method used in geostatistics for analysing data distributed in two dimensions, kriging, was used to visualise the distribution of *R. solani* AG3 inoculum from soil testing data from field 7. The kriging model specifies how successive measurements of a variable in space are correlated with each other. In order to determine the distribution pattern for *R. solani* in the intensively sampled area, a K-statistic was calculated. The K function for a completely random pattern is given by:

$K(s) = \pi \times s^2$, where s is the distance between points.

The K statistic for a clustered pattern will tend to be larger than the values given by the above expression.

3.2. Inter-laboratory comparison

3.2.1. Sample preparation

Soil samples for testing consisted of soils either spiked or naturally infested with *Colletotrichum coccodes*, *Rhizoctonia solani* AG3-PT or *Spongospora subterranea*. Tuber samples consisted of macerated peel spiked with *Pectobacterium atrosepticum*. Samples were prepared for three different notional levels (low, medium and high) to produce a suitable range for testing. The soil used in the soil test samples, M2008, was a sandy loam soil (pH6.3) collected from a potato field at tuber harvest. The soil was thoroughly air dried (2-3 days at 20ºC) and then kept at 4ºC until required.

Rhizoctonia test samples were prepared by spiking the M2008 soil with sclerotia. Inoculum was prepared by growing cultures of *Rhizoctonia solani* AG3-PT (isolate Rs08JL) for 5 weeks at 20ºC on potato dextrose agar (PDA) in 90 mm Petri dishes. After this time, sclerotia were visible over 50% of the plate. Sclerotia were removed using a scalpel and left to air dry over night. The sclerotia were then broken up using a scalpel to an average size of 2.2 mm in diameter. Sclerotia were added to the soil to produce the desired concentration. The low concentration sample consisting of 0.0006% sclerotia weight to soil weight, medium 0.006% and high, 0.06%

Thorough testing of M2008 soil revealed that it had adequate, stable natural levels of the *S. subterranea* suitable for use in the test as the low level without the need to spike. To achieve the medium and high test samples, the soil was spiked with *S. subterranea* infested tomato roots. Tomato roots were infected by growing tomato seedlings (cv. Moneymaker) in soil known to be infested with pathogen. After 3 weeks, the seedlings were harvested, washed, and a sub sample of the roots were tested by real-time PCR for presence/absence of *S. subterranea*. Roots with high levels of infection were air dried overnight and added to soil at 0.34% weight roots: weight soil for medium concentration samples and 0.8% for high concentration samples.

Thorough testing of the M2008 soil also revealed that it had naturally low levels of *C. coccodes* and was suitable for use as the low concentration sample. For the medium and high level samples, 90 mm Petri dishes of PDA were grown for 4 weeks at 20ºC. A mixture of conidia and microsclerotia were then removed with a scalpel and left to air dry for two hours. This material was added to soil at 0.14% (weight *C. coccodes*: weight soil) for the medium concentration and 0.42% for the high concentration.

Pectobacterium atrosepticum (isolate number P3986) was added at three different concentrations (high, medium and low) to 10 ml PB7 (2ml Tetrasodium pyrophosphate in 100ml Phosphate buffer pH7). The spiked PB7 solution was added to a Bioreba grinding bag and kept on ice for the duration of its use. Ten potato cores and potato peelings up to 10g was added to each grinding bag. The batch of potatoes was previously tested by real-time PCR and was free of *P. atrosepticum*. A mallet was used to break up the potato cores in each bag before grinding on a large Homex grinder. 800µl was removed from each bag and added to the appropriate labelled tube for freeze drying prior to dispatching to participating laboratories.

3.2.2. z-scores

A z-score is the standardised measure of bias and relates the difference, or error, in a result to the designated standard deviation of the results for the test samples (Equation 1).

$$\frac{X - \bar{X}}{\sigma^*}$$ Equation 1

Where, X is the lab sample mean, $\bar{X}$ is the assigned sample mean and σ^* is the designated standard deviation

The z-score reflects 'best practice' or fitness for purpose. In a normal distribution only about 1 in 20 results will be outside two standard deviations from the mean hence z-scores of $|z| \leq 2$ are considered satisfactory.

3.2.3. Distribution of samples

Samples were thoroughly homogenized prior to sending out and tested by real-time PCR prior to dispatch to ensure adequate quality. Each sample was identified by a unique code that did not reveal the amount of inoculum. Aliquots of 200g of soil and 800 µL of spiked tuber peel were sent to participating laboratories (SAC, SCRI, Fera). On arrival, the soil samples were again homogenized and 3 x 60g sub samples (SAC and SCRI) or 1 x 50g sub samples (Fera) taken for each level and frozen at –20C for stable storage prior to testing. Lyophilised peel samples were re-suspended in 100 µL dH_2O then processed as described in 'DNA testing' below. In addition, sample DNA extracts were distributed.

3.2.4. DNA Testing

Participating labs tested soils and peel extracts for DNA levels using their own methods. Two replicate extracts were produced for each test sample and each sample was tested in triplicate by quantitative PCR. Each participating lab returned the results (amount of DNA/g soil sample or pg DNA/uL for spiked peel samples). Labs aimed to return results to Fera within 3 weeks of receipt of samples. Fera tested their samples blind only after the other participating labs had returned sample results.

3.2.5. Data Analysis

For the inter-laboratory comparison trial, a consensus mean was calculated for each sample level (over the testing period). Results from each laboratory were compared to the consensus mean to provide an estimation of strength and direction of bias (see z-scores, below). The data for the DNA shelf-life testing was analysed using an ANOVA for repeated measures using Genstat version 11 (VSN International). To determine the distribution of *R. solani* AG3 in soil at a field (Field 7), data were analysed by Fera by

mapping Kriged estimates of variance and calculating the K-function. Analyses were done using Genstat version 11 (VSN International).

3.2.6. Sample storage experiment

Samples were prepared as described in 'Sample preparation' (above). Initial amounts of DNA were prepared to provide levels that equated to the 'medium' level samples for each soil-borne pathogen. For *Rhizoctonia solani* AG3PT samples, sclerotia were added to produce a concentration of 0.05% sclerotia per weight of soil. For *S. subterranea*, diced roots of *S. subterranea*-infested tomato was added to produce a concentration of 0.22% weight of roots per weight of soil. For *C. coccodes*, sclerotia, hyphae and conidia were added to produce a starting concentration of 0.03125% inoculum per weight of soil. Samples were tested four times over a 28 day period. Samples were either stored at room temperature (c. 18℃) or in a controlled environment store at 4℃. At each sampling time, three 50 g samples were taken and frozen at –20^{o}C prior to DNA extraction and real-time PCR using Fera's standard methods to determine whether sample inoculum was stable within the period that samples would be in transit prior to testing by all participating laboratories.

3.3. Effect of environmental conditions on powdery scab disease development:

Trials were planted at 2 sites in 2008 (East Loan, SCRI, Dundee and Woodland Field, SAC Aberdeen). Trial details are summarised in Table 3.

	Site 1: SCRI	**Site 2: SAC**
Cultivars Planted	Agria + Nicola	Estima + Nicola
Date of Planting	14 May 2008	8 May 2008
Replicates	3	4
Irrigation	Trickle tape	Trickle tape
Number of samples/date	4 plants	5 plants
Soil inoculum level (Sporeballs/g soil)		
Pre-planting	Mean value = 31 SEM = 5.80	Bed A (reps 1+2) = 74 Bed F (reps 3+4) = 155 Mean = 115
At harvest	Plot 1 = 9 Plot 2 = 13 Plot 3 = 6 Plot 4 = 7 Plot 5 = 33 Plot 6 = 22 Mean = 14.88 SEM = 4.18	Bed A (reps 1+2) = 44 Bed F (reps 3+4) = 117 Mean = 80.5

TABLE 3. SUMMARY OF POWDERY SCAB TRIALS

Week number	SCRI sampling dates	SAC sampling dates
1	-	12/6/08
2	-	19/6/08
3	-	25/6/08
4	1/7/08	2/7/08
5	8/7/08	10/7/08
6	16/7/08	17/7/08
7	22/7/08	24/7/08
8	29/7/08	30/7/08
9	5/8/08	7/8/08
10	12/8/08	14/8/08
11	-	21/8/08

TABLE 4. SAMPLING DATES IN THE POWDERY SCAB TRIALS

Trials were planted according to the EU standard protocol where at least one susceptible (Agria, Estima or Kennebec) and one medium susceptible cultivar (Desiree, Nicola or Saturna) should be planted, with Agria and Nicola being the preferred cultivars. Cultivars Agria and Nicola were planted at SCRI and Estima and Nicola at SAC. Both sites were inoculated with *S. subterranea*. At SAC, a slurry made from tubers with powdery scab symptoms was applied to the site using a hose and at SCRI, dried peelings from scabby tubers were finely ground and mixed with vermiculite, before being spread evenly across the trial site. No seed treatments were applied at either site. Soil nutrient analysis was made at SAC prior to planting. Irrigation was applied up to 4 weeks after tuber initiation (25mm was applied when the soil moisture deficit reached 18mm). Herbicides, late blight and aphid control were applied as per standard practice.

Environmental conditions were monitored at both sites using in-field monitoring equipment and met-station data. The following parameters were measured at least once per day:

- air temperature
- air humidity
- precipitation
- soil temperature (in the centre of a row with a susceptible cultivar)
- soil moisture content

3.3.1. Testing soil contamination by *S. subterranea*

Before planting, soil samples were taken using standard soil sampling procedures (1kg soil samples made up of 100 x 10g cores, of the equivalent sample according to plot size). At SCRI 3 x 60g samples of soil were taken from the 1kg sample and DNA was extracted from soil samples and tested for *S. subterranea* according to the methods of Brierley *et al* (2009) and Van de Graaf *et al* (2003). At SAC, *S. subterranea* DNA was extracted from 1 x 60g sub-sample of soil and quantified according to the methods described above. After harvest, the procedure was repeated but with samples taken from each plot (SCRI) or the area of each variety within each bed (i.e. 4 samples) (SAC) and tested for contamination. Soil inoculum levels before planting and after harvest are given in Table 3.

3.3.2. Plant Sampling

The EU standard protocol states that 24 tubers/plot of each cultivar x 3 replicates should be planted and samples of at least 3 plants/replicate should be taken once a week for 6 weeks starting after tuber set.

At SCRI, 4 plants/rep x 4 replicates were taken from each cultivar at 7 sampling dates commencing at tuber set (3 weeks after 50% emergence), and weekly samples thereafter. At SAC, 5 plants/rep x 4 replicates were taken from each cultivar at 11 sampling dates commencing at 50% emergence and weekly samples thereafter. Sampling dates are shown in Table 4.

Plants were dug carefully in order to catch as much of the root system as possible. Samples were placed in plastic bags immediately and taken to the laboratory. If the samples were not examined/dealt with immediately they were stored in a cold room. Roots were carefully separated from tubers, stems and stolons, and washed to remove soil but without damaging root tissue. If necessary the root ball was soaked in water to loosen the soil. Roots were then dried and weighed and assessed for root galls using the gall assessment key (Appendix I).

At SAC, root samples were also assessed microscopically for infection by *S. subterranea*. Root sections taken at each sampling date from each of the 2 cultivars Estima and Nicola were stained using 0.1% Cotton blue and examined for infection at 5 individual points. The percentage area of the root section infected was estimated at each point and a mean value calculated.

Tubers from each plant were washed carefully to remove all soil and all tubers in each sample were assessed for powdery scab using the standard tuber scoring table (Appendix 1). Also see the *Spongospora* website: http://www.spongospora.ethz.ch/LaFretaz/scoringtable.htm).

When lesions were very small, microscopic examination was required. If lesions could not be confirmed as powdery scab, tissue from within typical lesions was scraped onto a slide, stained and examined for sporeballs.

3.3.3. Real-time PCR assessment of samples

At each sampling date, and from each site, a root and tuber sample from one plant from each plot that had previously been scored for disease as described above was also tested for the presence of *Spongospora subterranea* DNA. The whole root system from each plant and tuber peelings taken from symptomless tubers from the same plant were collected and processed as follows:

3.3.4. Processing of root material

The fresh weight of each root sample was recorded after defrosting. At SCRI, the whole root sample was chopped up and placed in a 50 ml tube and freeze dried, then ground in liquid nitrogen. The dried weight of each sample was recorded and extraction buffer was added to each sample to a total volume of 30 ml. In the case of very large root samples the volume was increased to 45 ml (a note of the volume to which each sample was made up to was recorded). After thoroughly mixing the sample, three 1.5 ml aliquots were taken from each sample for DNA extraction. Each aliquot was centrifuged

(Eppendorf centrifuge 5415 D) at 6000rpm (2600 g) for 5mins after which the supernatant was removed and kept.

At SAC, root tissue was not freeze dried; DNA was extracted from the root samples immediately after defrosting, as per the procedure described above, with one slight modification; 10ml of extraction buffer was added to each sample instead of the 30ml required for a freeze dried sample.

3.3.5. Processing of tuber peel material

Each sample of tuber peel (weighing between 7 and 70 g) was placed into a Bioreba grinding bag (Long Special Universal bags: 15 x 28.5 cm Cat. No. 470100b) and the weight noted prior to the addition of 15 ml SPCB extraction buffer (10ml SAC). The sample was then pulverised to give an oat-meal consistency using a Homex grinder. Three 1.0 ml aliquots were taken for DNA extraction.

3.3.6. DNA extraction from root and tubers:

Cold $CHCl_3$ was added to each aliquot (in equal volume to that of the sample) in a fume hood. Each sample was vortexed twice and centrifuged at 13000 rpm (11,000 g) for 4 min. In a fume hood the aqueous phase was removed to a new tube and 90 µl of 3M NaOAc and 0.9 ml Isopropanol was added. Tubes were vortexed and incubated at room temperature for at least 1 hour then centrifuged at 13000 rpm (11,000 g) for 4 min. The supernatant was removed and the pellets washed in 150 µl 70% EtOH and centrifuged at 13000 rpm (11,000 g) for 2min. The EtOH was removed with a pipette and the pellet allowed to air-dry for 10 min before being re-suspended in 150 µl of 1xTE. The tube was vortexed and left overnight in fridge to help dissolve the pellet then stored at -20ºC in the freezer.

DNA of *S.subterranea* in each DNA sample was quantified according to the method of Van de Graaf *et al* (2003). Three DNA extractions per sample were made and each DNA sample was replicated twice in the real-time PCR assay

3.3.7. Calculation of DNA per g root and tuber peel

3.3.7.1. Root samples

The amount of DNA (pg DNA / g root) was calculated as follows: the proportion of the initial sample taken in each aliquot for DNA extraction was first determined, e.g. if 3 g dry weight of root material was suspended in a 30 ml suspension, then 0.15 g root dry weight would be contained in each 1.5 ml aliquot of the suspension. If the DNA extracted from this aliquot was resuspended in 150 µl, and 2 µl of this DNA was used in the PCR assay then the amount of DNA can be calculated on a DNA/ g dry weight basis.

3.3.7.2. Tuber peel samples

Similarly, pg DNA / g tuber peel can be calculated: if 15 g fresh weight of tuber peel material was macerated in 15 ml buffer, then there would be 1 g fresh weight in each 1.0 ml aliquot sampled. If the DNA extracted from this aliquot is resuspended in 150 µl, and 2 µl of this is used in the PCR assay then the amount of DNA can be calculated on a DNA/ g fresh weight basis.

3.4. Multiplexing of potato pathogens

Real-time PCR assays for many soil-borne pests and pathogens of potato were assessed in project R253 for suitability for multiplexing (to allow multiple pathogens to be detected in a single DNA sample), i.e. they worked using a standard set of real-time PCR cycling conditions. In this project, the suitability of assay combinations for *R. solani*, *S. subterranea* and *C. coccodes* were assessed. The advantages of multiplexing are savings in time and assay costs.

To increase the possibilities for combining fluorescent probes within a multiplex, the original probe sequences (Cullen *et al* (2002); Lees *et al* (2002); and van de Graaf *et al* (2003) for *C. coccodes*, *R. solani* and *S. subterranea,* respectively) were ordered with a black hole quencher (BHQ) at the 3' end rather than the fluorescent TAMRA as in original assays, and with combinable fluorescent probes at the 5' end (see Table 5). For each pathogen the assay was carried out as a single-plex, then in combination with the two other pathogens. A standard dilution series of target DNA was run in triplicate, and variations between the sensitivity of the assay and the standard curve (R^2) were compared between the target pathogen in a single-plex and in multi-plex combinations. TaqMan Universal PCR Master Mix (PE Applied Biosystems) was used, in addition, two master mixes marketed specifically as suitable for multiplexing were also assessed, PerfeCTa Mulitplex qPCR SuperMix (Quanta Biosciences) and QuantiTect Multiplex PCR Kit (Qiagen).

Assay		Primer/probe sequence and labels
C. coccodes	Forward	5'-TCTATAACCCTTTGTGAACATACCTAACTG-3'
	Reverse	5'-CACTCAGAAGAAACGTCGTTAAAATAGAG-3'
	Probe	TAMRA 5'-CGCAGGCGGCACCCCCT-3'BHQ
R. solani	Forward	5'-AAGAGTTTGGTTGTAGCTGGTCTATTT-3'
	Reverse	5'-AATTCCCCAACTGTCTCACAAGTT-3'
	Probe	FAM 5'-TTTAGGCATGTGCACACCTCCCTCTTTC-3'BHQ
S. subterranea	Forward	5'-CCGGCAGACCCAAAACC-3'
	Reverse	5'-CGGGCGTCACCCTTCA-3'
	Probe	TexasRed 5'-CAGACAATCGCACCCAGGTTCTCATG-3'BHQ

TABLE 5. ASSAY PRIMERS AND PROBES USED IN MULTIPLEX ASSAYS.

4. RESULTS

4.1. Evaluation of sampling strategies for detection of *Rhizoctonia solani* AG3

4.1.1. Results of initial intensive sampling

(ie. 100 cores per 4 ha on 10 x 10 grid) carried out in Aberdeenshire (Field 7; 3&4 June 2008) and Morayshire (Field 16; 11&12 December 2008).

Results of tests carried out to detect *R. solani* AG3 in an intensively sampled field in Aberdeenshire (Field 7) are shown in Table 6. Because two centres carried out the testing on half the samples, the results are presented as Ct values for comparability. The inter laboratory comparison component of this project found that different laboratories can generate different figures for pg DNA/ g soil from their Ct values based on variations in their standard curves. Therefore to eliminate these potential differences from a single data set derived from two different laboratories, Ct values have been reported. This allows a direct comparison of results.

The pathogen was more prevalent in some parts of the 4ha area sampled and absent in other parts. It was detected in 47% of locations and the lowest Ct value (which represents the greatest concentration of the pathogen in the soil) was 33.8. However, just under half of the locations where the pathogen was detected have Ct values of 39, which indicates only low levels of the pathogen were detected.

90	**40.0**	**40.0**	**40.0**	**40.0**	**39.9**	**40.0**	**40.0**	**39.8**	**40.0**	**40.0**
80	**39.4**	**40.0**	**40.0**	**40.0**	**39.6**	**36**	**39.8**	**40.0**	**39.1**	**40.0**
70	**40.0**	**40.0**	**40.0**	**39.2**	**40.0**	**40.0**	**37.7**	**39.8**	**39.5**	**40.0**
60	**39.4**	**40.0**	**34.7**	**40.0**	**39.9**	**40.0**	**37**	**40.0**	**36.8**	**40.0**
50	**39.8**	**40.0**	**38.9**	**37.9**	**40.0**	**38.9**	**37.6**	**37**	**33.8**	**38.5**
40	**37.3**	**37.7**	**36.3**	**37.5**	**40.0**	**39.9**	**40.0**	**40.0**	**40.0**	**40.0**
30	**39.1**	**40.0**	**37.9**	**40.0**	**40.0**	**38.9**	**40.0**	**40.0**	**40.0**	**40.0**
20	**40.0**	**39.7**	**40.0**	**39.3**	**40.0**	**40.0**	**39.5**	**35.2**	**40.0**	**40.0**
10	**37.6**	**39.6**	**40.0**	**40.0**	**40.0**	**38.3**	**37.5**	**40.0**	**40.0**	**40.0**
0	**35.2**	**40.0**	**38.5**	**37.6**	**40.0**	**35.6**	**40.0**	**39.7**	**39.7**	**39.8**
Sample no.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**

TABLE 6. CT VALUES FOR INTENSIVE SOIL SAMPLING OF FIELD 7 (ABERDEENSHIRE). EACH VALUE REPRESENTS SAMPLING FROM A 2X2M AREA AT A 20X20M GRID POINT. SHADED VALUES DETERMINED BY SAC AND UNSHADED BY SCRI.

At the second intensively sampled site in Morayshire (Field 16), despite the sampling taking place only 4 months after a potato crop, *R. solani* AG3 was detected in just 2% of samples (Table 7). This suggests that inoculum levels had fallen rapidly since harvest.

Results for *C. coccodes* and *S. subterranea* from the samples tested by SCRI from both fields 7 and 17 are shown in Table 8.

90	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**39.9**	**40.0**
80	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**39.5**
70	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**
60	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**
50	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**
40	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**
30	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**
20	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**
10	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**
0	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**	**40.0**
Sample no.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**

TABLE 7. CT VALUES FOR INTENSIVE SOIL SAMPLING OF FIELD 16 (MORAYSHIRE). EACH VALUE REPRESENTS SAMPLING FROM A 2X2M AREA AT A 20X20M GRID POINT. SHADED VALUES DETERMINED BY SAC AND UNSHADED BY SCRI

***S. subterranea* (sporeballs/g soil)**

Field 7

90	16.5		23.0		47.5		6.2		4.6	
80	2.3		36.4		2.6		41.3		9.0	
70	108.7		18.6		30.0		0.4		74.2	
60	0.0		33.1		6.0		41.7		37.6	
50	15.4		11.0		4.4		0.5		7.4	
40	0.0		0.3		0.0		0.3		0.2	
30	0.0		5.2		23.9		0.0		0.1	
20	0.0		4.1		0.0		0.0		0.0	
10	6.8		30.2		0.5		0.1		11.5	
0	22.7		0.5		0.0		31.3		0.1	
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**

Field 16

90	0.4		5.2		0.2		7.1		1.8	
80	14.7		2.9		210.6		7.3		0.2	
70	2.2		0.5		3.1		0.7		0.5	
60	0.0		6.3		0.0		1.3		0.5	
50	16.1		0.0		0.3		0.6		0.0	
40	0.5		0.8		1.4		4.6		0.0	
30	0.6		1.2		1.4		0.5		1.2	
20	0.9		3.2		1.9		1.8		0.8	
10	3.1		0.9		0.0		0.7		0.1	
0	0.2		0.1		1.9		0.7		0.1	
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**

***C. coccodes* (pg DNA/g soil)**

Field 7 Very little DNA was detected. Data not shown

Field 16

90			Not		Tested					
80										
70	276		0.0		0.0		0.0		0.0	
60	0.0		0.0		0.0		1200		298	
50	0.0		0.0		0.0		0.0		0.0	
40	0.0		0.0		0.0		0.0		0.0	
30	0.0		0.0		0.0		0.0		7415	
20	0.0		955		0.0		1307		0.0	
10	348		0.0		0.0		0.0		0.0	
0	0.0		79		713		0.0		0.0	
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**

TABLE 8. SPOREBALLS OF *S. SUBTERRANEA* AND DNA VALUES FOR *C. COCCODES* DETECTED IN 50% OF SOIL SAMPLES (SCRI SAMPLES) FROM FIELDS 7 AND 16

4.1.2. Spatial Distribution of *Rhizoctonia solani* AG3

Using results of initial intensive sampling (3&4 June 2008; 100 cores per 4 ha on 10 x 10 grid) carried out on Field 7 the mapped krigged estimates show that inoculum tends to be smeared in a north-south alignment (Fig. 2). Clustering is evident at the 1 to 2 Ha scale. This is borne out by the K function.

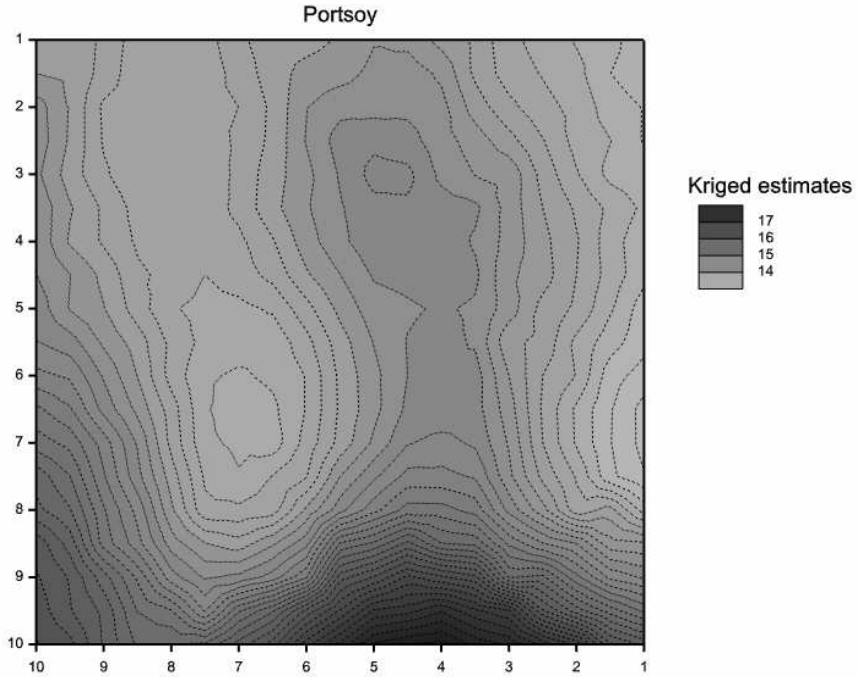


FIGURE 2. KRIGGED ESTIMATES (VARIANCE VALUES) PLOTTED AS A TWO DIMENSIONAL CONTOUR MAP SHOWING THE LIKELIHOOD OF FINDING *RHIZOCTONIA SOLANI* AG3 DNA IN SOIL SAMPLED IN A 10 X 10 REGULAR GRID OVER A 4 HA AREA (FIELD 7, ABERDEENSHIRE). DARK PATCHES REPRESENT HIGHER PROBABILITIES OF FINDING PATHOGEN INOCULUM THAN LIGHT PATCHES.

In Field 7, the distribution of *R. solani* AG3 inoculum in soil was aggregated where samples were collected at scales of less than 3 Ha (Table 9). At around 3 Ha, the distribution was more or less random. At scales of greater that 3 Ha, the distribution was uniform.

Approximate area (Ha)	K function	K(S)	Distribution of inoculum
0.5	0.207	0.126	Aggregated
1.0	0.488	0.283	Aggregated
2.0	1.075	0.785	Aggregated
3.0	1.501	1.539	Random
4.0	1.827	3.142	Uniform

TABLE 9. DISTRIBUTION OF *RHIZOCTONIA SOLANI* AG3 IN FIELD SOIL (FIELD 7) SAMPLED IN DECEMBER 2008 ESTIMATED BY COMPARING RIPLEY'S K FUNCTION WITH K(S) THE MEASURE OF COMPLETE SPATIAL RANDOMNESS.

4.1.3. Evaluation of sampling methodologies and depth of sampling

(Results of comparison of 5 sampling protocols using samples collected from Field 7 in Aberdeenshire (14 January 2009), Field 16 in Morayshire (14 January 2009) and Field 4 in Morayshire (14 January 2009)).

As found with the intensive sampling, *R. solani* was not detected in Field 16 (Morayshire), irrespective of sampling method. In the other two fields, the pathogen was detected but the level of detection varied both between sampling methodology and testing centre (Table 10). Taking the mean values, the pathogen was detected (by at least three out of four field/testing organisation combinations) where the sampling was systematic (method 3) or where 200 or more sampling cores were taken (methods 4 and 5). The Australian methodology was interesting in that, although *R. solani* was detected by one testing organisation in each field, the levels detected were relatively high. Dividing the 4 ha block into 1 ha units and testing samples from each unit and taking the mean was the most effective sampling method but was considered impractical.

Where different sampling depths were compared using the standard sampling method, *R. solani* was detected in the top soil but not below 5cm (Table 10).

Results for *S. subterranea* varied between sampling methods with no particular method showing more consistency (Table 10). Irrespective of sampling method, the relative level of contamination in each of the three soils was consistent. This was also broadly true for *C. coccodes*.

Sampling method		pg DNA / g soil					
		Field 4 (Morayshire)		Field 7 (Aberdeenshire)		Field 16 (Morayshire)	
a) *R. solani*		**SAC**	**SCRI**	**SAC**	**SCRI**	**SAC**	**SCRI**
1a. Standard (top 5cm soil) 100 bulked cores/4 ha		0	205	1127	0	0	0
1b. Standard (5-15cm soil depth) 100 bulked cores/4ha		0	0	0	0	0	0
2. Australian 33 x 3 bulked cores/4ha		0	1664	5153	0	0	0
3. New PCN 400 bulked cores/4 ha		0	237	267	140	0	0
4. Experimental 1: 200 cores bulked/4ha		108	2444	0	31	0	0
5. Experimental 2:100/cores bulked/1ha (= 400 cores/4 ha)	A	4640	0	4470	0	0	0
	B	885	100	197	448	0	0
	C	209	242	0	0	0	0
	D	1160	145	0	0	0	0
Mean		1724	122	1167	112	0	0
b) S. subterranea							
1a. Standard (top 5cm soil) 100 bulked cores/4 ha			29.7		4.7		1.2
1b. Standard (5-15cm soil depth) 100 bulked cores/4ha			18.0		1.6		0.2
2. Australian 33 x 3 bulked cores/4ha			36.5		1.3		0.6
3. New PCN 400 bulked cores/4 ha			19.5		4.4		2.9
4. Experimental : 200 cores bulked/4ha			45.9		16.7		0.4
5. Experimental 2: 100/cores bulked/1ha (= 400 cores/4 ha)	A		36.2		5.8		1.6
	B		56.0		2.9		5.9
	C		13.4		205.4		0.4
	D		5.8		0.94		0.4
Mean			27.8		53.7		2.1
c) C. coccodes							
1a. Standard (top 5cm soil) 100 bulked cores/4 ha			39.4		18.4		0
1b. Standard (5-15cm soil depth) 100 bulked cores/4ha			49.9		0		0
2. Australian 33 x 3 bulked cores/4ha			48.1		0		0
3. New PCN 400 bulked cores/4 ha			503.1		0		0
4. Experimental 1: 200 cores bulked/4ha			90.5		0		0
5. Experimental 2: 100/cores bulked/1ha (= 400 cores/4 ha)	A		0		15.1		0
	B		437.5		59.2		0
	C		0		0		0
	D		0		0		0
Mean			109.4		18.6		0

TABLE 10. COMPARISON OF RESULTS OF SOIL TESTING FOR A) *R. SOLANI* AG3, B) *S. SUBTERRANEA* AND C) *C. COCCODES* IN 3 FIELDS USING FIVE DIFFERENT SAMPLING METHODOLOGIES (JANUARY 2009) AND A COMPARISON OF THE DETECTION OF THE PATHOGEN IN THE TOP 5CM SOIL COMPARED TO THE 10CM BELOW THIS. VALUES ARE MEANS OF THREE SUB-SAMPLES FOR *R. SOLANI* AND TWO SUB-SAMPLES FOR THE OTHER PATHOGENS, ANALYSED IN DUPLICATE USING REALTIME PCR.

4.1.3.1. Repeat evaluation of two methods

Where repeat samples were taken from Fields 4 and 7 (28 & 29 March 2009), using the standard method but retaining only the top 5cm soil (method 1) and the Australian method (method 2) *R. solani* was detected in Field 4 (Morayshire) and at relatively high levels of contamination. However, the pathogen was not detected by either testing organization in Field 7 (Table 11).

	pg DNA / g soil			
	Field 4 (Morayshire)		Field 7 (Aberdeenshire)	
Sampling method	**Mean soil contamination**	**Standard deviation**	**Mean soil contamination**	**Standard deviation**
1. Standard - top 5cm soil	99348	31171.3	0	0
2. Australian	10083	4498.2	0	0

TABLE 11. DETECTION OF *R. SOLANI* IN REPEAT SAMPLING OF TWO FIELD SOILS USING TWO METHODS OF SAMPLING IN MARCH 2009 (MEAN OF SAC & SCRI RESULTS)

4.1.4. Comparison of results (all soil samples) between SAC and SCRI

Overall, there was considerable inconsistency between the results for SAC and SCRI. In Field 4, SAC's data at the first sampling date (Table 10) only detected *R. solani* using methods 4 and 5, whereas SCRI detected the pathogen with all methods. Upon re-testing (Table 11) both organisations detected the pathogen using methods 1 and 2. In Field 7, the pathogen was detected more easily by SAC than SCRI (Table 10), whereas at the second sampling date neither organisation could detect the pathogen. The possible reasons for this are discussed from page 47 onwards.

4.1.4.1. R. solani Anastomosis Groups present in soil (samples collected during a previous project)

Before commencing the testing of soil samples collected during the original diagnostics project (R253) for *R. solani* anastamosis groups other than AG 3, the assays developed at FERA for AG 2.1, 4 and 5 were assessed on isolate collections at SCRI.

Six of the eight AG 2.1 isolates tested positive using the AG 2.1 assay (Table 12); however the sensitivity of the assay was relatively low. Only one of the isolates characterised as AG 4 tested positive (R113) using the AG 4 assay. For this isolate, the assay was not very sensitive, and DNA concentrations of less than 20 pg /µl were not amplified. The AG 4 (R113) isolate along with an isolate characterised as AG 2-1 (R129), also cross-reacted with AG 5 assay, albeit only at very high DNA concentrations (200 ng / µl). The four isolates characterised as AG 5 tested positive using the AG 5 assay, however only DNA at concentrations of 20 pg / µl or more were amplified by the assay.

AG group	Isolate ID	Origin	Assay AG 2.1	AG 4	AG 5
AG 2-1	R121	Australia – Potato	+	-	-
	R100	UK – Potato	-	-	-
	R103	UK – Potato	+	-	-
	R129	Australia – Potato	+	-	+ (low)
	R40	Holland – Tulip	+	-	-
	R41	Holland – Cauliflower	+	-	-
	R63	Japan – Cabbage	+	-	-
	R67	Japan – Soil	-	-	-
AG 4	R112	UK – Cauliflower	-	-	-
	R113	UK – Broccoli	-	+	+ (low)
	R148	Australia – Cauliflower	-	-	-
AG 5	R110	UK – Potato	-	-	+
	R91	Japan – Sugar beet	-	-	+
	R92	Japan – Sugar beet	-	-	+
	R111	UK – Couchgrass	-	-	+

TABLE 12. *R. SOLANI* ISOLATES TESTED WITH FERA'S AG 2.1, 4 AND 5 REAL-TIME PCR ASSAYS; (+) INDICATES THAT THE ISOLATE WAS AMPLIFIED, + (LOW) INDICATES THAT ONLY VERY HIGH CONCENTRATIONS OF ISOLATE DNA TESTED POSITIVE WITH THE ASSAY.

4.1.4.2. Re-testing R253 soils for AG 2.1, AG 4 & AG 5

DNA extracted from 39, 40 and 29 soils originally sampled in 2005, 2006 and 2007 respectively and tested for *R. solani* AG 3, were retested for *R. solani* AG 2.1.

Four out of the 39 2005 soils were found to have detectable levels of AG 2.1. Of these 4, 3 were soils in which no AG 3 had been detected and in which no disease developed in the crop. One soil in which AG 2.1 was detected also had AG3 present, AG 3 had also been detected on the seed which was planted, but there were no visual disease symptoms on the progeny tubers.

Two out of the 40 2006 soils tested were found to have detectable levels of AG 2.1. One of these soils had no detectable AG 3 in either the soil or on the seed and disease did in fact develop in this crop. The other soil in which AG 2.1 was detected also had detectable levels of AG 3 in both the soil and on the seed, but no disease developed in this case.

A single soil out of the 29 2007 soils tested was found to have detectable levels of AG 2.1. This soil also contained AG 3 inoculum and disease did develop in the crop.

	Soils testing positive for AG 2.1 (total number of soils tested)	Soils in which only AG 2.1 detected	Soils in which both AG 2.1 and AG 3 detected
2005	4 (39)	- - -	-
2006	2 (40)	+	-
2007	1 (29)		+

TABLE 13. THE OCCURRENCE OF BLACK SCURF ON PROGENY TUBERS IN CROPS GROWN IN SOILS IN WHICH AG 2.1 (BUT NO AG 3) WAS DETECTED, AND IN SOILS WITH BOTH AG 2.1 AND AG3: (+) DISEASE, (-) NO DISEASE.

There were few incidences of AG 2.1 in the soil samples tested, 7 out of a total of 108. Where AG 2.1 was detected we are unable to draw any conclusions as to whether it caused disease or not, with only an single instance where its detected without the presence of AG 3 could be associated with disease. Of 35 soils tested for AG 4 and AG 5, all were negative.

4.2. Inter-laboratory comparison

There was variation in the amount of DNA detected between the three participating labs (Table 14a to d). In general there was good agreement between the labs for the levels of DNA detected. However, the similarity in results from one lab to the next varied depending on the target pathogen. For *R. solani* AG3 and *P. atrosepticum*, all labs were able to detect target DNA at all three levels within acceptable limits (i.e. the z-scores for each lab were ≤2). For *C. coccodes*, labs A and C were able to detect target DNA well within the acceptable limits for all three pathogen levels. However, Lab B failed to detect *C. coccodes* in the 'low inoculum' samples and returned a result for the high inoculum level that was outside acceptable limits (the z-score was -4.2). For *S. subterranea*, all participating labs failed to detect target DNA within acceptable limits in the 'low inoculum' samples but were able to detect target DNA accurately at moderate and high levels.

In order to determine whether the variability between labs was due to different extraction methods or as a result of post-extraction processes, aliquots of Fera's DNA extracts for *C. coccodes* were distributed to the labs for repeat testing. The results from the repeat testing are shown in Table 15. This revealed that variability, as indicated by the z-scores, between labs was lower in Lab B than when raw samples had been tested. However, the z-scores for Lab C were higher than when raw samples were tested. Lab B was able to detect pathogen DNA in the 'low inoculum' extract samples.

4.2.1. DNA shelf-life test

In soil spiked with one of three soil-borne pathogens (*R. solani* AG3, *C. coccodes* and *S. subterranea*), DNA levels declined by approximately 50 to 60% from three to seven days after inoculation ($P<0.003$) (Table 16). From 7 to 28 days, levels of DNA showed no further reduction ($P=0.375$). There was no difference in DNA levels over the duration of the experiment whether samples were stored at either 4^{o}C or 18^{o}C ($P=0.314$).

a

Laboratory	Target pathogen/level					
	C. coccodes low assigned value	Log DNA/sample 1.8979	*C. coccodes* Med assigned value	Log DNA/sample 4.6912	*C. coccodes* High assigned value	Log DNA/sample 5.205
	result	z-score	Result	z-score	Result	z-score
A	1.762	-0.3	4.871	0.3	5.201	0.0
B	-3.000	**-11.3**	3.795	-1.3	3.097	**-4.2**
C	2.311	1.0	4.901	0.3	5.226	0.0

b

Laboratory	Target pathogen/level					
	R. solani low assigned value	Log DNA/sample 3.7233	*R. solani* Med assigned value	Log DNA/sample 4.7643	*R. solani* High assigned value	Log DNA/sample 5.5935
	result	z-score	Result	z-score	Result	z-score
A	3.439	-0.3	4.604	-0.3	5.511	-0.2
B	4.469	0.7	5.599	1.7	6.665	2.0
C	3.060	-0.7	5.275	1.1	6.300	1.3

c

Laboratory	Target pathogen/level					
	S. subterranea low assigned value	Log DNA/sample 1.1408	*S. subterranea* med assigned value	Log DNA/sample 2.9039	*S. subterranea* high assigned value	Log DNA/sample 3.1372
	result	z-score	Result	z-score	Result	z-score
A	2.350	**4.6**	3.481	0.5	3.708	1.0
B	1.830	**2.6**	2.377	-0.5	2.722	-0.7
C	0.557	**-2.2**	1.920	-0.9	2.981	-0.3

d

Laboratory	Target pathogen/level					
	P. atrosepticum low assigned value	Log DNA/sample 2.801	*P. atrosepticum* med assigned value	Log DNA/sample 4.552	*P. atrosepticum* high assigned value	Log DNA/sample 6.304
	result	z-score	Result	z-score	Result	z-score
A	3.027	1.5	4.755	1.4	6.338	-0.6
B	2.677	-0.1	4.476	0.0	6.424	1.0
C	2.700	0.0	4.418	-0.3	6.158	-0.4

TABLE 14A TO D. MEAN VALUES OF TARGET PATHOGEN (LOG_{10} PG) DNA/G SOIL AND Z-SCORES OBTAINED BY EACH PARTICIPATING LABORATORY USING THEIR METHODS FOR NUCLEIC ACID EXTRACTION AND DETECTION. A Z-SCORE IS THE OBSERVED STANDARD DEVIATION OF REPRODUCIBILITY FROM A COLLABORATIVE TRIAL AND REPRESENTS FITNESS FOR PURPOSE. IF A LABORATORY RECEIVES A Z-SCORE OUTSIDE THE RANGE $|z| \leq 2$ IT IS LIKELY THAT THIS IS DUE TO POOR PERFORMANCE. NUMBERS IN BOLD INDICATE NON-COMPLIANT Z-SCORES.

Laboratory	Target pathogen/level					
	C. coccodes low assigned value	Log DNA/uL 0.77	*C. coccodes* Med assigned value	Log DNA/uL 3.57	*C. coccodes* High assigned value	Log DNA/uL 4.08
	result	z-score	Result	z-score	Result	z-score
A	0.64	-0.4	3.75	0.7	4.08	0.0
B	0.83	0.2	3.70	0.5	3.93	-0.6
C	1.28	1.4	4.19	2.3	4.51	1.6

TABLE 15. MEAN VALUES OF TARGET PATHOGEN (LOG_{10} PG DNA/UL) OBTAINED BY EACH PARTICIPATING LABORATORY USING DNA EXTRACTS PROVIDED BY FERA. A Z-SCORE IS THE STANDARDISED MEASURE OF BIAS FOR A COLLABORATIVE TRIAL AND REPRESENTS FITNESS FOR PURPOSE. IF A LABORATORY RECEIVES A Z-SCORE OUTSIDE THE RANGE $|z| \leq 2$ IT IS LIKELY THAT THIS IS DUE TO POOR PERFORMANCE. NUMBERS IN BOLD INDICATE NON-COMPLIANT Z-SCORES.

	Time (days) after adding pathogen to soil/amount of DNA/g soil			
Target DNA	3	7	14	28
R. solani AG3[a]	216.2	125.1	108.5	107.1
C. coccodes[a]	11.1	6.3	6.1	7.0
S. subterranea[b]	319.9	88.2	56.7	78.2

a, level of DNA in ng/g soil; b, level of DNA in pg/g soil

TABLE 16. DECLINE IN THREE PATHOGENS WITH TIME AFTER ADDING TO SOIL

4.3. Effect of environmental conditions on powdery scab disease development

4.3.1. Results and Discussion

4.3.1.1. Root infection – microscopic examination (SAC)

No significant differences between cultivars for percentage root infection, as assessed microscopically, were observed over the 11 sampling dates. Root infection was observed at a low level in both cultivars at the earliest sampling date (12/6/08) and increased to a mean value of approximately 50% infection in each root section observed by the 10th sampling date (14/8/08) (Figure 3). Root infection levels reduced in both cultivars at the final sampling date but this could be due to root degradation making the scoring of samples more difficult.

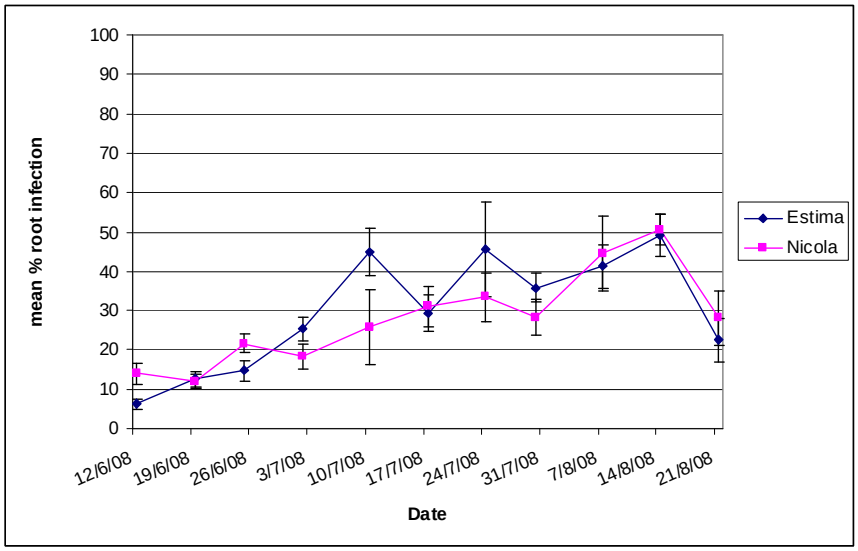


FIGURE. 3. PERCENTAGE AREA OF ROOT SAMPLE INFECTED (MEAN OF 5 SAMPLES) AS ASSESSED MICROSCOPICALLY AT 11 SAMPLING DATES IN CULTIVARS ESTIMA AND NICOLA SAMPLED AT SAC. STANDARD ERRORS OF MEANS ARE SHOWN.

4.3.1.2. Root galling

At the SCRI site, root galling was first observed on 22/7/08 and the severity of root galling increased over subsequent weekly sampling dates. At each date, cultivar Agria showed a significantly greater root gall severity than cultivar Nicola (Figure 4a). At the SAC site (Figure 4b), root galling was first observed on 17/7/08. However, in this case there was little difference in the severity of root galling between cultivars Estima and Nicola.

When both sets of data are plotted according to corresponding sampling weeks (Figure 4c) it can be seen that root galling was observed one week earlier (week 5) at SAC than at SCRI (week 6), with Agria showing significantly more galling than the other cultivars.

4.3.1.3. Powdery scab symptoms

Powdery scab symptoms were first observed at SCRI on 29/7/08 and at SAC on 31/7/08 (Figure 5a and b). When these dates are aligned (Figure 5c), this equates to week 8 of sampling (see Table 3) in each case.

At SCRI, powdery scab symptoms were significantly more severe on cultivar Agria than Nicola, as would be expected according to disease resistance rating and similarly, at SAC disease severity was higher on Estima than Nicola. Overall, Estima was the most susceptible cultivar followed by Agria and then Nicola. Higher levels of disease were recorded at SAC compared with SCRI according to the disease scores for Nicola (Figure 5c).

4.3.1.4. **S. subterranea** *detection in roots using real-time PCR.*

DNA of *S. subterranea* was detected in all cultivars at both sites from the earliest sampling dates (1/7/08 and 12/6/08 at SCRI and SAC, respectively) and there was a general trend for increasing detection of *S. subterranea* DNA in roots over time, see Figure 6. At SCRI, significantly more DNA of *S. subterranea* was detected in roots of cultivar Nicola compared with cultivar Agria at all sampling dates except the first (Figure 6a). This is in contrast with the results for root galling symptoms (Figure 4a) and tuber symptoms (Fig 5a); where in each case Agria had significantly more disease. At SAC there was little difference between cultivars in the amount of DNA detected.

4.3.1.5. S. subterranea detection in tuber tissue using real-time PCR

DNA of *S. subterranea* was detectable in symptomless tubers from the time of tuber formation (8/7/08) at both sites (Figure 7). Significantly more DNA was detected in symptomless tubers of cultivar Agria compared with Nicola at the SCRI site, whereas few differences in the amount of DNA detectable in tuber tissue of Estima and Nicola grown at SAC were noted.

In general it was observed that the SCRI samples seem to contain much higher levels of *S. subterranea* DNA than the SAC samples and therefore a comparative analysis of the calculations used was made in order to rule out experimental error. There was a similar relationship between the Ct (Critical threshold) values and the subsequent conversion to DNA concentration between SCRI and SAC. The conversion from sporeballs to DNA quantity only accounted for a small difference in values. SCRI found more DNA in samples than SAC i.e. many root and tuber samples produced Ct values in the range 20-25, whereas few Ct values <25 or <28 for root and tuber samples respectively were recorded at SAC. There was no difference in the amount of tissue or methods used for tuber extractions to account for this difference and we therefore conclude that the SCRI root samples genuinely contained more DNA than the SAC samples.

4.3.1.6. Dates of infection and disease symptom development.

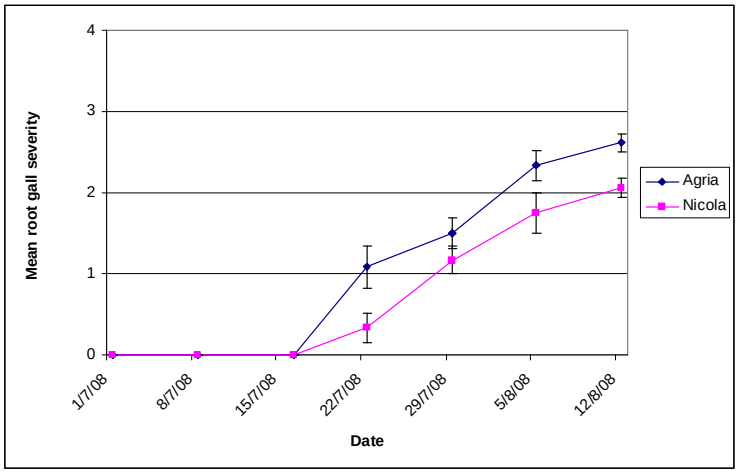
A summary of the dates at which root and tuber infections were detected by PCR and microscopy, and first root gall and powdery scab symptoms were visually observed, is given in Table 17. Despite the differences between cultivars and sites in terms of quantity of DNA detected and severity of symptoms, as described above, the dates at which infection occurred and symptoms developed were remarkably consistent between sites. Early sampling by SAC showed that roots were infected by *S. subterranea* at the very first sampling date (12/6/08) and this was confirmed using real-time PCR and microscopically. Although plants were not sampled at SCRI until 1/7/08 it is highly likely that they were also infected before that date, in line with the SAC samples as planting dates were very similar. Root galls were first observed at SAC on 17/7/08 and at SCRI on 22/7/08, suggesting that under the weather conditions experienced, tuber galls cannot be visualized until at least 5 weeks or more after root infection. Similarly, DNA of *S. subterranea* could be detected in tuber tissue as soon as tubers were formed (between 1/7/08-10/7/08) and tuber symptoms became visible 3 weeks later, occurring on the same date at both sites.

4.3.1.7. Weather data and inoculum level

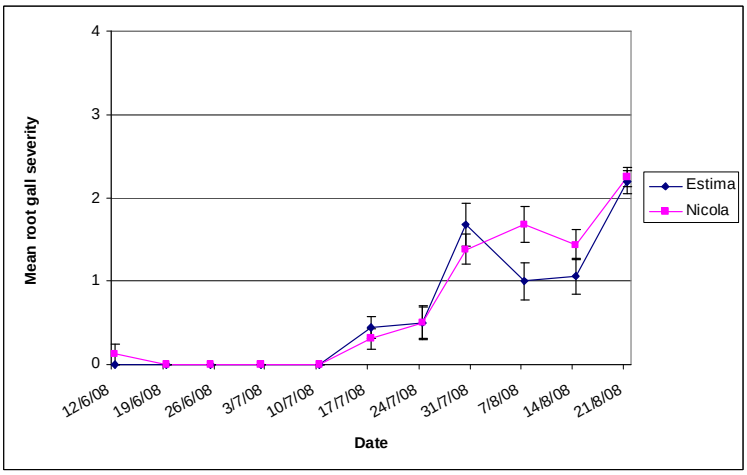
Mean soil temperatures (℃) and soil moisture deficits (mm) for each site are shown in Figures 8 a, b. The mean daily temperature was approximately 15℃ at both sites and each site was irrigated to ensure disease development. The SAC site was marginally cooler and wetter than the SCRI site, which may account for the differences in powdery scab severity, particularly where Nicola was shown to have more disease at SAC than SCRI. The weather conditions experienced should, from previous experience, have been conducive for the development of both root galls and powdery scab and this appears to be the case. The level of soil inoculum at SAC was on average slightly higher than at SCRI, although within the same order of magnitude. This may also have had some effect on disease levels, although it is not possible at present to draw any such conclusion. It is clear that the inoculum present at each site was sufficient to cause infection in all root and tuber samples assessed.

Powdery scab severity was higher at SAC than SCRI, and whilst this may be a result of the slightly cooler and wetter soil conditions, the level of inoculum pre-planting at SAC was also higher than at SCRI. Whilst the relationship between inoculum level and disease development still remains unclear, differences in inoculum level along with environmental conditions will be better understood when the results from more trials are completed. Indeed, additional trials planned for 2009 will specifically look at the effect of soil inoculum level on disease development.

a)



b)



c)

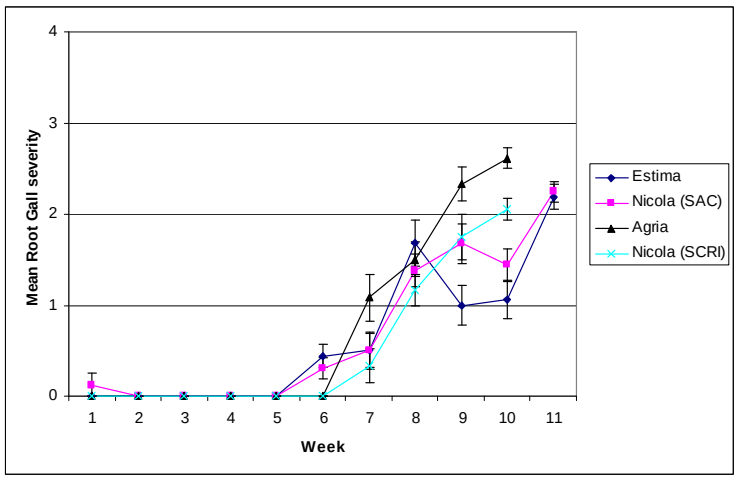
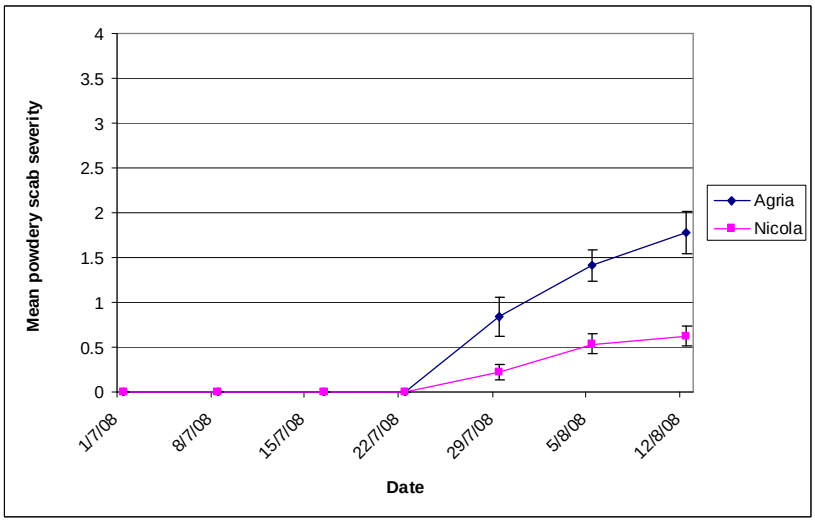
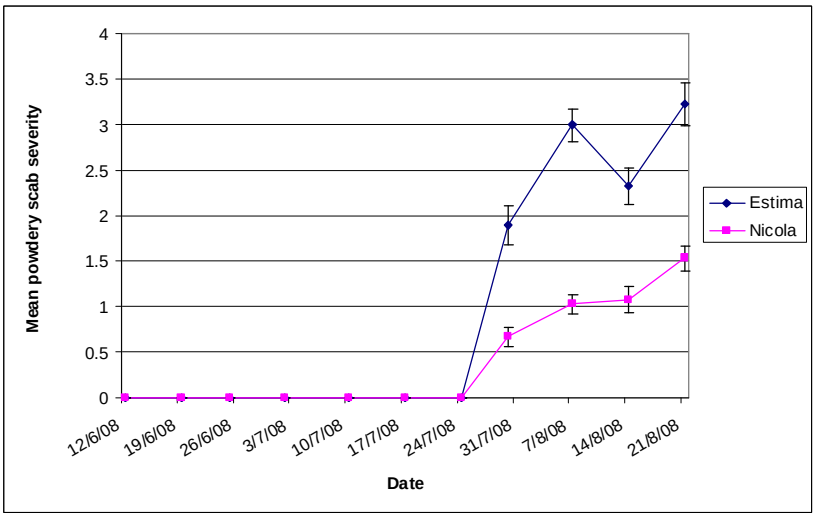


FIGURE. 4. MEAN ROOT GALL DISEASE SEVERITY ON A 0-4 SCALE OF INCREASING SEVERITY MEASURED AT WEEKLY INTERVALS ON A) CULTIVARS AGRIA AND NICOLA AT THE SCRI SITE B) CULTIVARS ESTIMA AND NICOLA AT THE SAC SITE AND C) DATA FROM BOTH SITES COMPILED AND ALIGNED ACCORDING TO SAMPLING WEEK. STANDARD ERRORS OF MEANS ARE SHOWN.

a)



b)



c)

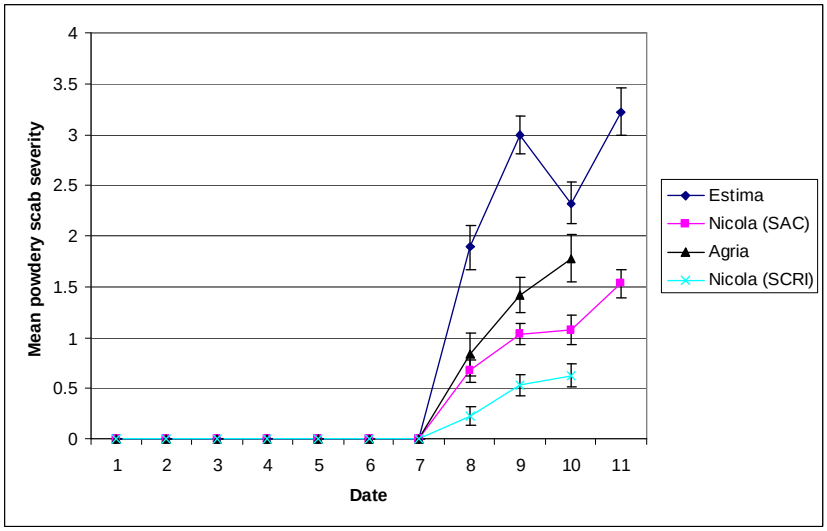
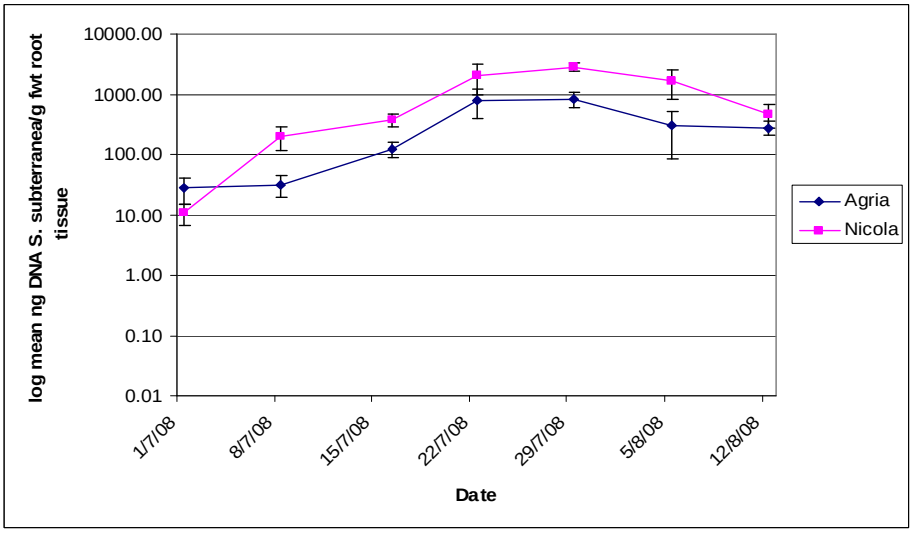
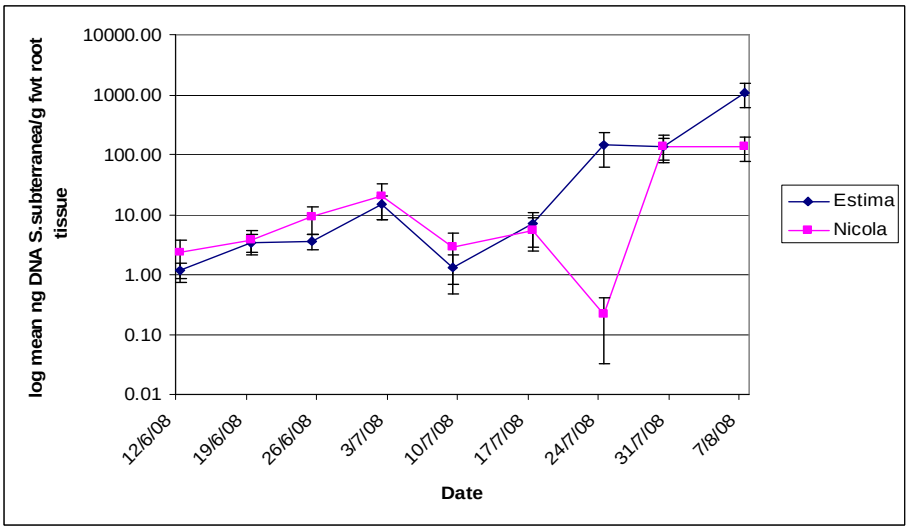


FIGURE. 5. MEAN POWDERY SCAB SEVERITY ON A 0-6 SCALE OF INCREASING DISEASE MEASURED AT WEEKLY INTERVALS A) AT THE SCRI SITE ON CULTIVARS AGRIA AND NICOLA B) AT THE SAC SITE ON ESTIMA AND NICOLA AND C) COMPILED SET OF DATA FROM BOTH SITES ALIGNED ACCORDING TO WEEKLY SAMPLING DATE. STANDARD ERRORS OF MEANS ARE SHOWN.

a)



b)



c)

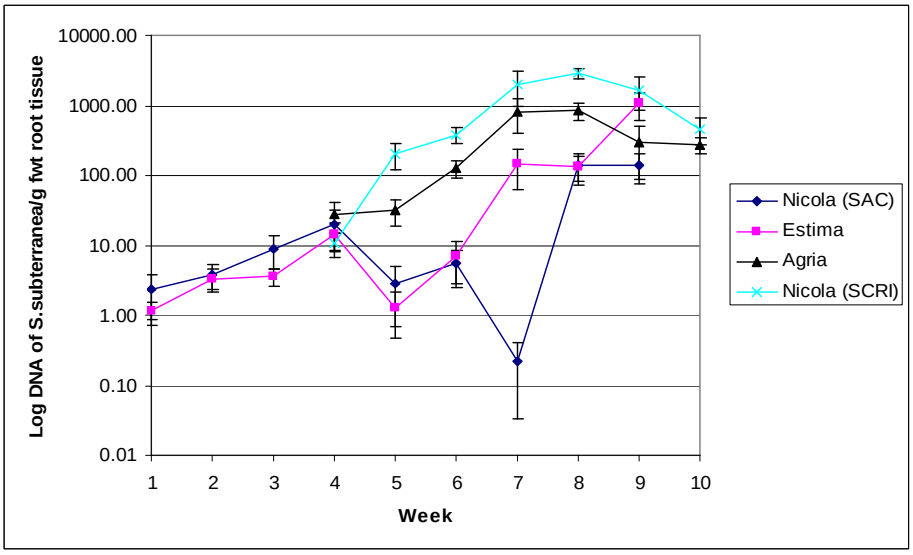


FIG. 6. AMOUNT OF *S. SUBTERRANEA* DNA (LOG NG DNA/G FRESH WEIGHT OF ROOT TISSUE) DETECTED IN ROOTS A) OF CULTIVARS AGRIA AND NICOLA AT SCRI OVER 7 SAMPLING DATES B) OF CULTIVARS ESTIMA AND NICOLA AT SAC OVER 9 SAMPLING DATES AND C) COMPILED SET OF DATA FROM BOTH SITES SHOWING ALIGNED SAMPLING DATES. STANDARD ERRORS OF MEANS ARE SHOWN.

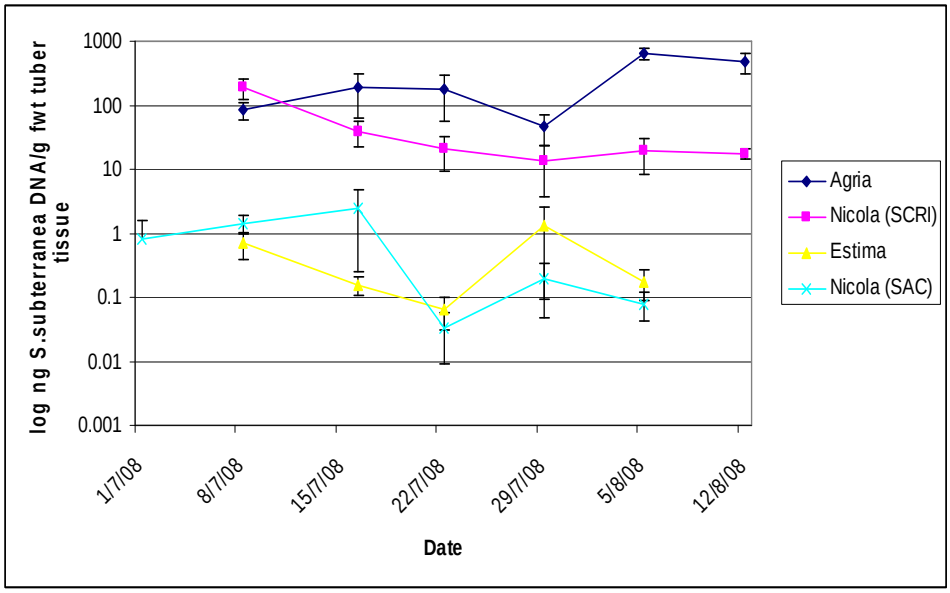
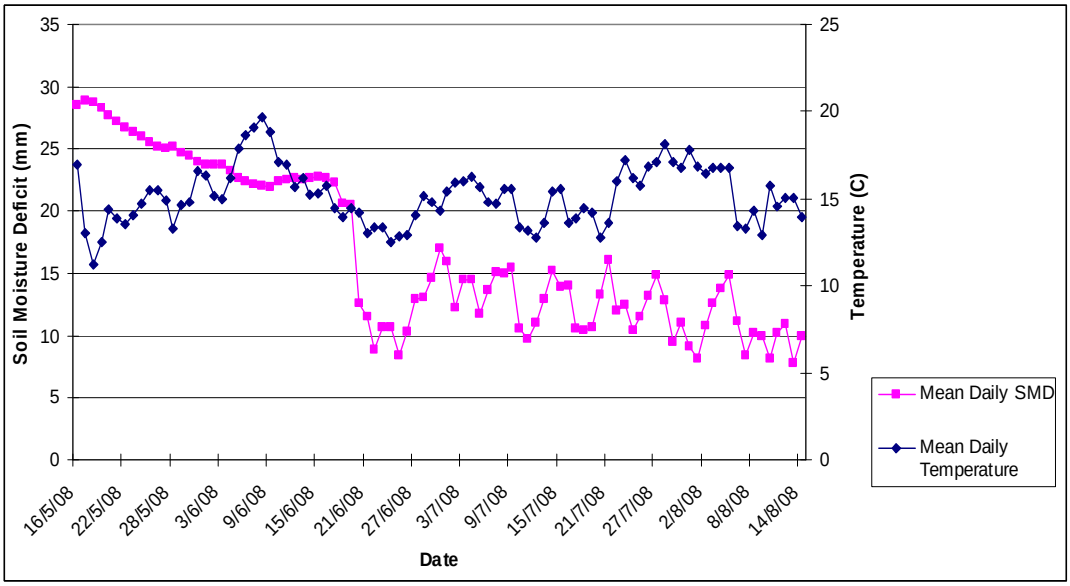


FIGURE. 7. LOG DNA (NG) OF *S. SUBTERRANEA* DETECTED PER G FRESH WEIGHT TUBER TISSUE IN SYMPTOMLESS TUBERS OF CULTIVARS AGRIA AND NICOLA (SCRI) AND ESTIMA AND NICOLA (SAC) AT 7 SAMPLING DATES. STANDARD ERRORS OF MEANS ARE SHOWN.

Week	1	2	3	4	5	6	7	8	9	10	11
SAC sampling date	12/6	19/6	25/6	2/7	10/7	17/7	24/7	30/7	7/8	14/8	21/8
SCRI sampling date	-	-	-	1/7	8/7	16/7	22/7	29/7	5/8	12/8	-
Root infection detected (DNA and microscopy)											
Estima	√√	√√	√√	√√	√√	√√	√√	√√	√√	√√	√√
Nicola (SAC)	√√	√√	√√	√√	√√	√√	√√	√√	√√	√√	√√
Agria	-	-	-	√	√	√	√	√	√	√	-
Nicola (SCRI)	-	-	-	√	√	√	√	√	√	√	-
Root gall symptoms observed											
Estima	x	x	x	x	x	√	√	√	√	√	√
Nicola (SAC)	x	x	x	x	x	√	√	√	√	√	√
Agria	-	-	-	x	x	x	√	√	√	√	-
Nicola (SCRI)	-	-	-	x	x	x	√	√	√	√	-
DNA detected in symptomless tubers											
Estima	-	-	-	-	√	√	√	√	√	√	√
Nicola (SAC)	-	-	-	√	√	√	√	√	√	√	√
Agria	-	-	-	-	√	√	√	√	√	√	-
Nicola (SCRI)	-	-	-	-	√	√	√	√	√	√	-
Tuber symptoms observed											
Estima	-	-	-	-	x	x	x	√	√	√	√
Nicola (SAC)	-	-	-	x	x	x	x	√	√	√	√
Agria	-	-	-	-	x	x	x	√	√	√	-
Nicola (SCRI)	-	-	-	-	x	x	x	√	√	√	-

TABLE 17. SUMMARY OF DATES (IN 2008) OF FIRST OBSERVATIONS OF DISEASE AND DETECTION OF *S. SUBTERRANEA* DNA IN ROOTS AND TUBERS ACCORDING TO SITE, SAMPLING DATE AND CULTIVAR. - = NOT TESTED OR NO TUBERS FORMED, √ = DETECTED, √√ = DETECTED BY PCR AND MICROSCOPY, X = NOT DETECTED.

a)



b)

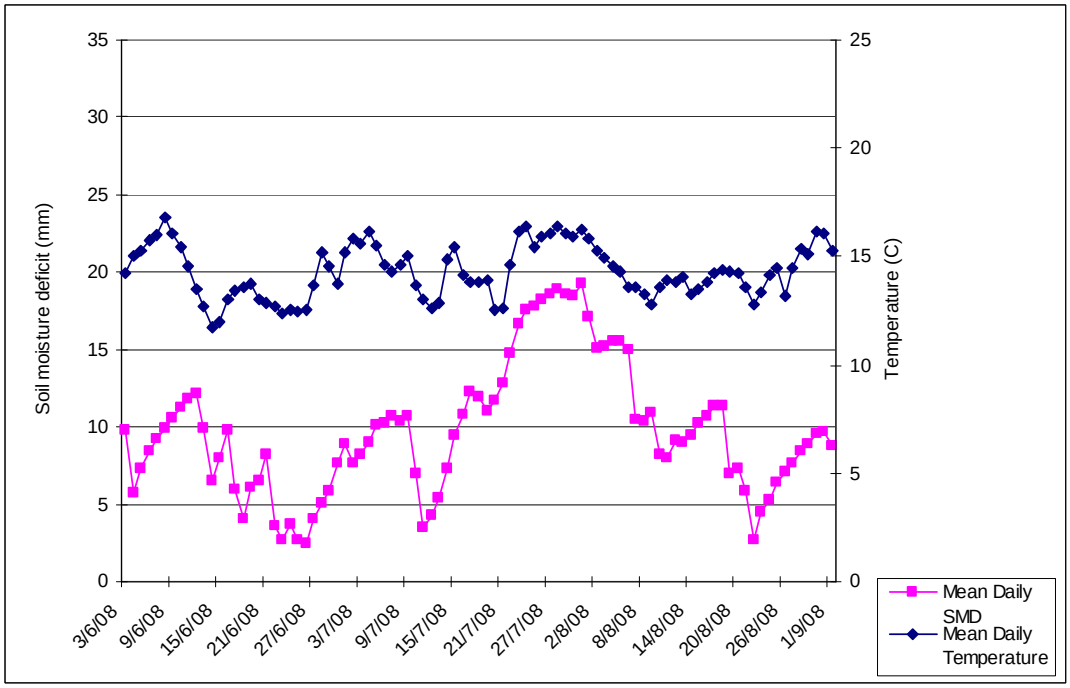


FIGURE. 8. MEAN DAILY SOIL MOISTURE DEFICIT (MM) AND TEMPERATURE (ºC) VALUES OVER THE DURATION OF THE TRIALS AS RECORDED AT A) SCRI AND B) SAC. TEMPERATURE VALUES ARE THE MEAN OF 24 HOURLY READINGS AND SOIL MOISTURE VALUES ARE THE MEAN OF 24 HOURLY READINGS FROM EACH OF 4 INDIVIDUAL PROBES.

4.4. Multiplexing of potato pathogens

Using the Applied Biosystems TaqMan Universal PCR Master Mix (Table 18) gave good results for *C. coccodes* and for *S. subterranea* in combination with each other (although there may have been a slight loss of sensitivity in the *C. coccodes* assay) and for *C. coccodes* in the presence of *R. solani,* however, the *R. solani* itself was not well amplified.

Using the PerfeCTa Mulitplex qPCR SuperMix improved the *R. solani* assay when multiplexed with *S. subterranea* (Table 19), but unfortunately reduced the efficacy of the *C. coccodes* assay, both in single-plex and multiplex assays. Similarly, using the QuantiTect Multiplex PCR Kit (Table 20) whilst improving the *R. solani* assay when multiplexed with *S. subterranea*, and performing well in the presence of *C. coccodes*, the *C. coccodes* assay itself was adversely affected by the master mix.

Depending upon the target pathogens of interest, certain multiplex assays are now available:

- *C. coccodes* and *S. subterranea* (Applied Biosystems TaqMan Universal PCR Master Mix)
- *S. subterranea* and *R. solani* (QuantiTect Multiplex PCR Kit)

- However more work would be required to find a suitable multiplex assay for *R. solani* in conjunction with *C. coccodes*.

Applied Biosystems – Universal MasterMix

C. coccodes

	DNA	*C .coccodes* single- plex	*C .coccodes* with *S.subterranea*	*C .coccodes* with *R.solani*
std1	2 ng	21.2	22.2	21.3
std2	200 pg	25.7	27.7	26.3
std3	20 pg	31.2	33.5	31.4
std4	2 pg	34.5	38.5	35.0
std5	200 fg	38.8	40.0	39.7
std6	100 fg	38.9	40.0	39.8
R^2		0.99	0.99	0.99

S. subterranea	sporeballs	*S. subterranea* single plex	*S. subterranea* with *C. coccodes*	*S. subterranea* with *R.solani*
std1	20000	22.3	22.5	22.1
std2	2000	28.8	28.5	27.3
std3	200	33.6	33.6	32.5
std4	20	36.7	36.9	35.6
std5	10	37.0	35.5	37.8
std6	2	40.0	39.5	38.3
R^2		0.93	0.91	0.91

R. solani

	DNA	*R. solani* single plex	*R. solani* with *C. coccodes*	*R. solani* with *S. subterranea*
std1	2 ng	24.1	24.0	26.2
std2	200 pg	29.8	32.4	34.6
std3	20 pg	34.6	35.3	36.0
std4	2 pg	37.8	37.6	38.7
std5	1 pg	37.4	37.6	39.4
std6	500 fg	40.0	39.3	40.0
R^2		0.94	0.84	0.81

TABLE 18. MULTIPLEX ASSAY USING APPLIED BIOSYSTEMS UNIVERSAL MASTERMIX. VALUES GIVEN ARE CT VALUES

PerfeCTa Mulitplex qPCR SuperMix

C. coccodes

	DNA	*C .coccodes* single- plex	*C .coccodes* with *S.subterranea*	*C .coccodes* with *R.solani*
std1	2 ng	26.6	27.6	25.6
std2	200 pg	31.1	32.7	31.9
std3	20 pg	38.2	39.2	38.6
std4	2 pg	40.0	40.0	40.0
std5	200 fg	40.0	40.0	40.0
std6	100 fg	40.0	40.0	40.0
R^2		0.98	0.99	0.99

S. subterranea

	sporeballs	*S. subterranea* single plex	*S. subterranea* with *C. coccodes*	*S. subterranea* With *R.solani*
std1	20000	21.0	20.3	20.4
std2	2000	26.5	26.4	26.0
std3	200	31.7	30.9	31.3
std4	20	35.7	34.2	37.9
std5	10	36.4	36.1	35.8
std6	2	40.0	36.8	40.0
R^2		0.98	0.93	0.93

R. solani

	DNA	*R. solani* single plex	*R. solani* with *C. coccodes*	*R. solani* with *S. subterranea*
std1	2 ng	23.9	24.1	24.1
std2	200 pg	30.2	30.3	32.0
std3	20 pg	33.8	37.2	35.0
std4	2 pg	37.2	40.0	36.0
std5	1 pg	37.6	40.0	37.7
std6	500 fg	40.0	40.0	40.0
R^2		0.95	0.98	0.92

TABLE 19. MULTIPLEX ASSAY USING PERFECTA MULITPLEX QPCR SUPERMIX. VALUES GIVEN ARE CT VALUES

QuantiTect Multiplex PCR Kit

C. coccodes

	DNA	*C .coccodes* single- plex	*C .coccodes* with *S.subterranea*	*C .coccodes* With *R.solani*
std1	2 ng	26.4	30.2	27.6
std2	200 pg	31.2	38.0	33.5
std3	20 pg	37.6	40.0	39.6
std4	2 pg	39.8	40.0	40.0
std5	200 fg	38.6	40.0	40.0
std6	100 fg	40.0	40.0	40.0
R^2		0.78	-	0.99

S. subterranea

	sporeballs	*S. subterranea* single plex	*S. subterranea* with *C. coccodes*	*S. subterranea* with *R.solani*
std1	20000	24.8	24.7	24.6
std2	2000	28.3	28.5	28.8
std3	200	31.7	31.8	32.2
std4	20	35.9	35.8	35.3
std5	10	36.5	36.5	36.5
std6	2	40.0	39.6	38.6
R^2		0.99	0.99	0.99

R. solani

	DNA	*R. solani* single plex	*R. solani* with *C. coccodes*	*R. solani* with *S. subterranea*
std1	2 ng	24.4	24.6	25.3
std2	200 pg	28.8	28.3	29.5
std3	20 pg	32.2	32.6	32.9
std4	2 pg	35.2	38.5	37.6
std5	1 pg	36.2	38.2	37.6
std6	500 fg	39.2	38.9	40.0
R^2		0.98	0.99	0.99

TABLE 20. MULTIPLEX ASSAY USING QUANTITECT MULTIPLEX PCR KIT. VALUES GIVEN ARE CT VALUES.

5. DISCUSSION

5.1. Detection of *R. solani* AG3

Different aspects related to the detection of *R. solani* in soil samples have been studied:

a) Timing of collection of soil samples
b) Soil sampling patterns (number of cores & sampling pattern)
c) Depth of soil sampling
d) Storage of soil samples
e) Volume of soil used for DNA extractions

5.1.1. Timing of collection of soil samples

In the fields where preliminary testing was carried out in early 2008 to identify two fields for intensive sampling, a crop harvested the previous autumn had exhibited black scurf. It can be assumed that these fields left mycelium and sclerotia in the soil after harvest. Yet the levels of *R. solani* AG3 detected in the soil just 4-5 months later were low in almost all fields. This was also true when, in the autumn of 2008, a field was identified for intensive sampling where a crop with black scurf had been harvested just three months earlier. In the intensive sampling, *R. solani* AG3 was detected at only two locations and at very low levels. This suggests that inoculum levels can decline very rapidly. In both the seasons when sampling was carried out, the autumn and winter weather was extremely wet and this may have been a cause of a rapid decline in inoculum as the pathogen is sensitive to soil conditions where carbon dioxide builds up (Blair, 1943).

5.1.2. Soil sampling patterns (number of cores & sampling pattern)

A range of sampling options were tested which represent practical and less practical options. There was a trend of an increase in detection where sampling points were increased. But even here, there was a lack of consistency between testing centres.

The analysis of *R. solani* AG3 DNA distribution in soil collected in a 200 x 200 m uniform grid in a field in Aberdeenshire suggests that sampling within an area of less than 3 Ha is not suitable for determining levels of soil-borne *R. solani*. This is because the inoculum at the scale of 0.5 to 2 Ha has an aggregated distribution. In practice, this means that samples collected at that scale would either overestimate or underestimate the amount of pathogen inoculum across a crop, or worse, miss the pathogen entirely. At the 4 Ha scale, the distribution of pathogen was uniform. The mean level of DNA would adequately represent the 'true' mean across the area sampled. Therefore, the current recommendation to sample a 4 Ha field is supported by this analysis. However, the analysis has only been done, so far, at one site, in one year and in the June following a potato crop. It is likely that the distribution of *R. solani* will vary on a field-by-field basis and is worth further investigation.

The population dynamics of *R. solani* AG3 after planting also need to be fully understood for it appears that the pathogen can develop very rapidly from low initial levels of inoculum.

5.1.3. Depth of soil sampling

One positive outcome of testing sampling systems was that *R. solani* AG3 appeared to be more prevalent near the soil surface (0-5cm) than at greater depth (5-15cm). This supports the contention that inoculum is sensitive to soil conditions, the upper layers being less prone to saturation. Ironically, in the initial soil sampling and the intensive sampling of two fields, the top 5cm of soil was rejected in favour of the deeper soil sample. In light of the sampling results described above, it is the intention that future sampling for *R. solani* in the project R422 will be modified to test soil from the top 5 cm of soil.

5.1.4. Storage & preparation of soil samples

The variability in results from field testing may occur at several points in the sampling/testing process. Firstly, whilst the sampling process ensures soil is taken from the requisite sampling points, the mixing of soil before sub-samples are made, either to send to other laboratories or prior to DNA extraction, is an inexact process, especially where soils are wet at sampling. Similarly, once dried, there is a risk that inadequate mixing before sub-sampling for testing may introduce further variation. Secondly, there is also a possibility that any delay in handling, unless dried or frozen immediately, may result in loss of inoculum through degradation in soil. Such a situation was recorded by Australian researchers (personal communication). In microbiological tests studying *R. solani* AG8 causing barley stunt in Morayshire, mycelium was been found by experience to be ephemeral in soil, easily broken down or rendered unviable when soil was disturbed (B Gray, personal communication). These findings suggest soil handling should be rapid after sampling.

R. solani AG3 exists in soil as either mycelium, usually living saprophytically on organic matter, or as sclerotia. The manner of sampling may not pick up organic matter where PCN spears are used. However, in the field sampling in this project used narrow bladed trowels and organic matter should have been obtained.

Another possible point in the testing process where inoculum may be inadvertently removed is when soil is sieved prior to taking a sub-sample for testing. The sieving process is to ensure stones are not part of a sub-sample that will be milled and to ensure only fine soil particles are present. The sieving process uses a fine sieve, often 2mm, and this might exclude sclerotia passing through, however sieving is not routinely used in the processing of soil samples at SCRI.

It is difficult to draw any conclusions about the survival of natural inoculum in soil from the results from the shelf-life trial because the experimental inoculum consisted of hyphae and sclerotia that were grown on artificial media (for *C. coccodes* and *R. solani*) or infected tomato roots (*S. subterranea*). It is likely that much of the artificial inoculum is short-lived in air-dried soil compared with natural inoculum

5.1.5. Volume of soil used for DNA extractions

All the evidence indicates that the soil diagnostic test can detect *R. solani* AG3 if it is present in a soil sub-sample. It follows that the larger the quantity of soil from which DNA is extracted the greater the chance of detecting the pathogen. Fera is able to extract DNA from a total sample weight of 0.25 kg soil whereas SAC and SCRI use a soil sub-sample size of 0.06 kg. Despite this no testing laboratory consistently detected *R. solani* AG3 in the original 15 field soil samples. The lack of consistency between labs in

the detection of *R. solani* in this initial testing comparison was primarily a result of very low levels of inoculum present, therefore all the results were below or very close to the threshold of detection and therefore inconsistently detected.

This lack of consistency in soil testing results also occurred when different soil sampling methods were compared (see notes in results section) but was less apparent in the intensive soil sampling carried out in the Aberdeenshire field (Field 7).

5.1.6. Inter lab differences in detection

Variability in testing results occurred between laboratories when similar samples were distributed for testing (see comments below). These differences included different steps in the testing procedure and differences in standard curves. However, the standard curves should normally conform to agreed parameters to ensure comparability. Overall, all labs consistently detected target DNA and the results do not appear to be greatly different (i.e. all low sample means are below medium sample means)

5.1.7. Summary

Overall, it was not possible to identify a single sampling procedure that consistently detected soil-borne inoculum of *R. solani* AG3. Advice from BioSS suggested that increasing sampling points or the number of tests would improve detection. To a degree this proved to be correct. Increasing sampling points would only improve the detection of other more uniformly dispersed soil-borne pathogens such as *C. coccodes* and *S. subterranea*. However, more intensive sampling would increase costs and the risks making a commercial test unviable.

Disease resulting from soil-borne inoculum probably starts from very low levels, increasing in response to root exudates from a newly planted potato crop. Just how low the level of inoculum can be in order to initiate disease has yet to be determined. Soil environment criteria must also be suitable for pathogen growth.

More understanding is also required of the spatial distribution and the population dynamics of *R. solani* over the period of time leading up to planting. The intensively sampled site in Aberdeenshire has added to our knowledge of the distribution of the pathogen at a single sampling time and with further intensive sampling data from the UK and collaborators in the International Diagnostics Project should lead to improved strategies for sampling.

One finding that will help detection is that *R. solani* AG3 tends to persist in the upper layers of soil and thus deep sampling is unnecessary. This finding will ensure that the sampling for *R. solani* can be comparable to that carried out for other pathogens.

Although no one sampling method was identified as optimum, in future sampling for *R. solani* by taking 200 cores rather than 100 and sampling only the top 5cm of soil will be adopted as the most cost effective method. This will be utilised in future R&D projects. In addition, care will be taken to standardise handling and testing procedures. Where possible, the time between sampling and receipt in the laboratory and the time between receipt and drying will be minimised. Standard curves for diagnostic testing will be agreed to fall within agreed parameters.

5.2. Inter-laboratory comparison

Results from the collaborative trial suggest that some variability in DNA values between labs is caused by steps in the qPCR quantification itself, possibly during the construction of the standard curves. This is supported by one laboratory returning a non-compliant result (with a z-score of 2.3 for the moderate inoculum sample) from Fera-supplied samples consisting of *C. coccodes* DNA extracts. However, the data also support the conclusion that gross errors in detection, for example not detecting pathogen, are likely to be due to problems during the extraction of nucleic acid.

Variability in results may also be attributed to error introduced when dividing the soil samples, either prior to sending the spiked soil samples to the participating laboratories, or by the laboratories themselves when sub-sampling the soil. Although care was taken by Fera to ensure that the samples were fully homogenized and stable prior to dispatch, samples with low levels of inoculum were prone to less uniformly distributed target pathogen DNA than medium and high inoculum levels. Table 21 shows the coefficient of variation (cv) for each of the samples. The cvs for all pre-dispatch samples were fit for purpose (i.e.≤10%) except for the *S. subterranea* low inoculum samples. The variability in DNA levels between sub-samples accounts for the poor reproducibility of results in the inter-laboratory comparison for these samples.

Target	Inoculum level/Coefficient of variation (%)		
	Low	Medium	High
C. coccodes	10.59	1.00	0.54
R. solani AG3	0.97	1.74	1.08
S. subterranea	22.05	5.80	8.35

TABLE 21. COEFFICIENT OF VARIATION FOR LOG DNA/ G SOIL IN SAMPLES PRIOR TO DISPATCH.

Differences in results between participating labs could have been due to each lab using different intervals before carrying out the test. However, this was ruled out because the shelf-life test showed that DNA was stable in the samples within the period from dispatch to testing. In addition, Fera independently tested the samples, as one of the participating labs in the inter-laboratory comparison, after all other participating labs had returned their results. There was no consistent reduction in the DNA values of Fera's results compared with the other labs' results.

Sclerotia were used in the inter-laboratory comparison for *R. solani* AG3 but it is still uncertain what proportion this type of inoculum makes up in soil. The evaluation of relative contribution of mycelium and sclerotia to soil-borne inoculum is worthy of study as these inoculum sources may have different properties. In the powdery scab inter-laboratory test infected tomato root pieces were used as inoculum. This differed from previous studies where sporeballs were the normal inoculum source. At certain times of the year it may be difficult to find enough tubers with powdery scab to supply sufficient amount of spore balls for spiking soils. The advantage of using infected tomato roots is that it is easy to artificially produce large amounts of material contaminated with *S. subterranea* DNA. The pathogen DNA in soil spiked with infected tomato roots has been found in this present study to have potential problems with stability. It was outside the scope of this study to compare the stability of infected root inoculum with that of sporeballs. However, it is likely that the DNA in sporeballs will be stable at room temperature for many months, possibly years. Another problem with spiking soil with infected tomato root pieces was that it was difficult to produce samples with uniformly distributed inoculum, particularly for the 'low inoculum' samples. This suggests that when

planning future inter-laboratory comparison trials, consideration should be given to using soils spiked with sporeballs as inoculum for *S. subterranea*.

Following on these preliminary inter-laboratory comparisons, a protocol for the preparation of samples for an inter-laboratory comparison trial has been prepared. This is described in Appendix 2.

5.2.1. Shelf life test

The amount of target pathogen DNA for C*. coccodes, S. subterranea, R. solani* and *P. atrosepticum* did not decrease at 4^{o}C and 18^{o}C for the period between 7 and 28 days after sample preparation. This showed that the samples are stable within the expected sample turnaround time (approximately three weeks from dispatch to testing).

It is difficult to draw any conclusions about the survival of natural inoculum in soil from the results from the shelf-life trial because the experimental inoculum consisted of hyphae and sclerotia that were grown on artificial media (for *C. coccodes* and *R. solani*) or infected tomato roots (*S. subterranea*). It is likely that much of the artificial inoculum is short-lived in air-dried soil compared with natural inoculum

5.3. Effect of environmental conditions on powdery scab disease development

A preliminary discussion is included in the results section. A full discussion of the results will be made when results of current trials and international trials are available. Conclusions from the first year of the GB trials are given below

5.4. Multiplexing of potato pathogens

We have demonstrated that assays for target pathogens can be multiplexed using the standard methodology employed for singleplex assays (at SCRI). The use of reagents marketed specifically for multiplexing did not perform as well as the standard Applied Biosystems MasterMix routinely used for real-time PCR assays at SCRI. The ability to multiplex will potentially provide savings in terms of both time and money for future research and testing laboratories. However not all target pathogens were multiplexed successfully, specifically, *R. solani* did not amplify successfully in multiplex reactions. Discussions with industry representatives and colleagues in Norway are on-going to provide an explanation as to why this may be so.

6. CONCLUSIONS

6.1. Evaluation of sampling strategies for detection of *Rhizoctonia solani* AG3

- It was not possible to identify a single sampling procedure that consistently permitted detection of soil-borne inoculum of *R. solani* AG3 in a diagnostic test.
- A range of sampling procedures has been tested. Increasing sampling points appeared to improve detection to a degree. This was a conclusion from examination of limited distribution data for *R. solani* from BioSS.
- However, more intensive sampling would increase costs and risks making a commercial test unviable.
- There was a lack of consistency in soil testing results. This may be due to:
    - Ineffective mixing of soil before sub-samples are taken
    - Loss of inoculum through degradation in soil between sampling and testing
    - Loss of inoculum if soil is sieved prior to testing
    - Differences in methods of testing between laboratories.
- *R. solani* AG3 appeared to be more prevalent near the soil surface (0-5cm) than at greater depth (5-15cm). This supports the contention that inoculum is sensitive to soil conditions.
- *R. solani* AG3 appears to be sensitive to soil conditions (particularly wet conditions), with inoculum declining rapidly in wet soil
- Disease resulting from soil-borne *R. solani* AG3 may develop from low levels of inoculum, which may be difficult to detect. Further work is required to improve understanding of this pathogen, the forms of inoculum present, its distribution and development once a potato crop is planted.
- The same samples in the intensive sampling and comparison of sampling procedures were also tested for presence of powdery scab and black dot. This additional testing has provided data on the distribution of these two pathogens in soil but also revealed that no sampling method showed improved consistency for detecting them over the current standard. The relative level of contamination in each of the three soils was consistent irrespective of sampling method, especially for powdery scab.

6.2. Spatial distribution of *Rhizoctonia solani* AG3

- At one site in Aberdeenshire, inoculum in June after harvesting potatoes the previous year was aggregated in patches of 0.5 to 2 Ha. In practice, this means that samples collected at that scale would either overestimate or underestimate the amount of pathogen inoculum across a crop, or worse, miss the pathogen entirely.

6.3. Inter-laboratory comparison trial

- Protocols have been developed for the production of spiked samples that are suitable for distributing to laboratories participating in an inter-laboratory comparison study.
- An inter-laboratory comparison study between three participating institutes was useful in highlighting areas where laboratory performance can be improved.
- There was generally good agreement between labs with respect to levels of target DNA reported. Differences arose where sample homogeneity was not adequate. However, one lab failed to detect *Colletotrichum coccodes* when present in soil at low levels (c. 100 pg DNA/g soil). This is likely to have been a problem with the soil extraction process.

- When sample extracts containing DNA of *C. coccodes* was distributed to participating laboratories, poor performance in one lab was likely to be attributable to differences between labs in producing standard curves.

6.4. Sample shelf-life test

There was a short-term decline in DNA levels in soils that had been spiked with pathogen (i.e. *C. coccodes*, *R. solani* AG3-PT and *S. subterranea*) inoculum. Between 7 to 21 days, the level of DNA remained stable at room temperature. Thus, samples are suitable for use in an inter-laboratory comparison study within the time required to dispatch and test samples.

6.4.1. Powdery scab

- Root galling was observed slightly earlier at the SAC site (week 5 of sampling) compared with SCRI (week 6). Agria showed significantly more gall symptoms than Nicola, and Estima the least. This is in agreement with previous EU trials where root galling was monitored across several sites.
- DNA of *S. subterranea* was detected in roots of all cultivars at both sites from the earliest sampling dates. At SCRI, significantly more DNA of *S. subterranea* was detected in roots of cultivar Nicola compared with cultivar Agria at all sampling dates except the first. This is in contrast with the results for root galling symptoms and tuber symptoms, where in each case Agria had significantly more disease. This may indicate that environmental conditions or host resistance mechanisms affect symptom development, not host infection.
- Root galls were not visualized until 5 weeks or more after root infection had occurred.
- Powdery scab symptoms were first observed in week 8 of sampling at both sites. Overall, Estima was the most susceptible cultivar followed by Agria and then Nicola. This is in accordance with previous results of EU trials. Higher levels of disease recorded at SAC compared with SCRI may be a result of slightly more conducive environmental conditions.
- DNA of *S.subterranea* was detectable in symptomless tubers from the time of tuber formation at both sites, showing that infection takes place at a very early stage of development. Significantly more DNA was detected in symptomless tubers of cultivar Agria (which also had more severe disease) compared with Nicola at the SCRI site. This may be because symptomless infections went on to cause disease in Agria.
- Tuber symptoms were first seen 3 weeks after infection was detected.
- The dates at which infection occurred and symptoms developed were remarkably consistent between sites.
- At both sites conditions were conducive to disease development. The mean daily temperature was approximately 15℃ at both sites. The SAC site was marginally cooler and wetter than the SCRI site, which may account for the differences in powdery scab severity, particularly where Nicola was shown to have more disease at SAC than SCRI. Results from international trials will add to the interpretation with regard to weather conditions.

6.4.2. Multiplexing

- Depending upon the target pathogens of interest, certain multiplex assays are now available:
- *C. coccodes* and *S. subterranea* (Applied Biosystems TaqMan Universal PCR Master Mix)
- *S. subterranea* and *R. solani* (QuantiTect Multiplex PCR Kit)
- However more work would be required to find a suitable multiplex assay for *R. solani* in conjunction with *C. coccodes*.

6.4.3. Anastomosis Groups

- The real-time PCR assays for *R. solani* AG 2.1, 4 and 5, developed at FERA when transferred to SCRI worked at relatively high DNA concentrations (200 pg DNA or more).
- Real-time PCR assays for *R. solani* AG groups have been developed at SARDI (Australia).
- Future laboratory reproducibility testing between laboratories participating in collaborative research will include comparisons of SCRI, FERA and SARDI assays.
- Soils collected during the R253 Diagnostics project were re-tested for AG 2.1, and 7 out of 108 had detectable levels of AG 2.1. However there was no relationship between the detection of AG 2.1 in soils and the occurrence of disease.
- Of 35 soils tested for AG 4 and AG 5, all were negative.

6.5. Future R&D

- Carrying out an inter-laboratory comparison with only three labs is problematic because the analysis of the data is difficult to interpret with so few participants. Also, this was the first year that such a study was undertaken for potato pathogens. The comparison study should be repeated with more labs in order to aid a full analysis of the data.
- The spatial distribution of *R. solani* AG3 was determined as aggregated at scales less than 2 ha. However, this analysis was done at only one site (Field 7, Aberdeenshire) in the December following a potato crop in 2008. Further analysis of samples collected in a uniform grid pattern at other sites and seasons should be undertaken in order to build up a picture of how *R. solani* AG3 is distributed in soil and how the inoculum distribution changes over time.
- Research is required into the epidemiology of *Rhizoctonia solani* to understand how inoculum develops from different initial levels, what factors influence inoculum development and the differences between seed- and soil-borne inoculum.
- During a study into the epidemiology, control measures can be applied to understand how they impact on inoculum development.

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8. APPENDICES

8.1. Appendix 1. Powdery scab scoring methods for tubers and root galls

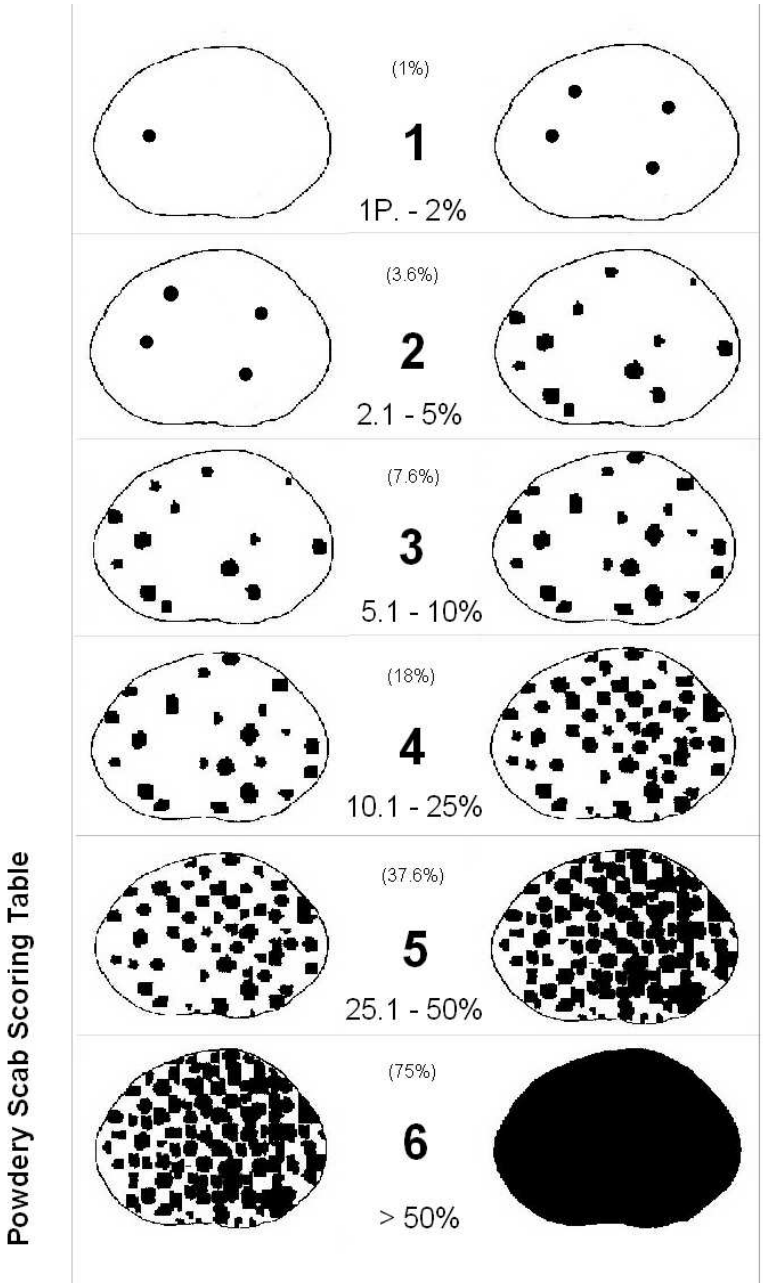

FIGURE 9. POWDERY SCAB SCORING SCALE

Score	Symptoms observed
0	no root galls
1	one or two root galls
2	several galls, mostly small (< 2 mm in diameter)
3	many galls, some > 2 mm in diameter
4	most major roots with galls, some or all > 4 mm in diameter

FIGURE 10. ROOT GALL SCORING KEY

8.2. Appendix 2 - Protocol for the preparation of samples for an inter-laboratory comparison trial (J. Peters and J. Woodhall)

8.2.1. *Rhizoctonia solani* AG3-PT and *Colletotrichum coccodes*

1. Remove suitable cultures of *Rhizoctonia solani* and *Colletotrichum coccodes* from long term store (at FERA, Rhizoctonia isolates are stored at -20ºC on infested barley grains; Colletotrichum isolates are stores at 4 ºC on agar discs in sterile distilled water). Incubate each isolate on three 90-mm petri-dishes containing potato dextrose agar (PDA) at 20ºC. Once sufficient hyphal growth has occurred (usually 7 days), check the colonies for purity microscopically and confirm the identity using an appropriate TaqMan assay or rDNA ITS sequencing.

2. Use the pure cultures inoculate numerous 90 mm petri-dishes of PDA. Incubate the cultures in the dark at 20ºC. After approximately 5 weeks, colonies will have produced sufficiently sized sclerotia for use. Approximately twenty petri-dishes are required per gram of sclerotia. Note, however, that production of sclerotia is highly variable between isolates.

3. Remove sclerotia using a sterile scalpel, care is required to avoid the additional removal of mycelia. Macerate the sclerotia using a scalpel blade and dry overnight between paper towels (this also allows any residual mycelia/agar present to dry up).

4. For *R. solani* AG3-PT, macerate the sclerotia to approximately 2 mm in diameter. Homogenise the sclerotia thoroughly and remove a 0.1 g sub-sample of the macerated sclerotia. Count the number of propagules within this sub sample. Measure the diameter of approximately 20 sclerotial propagules to ensure the average size is approximately 1 to 2 mm diameter. If the size deviates from this, continue macerating the material until the required size is achieved.

5. For *C. coccodes*, crush the dried sclerotia/hyphal mix to produce a fine powder.

6. Weigh the homogenized sclerotia and add to the appropriate weight of air-dried test soil (see below). The test soil consists of sandy loam field soil (pH 6.0 - 6.6) that has been air-dried for at least 3 days drying at room temperature that has been screened to ensure absence of all pathogens involved in this study. Mix the sample thoroughly.

7. Amount of culture material required for each inoculum level:
   Low concentration: 5×10^{-7} % sclerotia to soil (w/w);
   Intermediate level 5×10^{-5} % sclerotia to soil;
   High level 5×10^{-3} % sclerotia to soil.

8.2.2. Spongospora subterranea

1. *Spongospora subterranea* is an obligate parasite therefore inoculum needs to be prepared on live plant material. Soil known to be infested with *Spongospora subterranea* is used to infect tomato roots.

2. Tomatoes seeds (Cv. Moneymaker) are planted in shallow trays and grown for two weeks. The tomato seedlings are then transferred into larger pots (14 cm diameter, 2 l) containing the infested soil. Place pots in saucers and water as required.

3. After 3 weeks, harvest the seedlings, wash, and test three, 1 g sub-samples of roots ted by real-time PCR for presence/absence of *S. subterranea*.

4. Macerate roots with high levels of infection using a scalpel and air-dry overnight. When dry, crush the root-inoculum mix to produce a fine powder.

5. Homogenise the infected root material thoroughly, weigh then add to the appropriate weight of air dried test soil. The test soil consists of sandy loam field soil (pH 6.0 - 6.6) that has been air-dried for at least 3 days drying at room temperature that has been screened to ensure absence of all pathogens involved in this study. Mix the sample thoroughly.

6. Amount of culture material required for each inoculum level:
   Low concentration: 0.001% of root material is added to the soil (w/w);
   Intermediate level: 0.05% of root material is added to the soil (w/w);
   High level: 2 % of root material is added to the soil (w/w).

Note; it is likely that spore balls will be used to inoculate soil samples in future inter-laboratory comparison trials. This is because there was a high degree of variation in DNA levels between replicate samples when soil was inoculated with infected root fragments. Also, some of the participating labs express *S. subterranea* results as amount of spore balls/g soil.

8.2.3. Pectobacterium atrosepticum

1. Remove a suitable culture of *Pectobacterium atrosepticum* from long term store and plate out on nutrient agar. Confirm the identity of the culture by real-time PCR.

2. Touch a few colonies from a pure culture of *P. atrosepticum* with a bacteriological loop and use to inoculate 100 mL nutrient broth. Incubate the broth overnight at 37℃, agitating continuously. Carry out a 10-fold di lution series of broth plus bacterial suspension with PB7 (2ml tetrasodium pyrophosphate in 100ml Phosphate buffer pH7) from 10^{-1} to 10^{-9}. Flood-seed 100uL from the third, fifth and seventh dilutions, and spread onto the entire surface of each of three 90 mm petri-dishes containing nutrient agar. Incubate the petri-dishes at 37C and determine the number of colony forming units (CFU) per mL for each dilution.

3. Remove peel (from rose to stolon end) from potato tubers that are confirmed to be free of pectobacterial contamination. Place c. 10 g into an ELISA grinding bag (Bioreba Ltd). Add 9 mL PB7 to each bag, except for the negative control material

where 10 mL PB7 is added. Repeat this process until ten sets of cores/peel, one for each dilution plus a negative control, have been prepared.

4. Break up the potato peel using a mallet then add 1 mL from each of the pectobacterium dilution tubes to each of the bags (containing cores, peel and PB7) prior to homogenization using a large sample grinder (Homex Ltd).

5. The ELISA bags contain an internal filter which will separate any larger particles present. Transfer the filtrate from each bag to a 30 mL universal tube.

6. Transfer approximately 800 µL from the universal tubes to labelled 2 mL sample tubes. Freeze each aliquot at -80℃ for 3 hours, after which transfer the samples to a freeze drying and lyophilize overnight.

7. After freeze drying, seal the sample tubes and keep at 4℃ prior to dispatch.

8. Perform plate counts to determine the number of CFUs present in each dilution series. Use 10^3, 10^5 and 10^6 CFU/mL concentrations for the inter-laboratory comparison testing. In addition, determine the quality and homogeneity of the samples by real-time PCR testing of at least three samples at each dilution prior to dispatch.

8.2.4. Homogeneity testing and dispatch

1. On the same day that samples are prepared, remove three, 50 g subsamples of soil (or lyophilised inoculated sap samples for *P. atrosepticum*) for each inoculum level from the bulk test samples. Extract nucleic acid (using the method described in SOPs 'Nucleic acid extraction from large scale soil samples' and 'Nucleic acid extraction from tuber peel') and measure the amount of target pathogen DNA in duplicate. Homogeneity of the samples should be determined by ANOVA prior to dispatch. If the coefficients of variation are greater than 10%, the samples are unsuitable for use (ideally coefficients of variation should be <5%).

2. Label each sample for dispatch with a unique identification code that does not reveal the amount of inoculum in the sample. Dispatch c.250g aliquots of suitable soil samples, or lyophilized sap samples, for each level of inoculum (low, medium and high) to collaborating laboratories.

3. Soil samples should be kept by the preparation laboratory at room temperature until the partner laboratories acknowledge receipt of sample, after which they are placed at -20ºC until used in the comparative test.

8.3. Appendix 3 - Project deliverables

The deliverables listed below are those described in the original project application

a) An improved methodology for soil sampling which will encompass three major soil pathogens and other soil pests and diseases.

It has not been possible to identify a sampling regime that consistently ensures *R. solani* AG3 will be detected from a sample taken across a 4 ha field. There was a trend to improved detection by increasing sampling points but even this was inconsistent. Thus it has not proved possible to identify a uniform sampling strategy for *R. solani*, *C. coccodes* and *S. subterranea*. Whilst some detailed information of spatial distribution has been produced, more is required along with greater understanding of epidemiology in order to identify an improved sampling strategy for this pathogen.

b) Standardisation of testing methodology, consistency and validation between collaborators.

There was a degree of consistency between laboratories but not complete consistency. The inter-laboratory comparison has proved valuable in identifying how differences in test results may arise from different laboratories. The main outcome of this part of the project has been the development of a protocol for the preparation of samples for an inter-laboratory comparison trial. This will be used in an International Diagnostics project to compare testing in laboratories across the world.

c) An initial understanding of water relationships and population dynamics of infection by *S. subterranea*.

Consistent and detailed results have been found in this study on powdery scab carried out at two centres in Scotland. It has already provided unique information on the infection process and re-enforced existing knowledge on disease development. Analysis of the water relationships will require datasets from several years of trials and many locations (this is part of a European wide project) to be analysed before firm conclusions can be drawn.

d) An evaluation of the potential for multiplexing soil pathogen diagnostic tests

The potential for jointly testing two pathogens in diagnostic tests has been demonstrated and the most appropriate PCR Kits identified. Testing combinations of *C. coccodes* & *S. subterranea* and *S. subterranea* & *R. solani* are possible but more work is required for *C. coccodes* and *R. solani*.