Informing Management of Potato Diseases through Epidemiology and Diagnostics

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Report Authors: Jennie Brierley[1], Stuart Wale[2], James Woodhall[3], Jeff Peters[3], Alison Lees[1], Daan Kiezebrink[2]

[1]James Hutton Institute, Invergowrie, Dundee; [2]SRUC, Craibstone Estate, Aberdeen, [3]Fera, Sand Hutton, York.

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2. Preface

The research described in this final report was carried out as part of a levy body-funded international collaboration on potato disease diagnostics. The collaboration involved parallel research projects in Great Britain and Australia. Research groups in New Zealand and South Africa also contributed knowledge and information from on-going research programmes in their own countries. The British project was funded by Potato Council and consisted of researchers from the James Hutton Institute (Dundee), SRUC (Aberdeen) and Fera (York). The Australian research projects are part of the Australian Potato Research Program (APRP2) which is facilitated through Horticulture Australia Ltd (HAL) and funded by the Australian national potato levy. The British research project and on-going projects in New Zealand were considered as voluntary contributions to the Australian research programme and resulted in the leveraging of funds from the Australian Government. This allowed a comprehensive programme of work to be funded. The Australian researchers, from the South Australian Research and Development Institute (SARDI), Department of Primary Industries Victoria (DPIVic) and the Tasmanian Institute of Agricultural Research (TIAR), are due to complete their programme of work in 2015. The information in this report is mainly that resulting from the British research project, although reference is made to on-going Australian work in some sections.

3. Summary

This report describes work completed during a three year collaboration between research partners in GB and Australia. In both countries DNA-based tests to detect and quantify the amount of key potato pathogens had been developed prior to the start of the project. Funding was obtained to allow the researchers to collaborate on the generation and analysis of data on the use and interpretation of the tests and test results. The report focuses on the pathogens *Spongospora subterranea* (which causes powdery scab) and *Rhizoctonia solani.* The aim of the work was to better understand the factors which determine the amount of disease they cause and to use this information to try and improve the interpretation of diagnostic test results and therefore disease management. In the case of *R. solani*, field trials were also carried out to evaluate the impact of a range practical control options on disease development.

3.1. *Powdery scab*

State of knowledge at the start of the project

Diagnostic assays for the detection and quantification of *S. subterranea* had been developed and validated prior to the start of this project. Previous Potato Council and Horticulture Australia Limited (HAL)-funded work had shown that soil-borne inoculum is of greater importance in causing disease than seed-borne inoculum, but a relationship between soil inoculum concentration and the amount of disease on progeny tubers had not been shown in the field. This is attributed to the nature of the life cycle of *S. subterranea:* the pathogen is capable of very rapid multiplication through the formation of secondary zoospores in roots if conditions are suitable. Burnett (1991) concluded that initial soil pathogen concentration was of less importance than the build up of inoculum during the root multiplication phase. The impact of environmental factors (conducive conditions) on disease development was reviewed by Brierley *et al*., (2008) and Merz & Falloon (2009).

Work completed during the project

A “standardised” powdery scab field trial design was agreed by researchers following the Second European Powdery Scab Workshop in 2007. Trials based on this standard design have been carried out during this project. Additional DNA-based testing of samples was done to provide information on the timing of infection as well as symptom development. Nine trials have been completed: six in Scotland and three in Australia. In addition, a set of field trials and two controlled environment experiments were carried out in Scotland to investigate the relationship between initial soil inoculum levels (spore balls / g soil) and the development of powdery scab.

New information generated

3.1.1. Crop Infection and the relationship between soil inoculum level and powdery scab incidence and severity.

The mean daily soil temperature was remarkably similar through the growing seasons in all nine of the field trials. Mean daily temperatures rarely exceeded 20^{o}C at any site, even those in Victoria, Australia. Whilst the Australian soils were generally slightly warmer than the Scottish ones, at the time of tuber initiation, relatively warm soil temperatures were recorded at JHI (Dundee) in 2010, being at least comparable if not warmer than the Victorian sites during this period. Collectively the field trials have shown that where soil inoculum is present, root infection occurs very early; at plant emergence roots are already infected with *S.*

subterranea. Similarly, tubers are infected as soon as they are initiated. Symptom development, root galling and powdery scab lesions do not become apparent until some weeks after infection has taken place. As the timing of root and tuber infection and disease development across all nine sites did not differ markedly, statistical analysis could not identify specific temperature or moisture conditions that could be related to the timing or extent of infection and/or disease development. As a result, it has not been possible to make recommendations on factors such as timing of irrigation and how they can be modified to affect the extent of disease development.

The field trials involving field soils amended with different levels of *S. subterranea* inoculum showed that, under field conditions in Scotland there was a relationship between initial soil inoculum level and powdery scab incidence and severity at harvest. When soils from the field trials were used in pot trials in controlled environment studies (15^{o}C, soils kept constantly damp), there were differences in the outcome of the trials between years. In 2009, the results indicated that under the controlled conditions, the disease levels were less affected by initial soil inoculum compared to the disease levels recorded in the field. High levels of disease occurred from very low levels of initial inoculum in the controlled environment, although relatively low levels of disease occurred from the higher levels of inoculum. In 2010, there was very little disease in the controlled environment study across all the different inoculum levels.

Overall, the results indicate that DNA-based, real-time PCR assays can be used to reliably detect the presence of *S. subterranea* in field soil. When the pathogen is detected in soil there is the likelihood that the amount of disease on progeny tubers will be greater at higher levels of soil inoculum although under constant cool, wet conditions high levels of disease can develop even when soil inoculum levels are low. In a previous study (Potato Council- funded project R253) the real-time PCR assays were used to test symptomless seed tubers for the presence of *S. subterranea*. Out of 124 seed stocks sampled, 56 (45%) were found to have some level of symptomless contamination. It was not possible to determine if these stocks caused disease as they were predominantly planted into infested soils, however the work indicated that the real-time PCR assay can also be used to test seed for the presence of *S. subterranea*. This would be of use in situations where land is known to be free from powdery scab and introduction of the pathogen via infected/contaminated seed needs to be avoided.

3.1.2. Varietal susceptibility to powdery scab

The cultivar Nicola in Australia appears to be more resistant to powdery scab and root galling than it is in Scotland. The Nicola used in Australia has been verified by SASA to be the same cultivar as that used in GB. Therefore differences in its resistance may be attributed to factors other than genetic ones, but analysis of the data did not identify a key factor that could explain this difference.

In Scotland, Nicola was less resistant to root than to tuber disease, a finding which was also reported by Merz *et al.,* (2012), whilst Agria and Estima were relatively susceptible to both. Merz *et al.,* (2012) also reported large differences in root galling for the same cultivar between years. However, they were unable to link differences to the average temperatures (in August) at the two Swiss locations used in the study.

3.1.3. Post harvest soil inoculum levels

There was an elevation of soil inoculum levels during the field trials (i.e. from pre-planting to final harvest) at the JHI trial site in 2009 and 2010 and to a lesser extent in Victoria, Australia in 2009/2010, but not at other sites. There is no obvious link to production of root galling or powdery scab symptoms in the trials in which spore ball levels increased over and above that found in other trials. One factor which may influence the extent of spore ball contamination is the maturity of the lesions at the time of harvest, i.e. if tubers are harvested before pustules burst releasing spore balls in to the soil, then build up of inoculum may be lessened. The final harvest at JHI in 2008 was earlier than in subsequent years, and there was little build up of inoculum in this trial.

3.1.4. Recommendations

The project has provided new information on the timing of *S. subterranea* infection and additional information on the relationship between soil inoculum levels and powdery scab incidence and severity. This information has not substantially altered previous recommendations for management of the pathogen, for example those provided in the review of powdery scab strains and conducive conditions (Brierley *et al.,* 2008):

- Inoculum - assess the level of powdery scab on seed and in soil
- Conducive Conditions - wherever possible conducive conditions should be avoided.
    - • Select a free draining field
    - • Avoid over cultivating soil and creating too fine a tilth which results in slumping as this will increase the risk of prolonged soil saturation
    - • Avoid soil compaction either when cultivating soil or through vehicles wheels travelling through crops
    - • Avoid over irrigation leading to soil saturation.

- Carry Out Specific Control Measures
    - Utilise variety resistance. Where a risk of powdery scab is identified, plant moderately resistant or resistant varieties wherever possible.
    - Consider chemical control. If a soil analysis indicates the soil has greater than 6 mg/kg zinc, the risk of powdery scab is much lower. Where low soil zinc levels exist, applying zinc to soil may provide some limited control of powdery scab. However, trials have shown that this treatment works best where the disease pressure is lower. The maximum amount of zinc that can be applied annually is 15 kg elemental zinc per hectare. Ideally, it should be incorporated into soil before planting.
    - Extension of Authorisation for the use of fluazinam on seed tubers against *S. subterranea* allows some fluazinam-containing products to be used for the control of powdery scab. The product is to be applied as a tractor mounted downward directed drench in a minimum water volume of 200 litres per hectare, on the ridged bed prior to de-stoning or bed-tilling. No further products containing fluazinam can be applied to the haulm for control of potato blight.

3.2. *Potato Mop Top Virus (PMTV)*

Potato Mop Top Virus (PMTV) is a viral disease affecting potato. *Spongospora subterranea* is the vector of PMTV (Jones and Harrison, 1969). Initial transmission of

PMTV to potato occurs in the process of infection by the powdery scab pathogen *S. subterranea*. Therefore whilst infection by *S. subterranea* is not indicative of PMTV infection, potatoes cannot be infected with PMTV without also being infected with *S. subterranea.*

The "standardised" powdery scab field trial with additional testing of samples was used to study infection of potatoes by PMTV at the JHI site. Where PMTV infection was investigated in parallel with *S. subterranea,* PMTV was found to have infected potato roots at or shortly after emergence and tubers one week after initiation. PMTV infection in tubers was higher in Nicola than Agria. Although tubers of both cultivars became infected with PMTV, low incidences of spraing symptoms were observed in both cultivars. In both cultivars, PMTV infection in tubers occurred in the absence of powdery scab symptoms. In the inoculum level field trials, in both years, no difference in the level of spraing or PMTV infection was detected between inoculum levels.

3.3. *Rhizoctonia solani*

State of knowledge at the start of the project

Large uncertainties remain concerning the epidemiology of *R. solani*, despite it being the focus of research for many years. This is predominantly due to the dynamic nature of *R. solani* survival in soil- the fungus is able to survive as hyphae on suitable host tissue and as sclerotia. The latter are able to survive relatively long periods of time in a dormant state. The project was designed to address aspects of the biology of *R. solani* that impact on our ability to assess the risk of disease developing in potato crops. For example, *R. solani* exists as a number of anastomosis groups (AG), with AG 3 being the most dominant strain in UK potato crops, however little was known about the ability of other AG's to cause disease. *R. solani* exists as both sclerotia and hyphae, but little was known about how our ability to detect inoculum is affected by the form in which *R. solani* is present. Work had been carried out on the contribution made by both seed and soil-borne inoculum to stem and tuber disease, however, the results were confusing and often contradictory. Therefore, a programme of research, encompassing, controlled environment studies, field monitoring and field trials was carried out to:

- determine the relative importance of soil- and seed-borne inoculum levels in causing disease.
- determine the importance of soil type/soil treatments as a factor affecting disease development
- investigate whether the correlation between inoculum level and disease is influenced by AG.
- determine how *R. solani* exists in soil, with respect to propagule type and how this affects disease development and pathogen survival
- Evaluate and compare the practical disease control options currently available to growers.

Work completed during the project
Six field trials, seven controlled environment studies and a programme of testing/monitoring of tubers and field soils has been carried out in GB during the three year project.

New information generated
The researchers have produced a separate document (*Rhizoctonia solani* Reviews) which includes images of tuber symptoms caused by *R. solani*; reviews of the results presented here along with results from previous studies; and summaries of control measures for stem and tuber diseases caused by *R. solani*. The sections below refer specifically to the new information generated in GB as part of the current project.

3.3.1. Anastomosis Groups

Rhizoctonia solani exists as a number of anastomosis groups (AGs) that are known to infect different hosts: AGs 2-1, 2-2, 3, 4, 5, 8 and 9 have been associated with diseases of potatoes. AG3 occurs as different subgroups including AG3-PT which occurs on potatoes. In this report AG3 or AG3-PT are referred to according to which real-time PCR assay was used to detect\quantify the presence of AG3: The assay used at Fera is specific for AG3-PT whilst the assay used at JHI detects all subgroups of AG3. Experimental work comparing the pathogenicity of a number of isolates of different AGs, confirmed our previous understanding that in GB, AG 3-PT is the main causal agent of stem disease and black scurf on potato. However some isolates of AG2-1 and 5 and binucleate (ie with two nuclei per cell) *Rhizoctonia* (BNR) species also caused stem disease. The study also showed that various AGs of *R. solani* can cause the elephant hide symptom in progeny tubers and Koch's postulates*[1] were proven for AG3-PT and a BNR species causing elephant hide-type symptoms. Both AG3-PT and AG2-1 were commonly detected in potato seed stocks using real-time PCR. When commercial potato growing field soils were sampled pre-planting, AG2-1 was found more commonly than AG3-PT. However, in soil samples taken post-harvest, AG2-1 levels had often declined, but AG3-PT levels in soil increased, and AG3-PT was isolated from progeny tubers with black scurf symptoms. Whether AG3-PT was introduced on seed, or was present at undetectable levels prior to planting remains unclear.

3.3.2. Seed vs Soil inoculum

Two controlled environment experiments were carried out to investigate the effect of seed inoculum (based on the surface area covered with black scurf) and soil inoculum (based on soil inoculated with varying amounts of *R. solani* sclerotia and/or hyphae) on disease development. The results showed that in the absence of soil inoculum, the effect of seed inoculum was evident. Low levels of seed inoculum did not cause extensive black scurf on progeny tubers, but higher levels of seed inoculum (*c.* 10-15% surface area with black scurf symptoms) caused high levels of both stem canker and black scurf. In both experiments, even the very low levels of soil inoculum (0.00005g sclerotia / g soil) used caused disease.

3.3.3. Propagule Type

A third controlled environment experiment was carried out to compare the effect of different propagule types (hyphae versus sclerotia) as sources of *R. solani* inoculum

*[1] Koch's postulates: the method for identifying the micro-organism causing a disease. It is based on four criteria that must be met before a micro-organism can be confirmed as the cause of a disease/symptom.

and to determine the impact on disease development. The results showed that there were differences between inoculum types in terms of detection (by real-time PCR) and the ability to produce disease. Hyphal inoculum was detected at lower levels than sclerotia. This is likely to be because sclerotia were not as uniformly distributed in soil at low inoculum levels as hyphae. Hyphal inoculum at the lowest level caused disease in stems but did not produce black scurf. This could be because at low levels, inoculum did not survive long enough in compost to initiate disease on stolons or tubers. At equivalent levels of inoculum in compost, sclerotia produced more severe black scurf than hyphae. In general, stem canker and black scurf incidence and severity were higher in sclerotia-inoculated pots than in compost inoculated with hyphae.

3.3.4. Detection of soil inoculum

Soil type influences the likelihood of detection of *R. solani* in field soils. In a controlled environment experiment, different soil types were all inoculated with 0.00003g sclerotia per g soil. When real-time PCR was applied to samples of the soils the test results varied between zero and 1,000 pg DNA/ g soil. However black scurf developed on progeny tubers grown in all but one of the eleven soils inoculated at this level. The inoculum added to the soils was therefore below the threshold of detection in some soil types, but sufficient to cause disease. In the field trials to evaluate control options soil-borne inoculum was only detected pre-planting in two of the five trials nevertheless, disease developed in each trial. In one trial in 2010, stem canker was present at the first sampling date (June 2010) whilst soil borne inoculum remained undetected until mid-August. As mini-tubers were used as the seed, soil inoculum was assumed to be responsible for the early infection of stems, despite it being undetectable.

The results from the controlled environment experiments and field trials indicate that current methods cannot reliably detect sclerotia in field soils at levels which are sufficient to cause disease. This is assumed to be primarily due to the size of the soil sample which will not necessarily pick up low levels of unevenly distributed sclerotia within a given field. However, when *R. solani* inoculum is detected in soil samples there is a strong likelihood that disease will develop on the crop. Therefore although a negative soil test result cannot be taken to indicate that *R. solani* is not present/there is no disease risk, a positive test result does indicate that there is a risk of disease developing. The relationship between the amount of inoculum detected in soil and the amount of disease that develops has not been defined. In two of the three controlled environment experiments mentioned above, the incidence and severity of disease (black scurf) was greatest (100% incidence) at the highest soil inoculum level. The amount of *R. solani* AG3 DNA / g soil detected at planting varied between the two trials and ranged from ~10 to 5,000 pg DNA /g soil. In the other experiment, the highest soil inoculum level (~25,000 pg DNA /g soil) resulted in less black scurf than the lower soil inoculum levels (~1,000 pg DNA /g soil). However, it was observed that the high soil inoculum level decreased rapidly after planting and this may have contributed to the lack of relationship between soil inoculum level and tuber disease. There was no consistent trend for higher soil inoculum levels (at planting) to result in higher levels of stem disease in the controlled environment experiments. The work has highlighted the difficulties in establishing the sufficiently low levels of soil inoculum required to study the relationship between inoculum and disease. Even the lowest levels tested (0.00005g sclerotia / g soil) resulted in disease and therefore the threshold for disease (under controlled conditions) must be below this level.

In a field trial in which additional organic matter was added to some plots, *R. solani* inoculum (either hyphae or sclerotia) was applied along the drills at planting. The sclerotial inoculum equated to 0.000005g sclerotia / g soil. Although inoculum was not regularly detected post planting disease still developed in the inoculated plots. Both of the inoculum types (in plots amended with organic matter) were associated with greater numbers of pruned stolons than any other treatments. Increasing the level of organic matter (from 0.2g/kg soil to 0.5g/kg soil) did not have a consistent effect on disease development/the persistence of the pathogen inoculum.

3.3.5. Monitoring changes in *R. solani* inoculum levels in soil

Two sources of data on changes in *R. solani* soil inoculum levels over time were available: samples taken from controlled environment studies; and monitoring of field soils. Monitoring of field soils (2 sites) suggested that AG3-PT may not survive well in the absence of the host crop. This is supported by the studies of soil from the field experiment where organic matter had been added to some plots. Sampling (main-plots only) in the December and February following potato harvest detected only a trace amount of inoculum in two (out of 4) samples. In the controlled environment studies where soil inoculum levels were monitored during the course of the experiments, there was no consistent trend as to whether inoculum levels increased or declined during the experiments.

3.3.6. Impact of soil moisture level on disease development

A controlled environment experiment, using soil inoculated with sclerotia, was carried out to study the effect of soil moisture on disease levels. The level of moisture applied had no effect on stem disease. At the end of the experiment, plants grown in pots where *R. solani* AG3-PT sclerotia had been added produced tubers with black scurf and elephant hide. The effect of moisture on tuber blemishing was evident with the low moisture treatment (20% volumetric water content) resulting in more black scurf than the high (40%) moisture treatment. Also, the low moisture treatment resulted in more elephant hide than either the moderate (30%) or high moisture treatments.

3.3.7. Control options

No single control measure consistently reduced disease on haulm or tubers. Of the options compared, Amistar most frequently reduced disease significantly, but depending on the symptom assessed, significant reductions were confined to one or two trials. The results suggest that fungicide treatments work best in locations where inoculum pressure is not high – at least initially.

3.4. *Soil sampling & diagnostics methodology*

In addition to the experiments focussed on specific pathogens, two other pieces of work were carried out which addressed the optimum soil sampling strategy for the soil borne pathogens studied; and the comparability of DNA test results between participating laboratories. Both aspects involved input from international collaborators in Australia and also from researchers from Plant and Food Research, New Zealand.

3.4.1. Soil sampling

Prior to the start of this project, some work had already been completed on soil sampling and the distribution of soil borne pathogens. During the project further work

in Australia**,** using fields (40-50ha) subject to pivot irrigation, was carried out. Data from this work and previously collected information were used to study the spatial variability of *R. solani* inoculum across intensively sampled grids. The analyses indicated that *R. solani* AG3 is either highly variable or stratified. In cases where the fields are highly variable for pathogen inoculum, it is vital that samples are collected throughout the field area. In addition, data become highly unreliable when collected further than 80 to 120 m apart. This strongly suggests that clusters of sample points should be collected at most every 50 m and ideally as close together as possible.

3.4.2. Diagnostic testing

Inter-laboratory comparison tests were carried out between all 5 international partners (three in GB, one in both Australia and New Zealand) to determine how each institutes' diagnostic results compared. These tests were conducted using soil samples that had been artificially spiked using each of three pathogens (i.e. *Rhizoctonia solani* AG3, *Colletotrichum coccodes* and *Spongospora subterranea*) by Fera to produce four categories of inoculum (designated 'nil', 'low', 'moderate' and 'high'). After, pre-testing to determine that the samples were fit for purpose, they were dispatched to partner institutes for testing. Each institute used their own sample extraction and testing methods. Analysis of the results showed that the degree to which samples varied (i.e. the coefficients of variation) within 'medium' and 'high' inoculum levels were generally within the accepted limit (<10%). However, 'low' samples always had coefficients of variation that were higher than 10%. This illustrates the difficulty in producing samples with low variation when pathogen inoculum levels are low but demonstrated that the inter-lab comparison samples were suitable for the purposes of the trial. There was reasonable agreement between all participating laboratories. This comparison study demonstrates that results generated from research and commercial testing carried out at participating laboratories in GB, Australia and New Zealand are comparable.

4. Experimental Section

4.1. *Powdery scab*

4.1.1. Introduction

Powdery scab may be seed-borne (Hide 1981; Read *et al.* 1995) or soil-borne (Letham *et al.* 1988; Merz *et al.* 2006) with the result that planting disease-free and uncontaminated seed-tubers in infested soil (Letham *et al.* 1988; Merz *et al.* 2006) or infected or contaminated tubers in uninfested soil (Falloon *et al.* 1996; de Nazareno and Boschetto 2002) can lead to the development of disease in progeny tubers. In un-infested soils, transmission of the pathogen from infected seed tubers to progeny does not appear to be straightforward. Burnett (1991) and Keiser *et al.* (2007) found no consistent correlation between the level of seed tuber infection and subsequent disease levels on progeny tubers. This finding is supported by the results of the Potato Council-funded project (R253) in which no consistent relationship between seed inoculum levels and progeny disease were observed in 113 monitored crops. Other studies have reported a lower incidence and severity of disease on progeny tubers compared to the seed stock from which they were grown (Braithwaite *et al.,* 1994; de Nazareno & Boschetto, 2002). Furthermore, a number of studies have shown that disease may not develop where temperature and/or soil moisture conditions are unfavourable, even where soils are known to be infested or diseased tubers are planted (Hughes *et al.* 1980; Christ and Weidner 1988; Nakayama *et al.* 2007).

Diagnostic assays for the detection and quantification of *S. subterranea* have been developed and validated in previously funded projects. Previous Potato Council and Horticulture Australia Limited-funded work shows that soil-borne inoculum is of greater importance in causing disease than seed-borne inoculum, but that a relationship between soil inoculum concentration and resulting disease on progeny tubers is difficult to establish in the field.

The biphasic nature of the life cycle of *S. subterranea* ensures that the pathogen is both persistent, with the production of resting spores, but also capable of very rapid multiplication through the formation of secondary zoospores in roots if conditions are suitable. This means that infection may not consistently be related to initial inoculum levels, but could be related better to secondary infection by zoospores if conditions are conducive to their formation. This may explain contradictory findings of a number of studies investigating the relationship between inoculum and disease. For example, van de Graaf *et al.* (2005), in inoculated pot experiments (5-50 spore balls/g soil), and Merz *et al.* (2006), in field trials correlating multiple spot sites of soil inoculum and disease, both found no evidence of a relationship between soil inoculum concentration and the incidence and severity of disease on tubers. Parker (1984) found that naturally occurring soil inoculum resulted in a disease incidence and severity similar to that obtained in pots to which additional inoculum had been added. Similarly, Christ (1989) compared field plots with natural inoculum levels to plots to which additional inoculum had been added and found no difference in disease levels between the two. In contrast, Qu *et al.* (2006) found a significant relationship between soil inoculum level and disease incidence when they tested 17 field soils in the USA and found them to be contaminated with *S. subterranea* at levels between 0 and 14,400 sporeballs/g of soil (determined using a competitive PCR assay). Shah *et al.* (2005) after adding spore balls to pots, found that a significant aysmptotic

relationship existed between inoculum concentration (ranging from 0-12,000 spore balls/g soil) and the severity of disease in tubers. Nakayama *et al.* (2007) tested 29 potato fields in Japan using a competitive PCR assay and found the highest spore ball density to be 105 spore balls / g soil. They found no evidence of a significant relationship between spore ball density in the soil and tuber disease severity, but did find a significant positive relationship between the amount of disease on the roots of plants and tuber disease severity. This is in accord with the conclusion of Burnett (1991) that initial soil pathogen concentration was of less importance than the build up of inoculum during the root multiplication phase. The Potato Council project (R253) found a relationship between soil inoculum concentration and disease (data from 3 years combined) in commercial fields, if soil inoculum was put into one of three categories, zero, < 10 sporeballs/ g soil and > 10 spore balls/ g soil. However, it is difficult to ascertain the direct affect of soil inoculum level from monitored crops where the results are confounded by many factors such as cultivar, soil type and different agronomic practices. Therefore the relationship between primary soil inoculum (spore ball) level and powdery scab development remains unclear. It is proposed that higher initial inoculum levels offer the pathogen a greater opportunity to infect and cause disease, thereby establishing a link between initial inoculum level and disease. However, due to the production of zoosporangia and secondary infections under favourable conditions, disease development may be high irrespective of initial inoculum levels. The impact on disease of both initial inoculum levels and secondary infection will be dependant upon environmental factors.

It has been established that powdery scab symptoms are particularly prevalent at temperatures between 12-15°C and root galls at approx. 17°C (van der Graaf *et al*, 2005). A more detailed evaluation of the effect that the pathogen/soil/environment interaction has on infection and disease development is important in terms of elucidating the epidemiology of the disease, and this could also lead to improved interpretation of the *S. subterranea* soil tests and timing of practices such as irrigation which may affect the extent of disease.

As a result of the Second European Powdery Scab Workshop (2007) a standard field trial protocol was established to allow comparison of results between countries. This trial has been coordinated by Ueli Merz in Switzerland and several other countries including Switzerland, France, The Netherlands and Norway participated. It was proposed that participating countries would complete three years of field trials to enable a range of environmental factors to be encountered and their affect on powdery scab development to be assessed. With Potato Council funding (R411) two trials were completed in Scotland in 2008. Whilst the standard core experimental factors being monitored at each site were visual symptoms, i.e. root galls and tuber scabs, in the Potato Council-funded trials, molecular diagnostics were used as an additional tool in determining time and level of infection to bring added value to the data. This allows the time of infection, as opposed to symptom development, to be correlated specifically with environmental conditions and this information is critical to our understanding of *S. subterranea* epidemiology. As part of the project reported here, the standardised trial plus the additional testing to determine the timing of infection was repeated at two sites in Scotland (JHI & SAC) in 2009. A combined DNA/RNA (NA) extraction procedure was employed to determine levels of not only *S. subterranea* but also PMTV, which was present in the powdery scab spore balls used to inoculate the field sites. In 2010, the trials were repeated at JHI and SAC, again with the inclusion of the PMTV monitoring. The Australian partners carried out the core trial with additional diagnostics at three sites, two in 2008/2009 and another

in 2009/2010. Therefore a total of nine standardised powdery scab field trials have been completed and are reported on here.

In addition, a set of field and controlled environment experiments were carried out to investigate the relationship between initial soil inoculum levels (spore balls), and the development of powdery scab. This study comprised four field trials to investigate the link between soil inoculum level and disease development. A second component of this study was to compare the development of powdery scab on progeny tubers when grown with different levels of soil inoculum but under conducive controlled environment conditions.

4.1.2. Field trials to evaluate factors that govern infection, multiplication and disease development of powdery scab.

Materials and Methods

Trials were planted at JHI and SAC in 2008, 2009 and 2010. Three trials have been completed in Australia, one in Tasmania and two in Victoria (Table 1).

Table 1. Field trial locations from British and Australian-funded research.

Year	Location	Country
2008*	JHI	Scotland
	SAC	Scotland
2008/2009	Tasmania	Australia
	Victoria	Australia
2009	JHI	Scotland
	SAC	Scotland
2009/2010	Victoria	Australia
2010	JHI	Scotland
	SAC	Scotland

*The trials in Britain in 2008 were carried out as part of a previous Potato Council-funded project (R411). It was considered that three years' data would be required to study powdery scab and therefore additional trials in Britain in 2009 and 2010 were also carried out.

Cultivars and cultivation

Trials were planted according to the standard protocol agreed at the Second European Powdery Scab Workshop where at least one susceptible cultivar (Agria, Estima or Kennebec) and one medium susceptible cultivar (Desiree, Nicola or Saturna) were planted, with Agria and Nicola being the preferred cultivars. Nicola was a consistent cultivar throughout all trials. See Table 2 for which cultivars were planted at each site.

No seed treatments were applied. Irrigation was applied up to 4 weeks after tuber initiation (25mm was applied when the soil moisture deficit reached 18mm) to ensure disease development. Herbicide, late blight and aphid control were applied as per standard practice

Soil inoculum
Trials were either planted on land known to be infested with *S. subterranea* or were inoculated with potato peel from tubers with powdery scab symptoms.

Before planting, soil samples were taken from the field trial areas to determine soil inoculum levels. Post harvest, soil samples were taken from each plot and soil inoculum levels determined. In the GB trials, the extraction of DNA from soil followed the method of Brierley *et al.* (2009) and the amount of *S. subterranea* in a sample was determined using the real-time PCR of van der Graaf *et al.,* (2003). Trials carried out in Australia used the methods of SARDI (Ophel-Keller *et al.,* 2008).

Environmental Monitoring
Environmental conditions were monitored at 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

Plant Sampling
The standard protocol states that 24 tubers/plot of each cultivar x 3 replicates should be planted and samples of at least 3 plants/rep taken once a week starting after tuber set, for 6 weeks. At all field trial sites these minimum requirements were exceeded (details in Table 2) except that SAC 2010, harvested 2 plants/sampling occasion.

Plants were dug carefully in order to catch as much of the root system as possible. Roots were carefully separated from tubers, stems and stolons and washed to remove soil but without damaging root tissue. Roots were then dried and weighed and assessed for root galls using the gall assessment key (See Appendix 1). 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 (available at 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.

Table 2. Summary of British and Australian-funded field trials.

JHI 2008*	Cultivars Planted	Agria + Nicola
	Date of Planting	14 May 2008
	Replicates	3
	Irrigation	Trickle tape
	Number of samples/date	4 plants
	Time of first sample	Tuber initiation
	Number of samples taken	7
SAC – 2008*	Cultivars Planted	Estima + Nicola
	Date of Planting	8 May 2008
	Replicates	4
	Irrigation	Trickle tape
	Number of samples/date	5 plants
	Time of first sample	Emergence
	Number of samples taken	11
Tasmania- 2008/ 09	Cultivars Planted	Kennebec + Nicola
	Date of Planting	21 October 2008
	Replicates	4
	Irrigation	Centre pivot
	Number of samples/date	4 plants
	Time of first sample	Emergence
	Number of samples taken	7
Victoria- 2008/ 09	Cultivars Planted	Kennebec + Nicola
	Date of Planting	1 December 2008
	Replicates	4
	Irrigation	Lateral irrigator
	Number of samples/date	4 plants
	Time of first sample	Emergence
	Number of samples taken	9
JHI 2009	Cultivars Planted	Agria + Nicola
	Date of Planting	7 May 2009
	Replicates	4
	Irrigation	Trickle tape
	Number of samples/date	4 plants
	Time of first sample	Emergence
	Number of samples taken	9

* Data from Potato Council-funded project R411.

SAC- 2009	Cultivars Planted	Estima + Nicola
	Date of Planting	29 May 2009
	Replicates	4
	Irrigation	Trickle tape
	Number of samples/date	4 plants
	Time of first sample	Emergence
	Number of samples taken	10
Victoria- 2009/ 2010	Cultivars Planted	Kennebec, Nicola, Desiree + Russet Burbank
	Date of Planting	16 December 2009
	Replicates	4
	Irrigation	Lateral irrigator every 3
	Number of samples/date	4 plants
	Time of first sample	Emergence
	Number of samples taken	9
JHI 2010	Cultivars Planted	Agria + Nicola
	Date of Planting	11 May 2010
	Replicates	4
	Irrigation	Trickle tape
	Number of samples/date	4 plants
	Time of first sample	Emergence
	Number of samples taken	9
SAC 2010	Cultivars Planted	Estima + Nicola
	Date of Planting	24 May 2010
	Replicates	4
	Irrigation	Trickle tape
	Number of samples/date	2 plants
	Time of first sample	Emergence
	Number of samples taken	10

Real-time PCR assessment of samples

At each sampling date, 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 *S. subterranea* DNA. The whole root system from each plant and tuber peelings taken from symptomless tubers from the same plant were collected and processed.

The method of DNA extraction used by JHI and SAC in 2008 and the combined extraction of DNA and RNA, nucleic acid (NA), used by JHI and SAC in 2009 and 2010 to enable the co-investigation of *S. subterranea* and PMTV infection are provided in Appendix 2. The method of DNA extraction from root and tuber peel varied slightly between the participating laboratories and therefore absolute comparisons of the level of DNA between sites may not be robust, but patterns of DNA levels in roots and symptomless tubers over time are valid across sites.

At final harvest, all remaining progeny tubers were assessed for powdery scab.

Results and Discussion

The results of the assays of soil inoculum levels determined pre-planting and post harvest at all nine sites are presented in Table 3.

Table 3. Soil inoculum levels, mean sporeballs/ g soil (SEM) pre-planting and post harvest at each site.

	Pre-planting (whole trial area)	At harvest (whole trial area)	At harvest susceptible cultivar plots	At harvest resistant cultivar plots
JHI 2008	31 (5.8)	15 (4.2)	14 (4.1)	16 (8.3)
SAC 2008	115**	81	Data not collected	Data not collected
Tasmania * 2008-09	44 (1.6)	37 (3.4)	44 (2.4)	31 (2.2)
Victoria * 2008-09	26	27 (5.8)	27 (7.1)	27 (5.2)
JHI 2009	51 (8.0)	362 (43.2)	281 (28.1)	443 (60.4)
SAC 2009	116.5**	-	-	-
JHI 2010	3 (0.6)	98 (14.2)†	122(22.4)†	81 (11.9)
SAC 2010	3.5**	-	-	-
Victoria * 2009-10	3 (0.2)	19 (5.7)	(a) 46 (16.1) (b) 19 (7.9)	(c) 9 (1.0) (d) 2 (0.5)

*PCR data from Tasmania and Victoria were reported as pg DNA/ g soil, this has been converted to spore ball equivalents using JHI's conversion factor of 90 pg DNA/ spore ball.
† Post harvest a single Agria plot had an inoculum level of 4,155pg DNA / g soil and was omitted from the means presented here.
(a) Kennebec, (b) Desiree, (c) Russell Burbank, (d) Nicola
** Only one sample was taken across the whole pre-planting area therefore SEM values are not available.

To enable a comparison of results across sites, the timing of infection and development of disease symptoms have been normalized to weeks after planting (WAP), as timings of infection and disease expression are based on weekly observations.

Table 4. Date of planting and approximate dates of plant emergence and tuber initiation at each site (weeks after planting, WAP).

	Date of planting	Date of emergence (WAP)	Date of tuber initiation (WAP)
JHI – 2008	14 May	10 Jun (3)	8 Jul (7)
SAC – 2008	8 May	12 Jun (4)	2 Jul (7)
Tasmania- 2008/09	21 Oct	19 Nov (4)	10 Dec (7)
Victoria- 2008/09	1 Dec	22 Dec (3)	29 Jan (8)
JHI – 2009	7 May	4 Jun (3)	23 Jun (6)
SAC – 2009	29 May	6 Jul (5)	14 Jul (6)
JHI – 2010	11 May	9 Jun (4)	29 Jun (6)
SAC – 2010	24 May	21 Jun (3)	13 Jul (7)
Victoria- 2009/10	16 Dec	5 Jan (2)	27 Jan (5)

Emergence and tuber initiation occurred at similar times after planting at all sites except Victoria 2009/10, where both emergence and tuber initiation were earlier.

Data analysis

In association with BioSS, all data sets were put into a standard format allowing them to be fully interrogated for trends and the identification of factors, for example, mean, minimum and maximum soil temperatures, soil moisture deficit, soil inoculum level, affecting the timing and extent of infection and disease development.

As the timing of root and tuber infection and disease development across all nine sites did not differ markedly, the analysis could not identify specific temperature or moisture conditions that could be related to the timing of disease. Therefore, the results below are descriptive rather than fully analytical.

Differences in the extent of root galling (recorded at the final sample harvest taken at each site) and powdery scab severity (at final harvest) between cultivars and sites were analysed. Sites and cultivars which differed by more than 2X standard error of the mean (SEM) are considered significantly different ($p<0.05$).

Environmental conditions

The mean daily soil temperature was remarkably similar through the growing seasons in all nine of the field trials. Mean daily temperatures rarely exceeded 20^{o}C at any site, even those in Victoria, Australia. Whilst the Australian soils were generally slightly warmer than the Scottish ones, at the time of tuber initiation, relatively warm soil temperatures were recorded at JHI 2010, being at least comparable if not warmer than the Victorian sites during this period. SAC 2008 had the coolest soil temperatures during the period of tuber initiation.

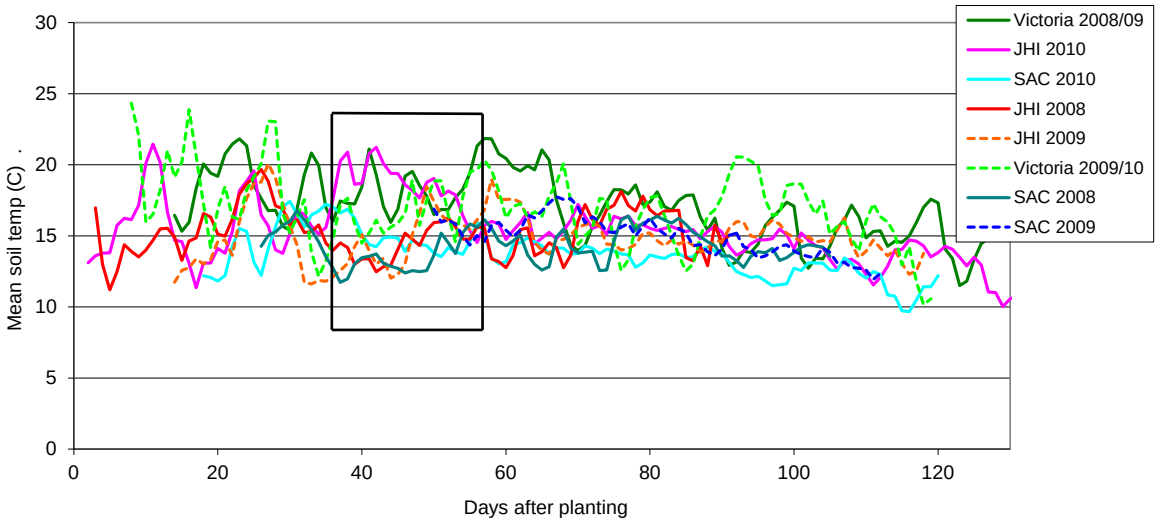


Figure 1. Mean daily soil temperature recorded at all nine of the field trial sites. The box represents the period of time over which tuber initiation took place across the 9 sites.

All the sites were irrigated, and this may mitigate differences in infection and disease development between sites that might otherwise have become evident. Soil moisture was recorded as the soil moisture deficit (mm) at eight of the nine sites (Tasmania used a different logging system). Other than in Victoria 2009/10 which had somewhat drier soil, all sites had similar soil moisture levels, with SAC being the dampest (Figure 2).

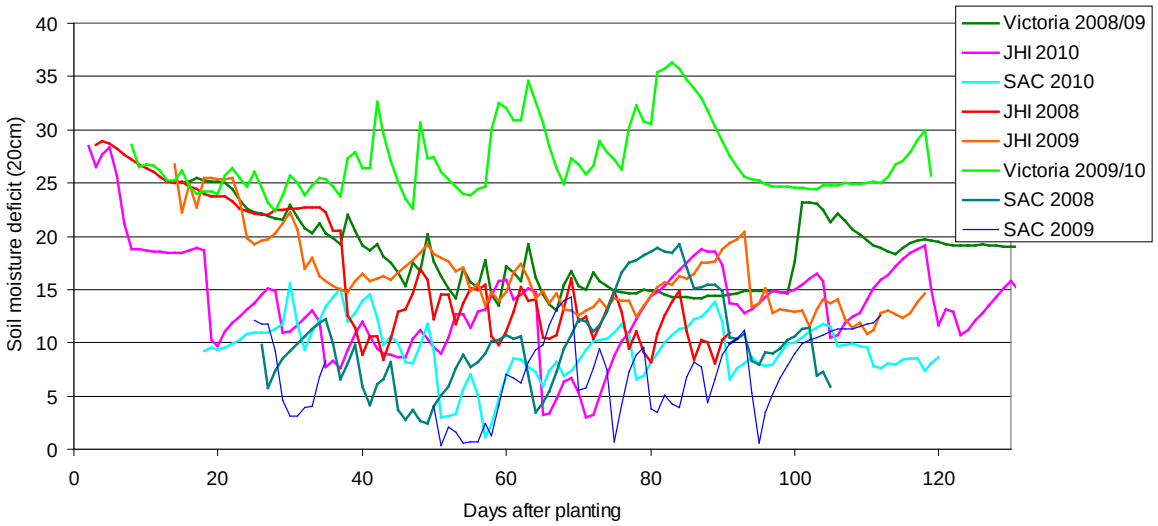


Figure 2. Mean daily soil moisture deficit (mm) recorded at 20 cm depth at eight field trial sites.

The timing of infection and symptom development

The timing and extent of root infection, root galling and scab symptoms are shown for each trial in Figures 3-11. Root infection was detected at each site when the first sample harvest was taken i.e. the date of the root infection in Table 5. Root infection occurred earlier in Victoria (2 or 3 WAP) than either Tasmania or Scotland (4 or 5 WAP). In 2008 at JHI, the first sample was taken relatively late (at tuber initiation rather than emergence) and hence whilst the first root sample was infected, infection probably occurred somewhat earlier than recorded (6 WAP). The early timing of root infection in the Victoria field trials is associated with the earlier emergence of plants at this site, and hence the onset of sampling occurred earlier.

The development of root galls occurred over a three week time span at all sites (6 to 9 WAP); JHI and SAC in 2008 having the latest and Victoria 09/10 having the earliest observation of root galls after planting. At all sites root galling was not observed until at least 3 weeks after roots became infected, except at SAC in 2009 when it was only 2 weeks. Root galling generally increased during the season and the highest galling score was at the last sample harvest, with the exception of the 2009 season at JHI, where lower root galling scores where encountered at the end of the sampling season (Figure 7).

Root infection, measured as ng DNA/ g fwt root material, did not always increase steadily through the season, and in some cases the level of infection decreased at the later sample harvests as illustrated in Figures 3 and 7, during the 2008 and 2009 seasons at JHI. Lower levels of *S. subterranea* DNA in root tissue may be due to the infection cycle slowing down as roots age in the later stages of the season or conditions less favourable once irrigation stopped.

Table 5. Date of first appearance (weeks after planting) of root infection, root galls, tuber infection and powdery scab symptoms at each site.

	root infection (WAP) †	root galls (WAP)	tuber infection (WAP)	powdery scab (WAP)
JHI 2008	1 Jul (6)	22 Jul (9)	8 Jul (7)	29 Jul (10)
SAC 2008	12 Jun (4)	17 Jul (9)	2 Jul (7)	24 Jul (10)
Tasmania 2008/09	19 Nov (4)	17 Dec (8)	‡24 Dec (9)	-
Victoria 2008/09	22 Dec (3)	29 Jan (8)	29 Jan (8)	5 Feb (9)
JHI 2009	9 Jun (4)	7 Jul (8)	23 Jun (6)	23 Jun (6)
SAC 2009	6 Jul (5)	20 Jul (7)	14 Jul (6)	17 Aug (11)
JHI 2010	9 Jun (4)	7 Jul (8)	29 Jun (6)	13 Jul (8)
SAC 2010	28 Jun (4)	5 Jul (7)		2 Aug (11)
Victoria 2009/10	5 Jan (2)	2 Feb (6)	27 Jan (5)	17 Feb (8)

† This is also the date of the first sample harvest, which was taken at the time of near complete emergence; except at JHI 2008, JHI 2009, and SAC 2010 when it was 2, 1 and 1 weeks after emergence respectively.

‡ The first tuber sample assessed for infection using real-time PCR was taken 2 weeks after tuber initiation.

JHI: 2008

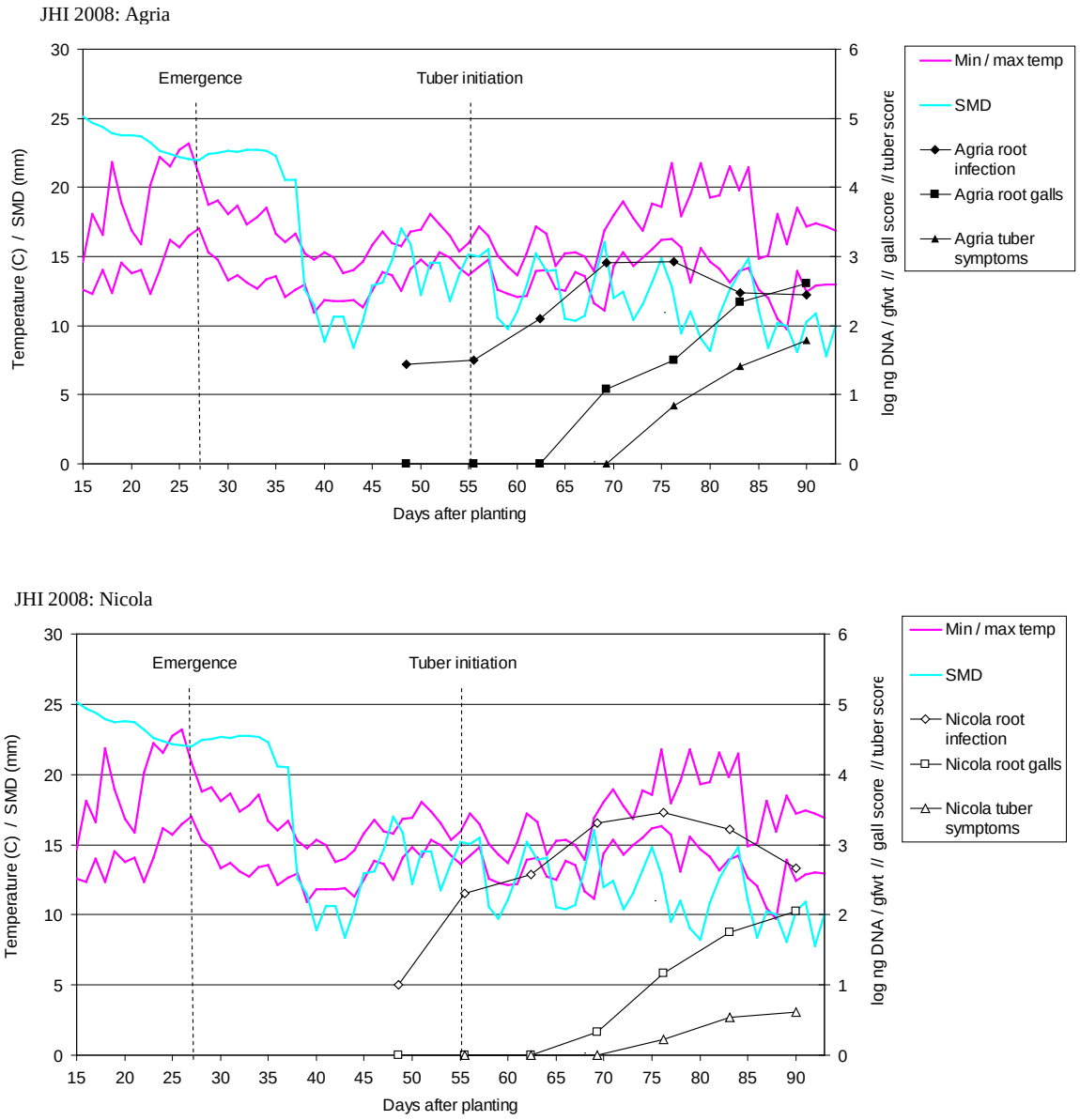


Figure 3. Minimum and maximum daily soil temperature (ºC) and mean soil moisture deficit (mm) values over the duration of the trial at JHI-2008. The mean root gall disease severity on a 0-4 scale of increasing severity, and mean powdery scab severity on a 0-6 scale of increasing disease, and the amount of *S. subterranea* DNA (log ng DNA/g fresh weight root tissue) measured at weekly intervals at the JHI site in 2008.

SAC -2008

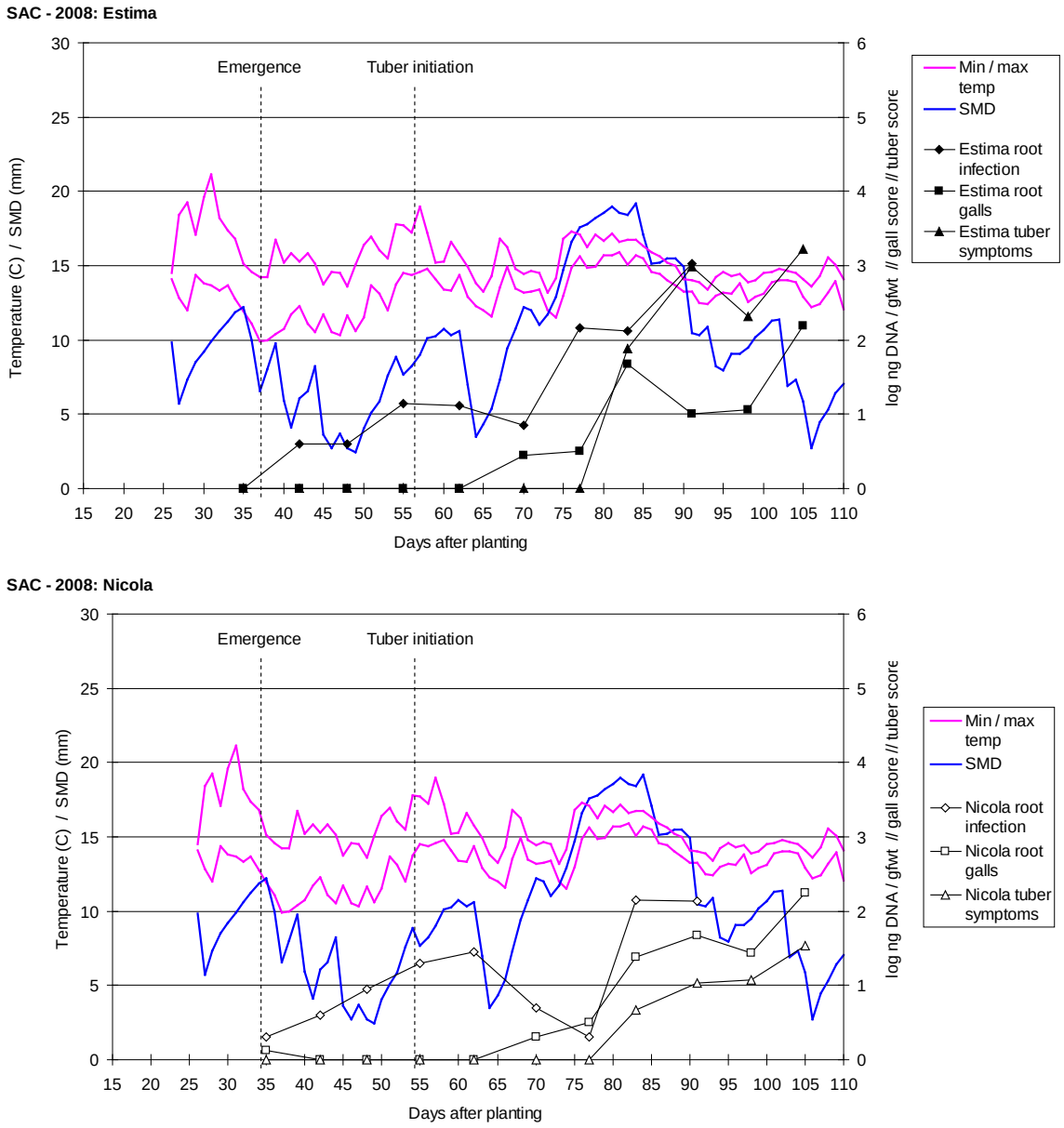


Figure 4. Minimum and maximum daily soil temperature (ºC) and mean soil moisture deficit (mm) values over the duration of the trial at SAC-2008. The mean root gall disease severity on a 0-4 scale of increasing severity, and mean powdery scab severity on a 0-6 scale of increasing disease, and the amount of *S. subterranea* DNA (log ng DNA/g fresh weight root tissue) measured at weekly intervals at the SAC site in 2008.

Tasmania- 2008/ 09

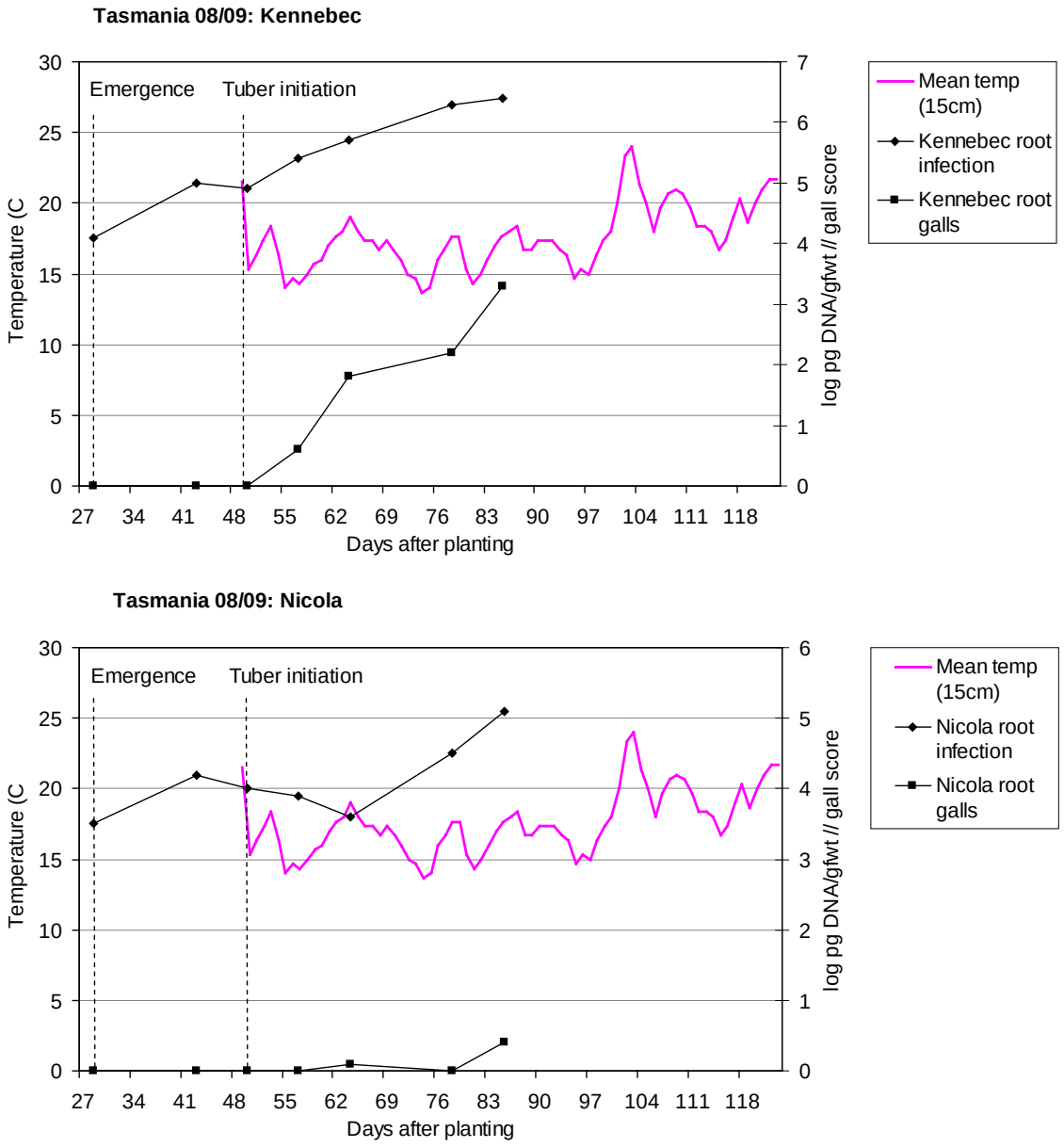


Figure 5. Mean daily soil temperature (ºC) values over the duration of the trial at Tasmania 08/09. The mean root gall disease severity on a 0-4 scale of increasing severity, and the amount of *S. subterranea* DNA (log pg DNA/g fresh weight root tissue) measured at intervals at the Tasmanian site in 2008/9.

Victoria- 2008/ 09

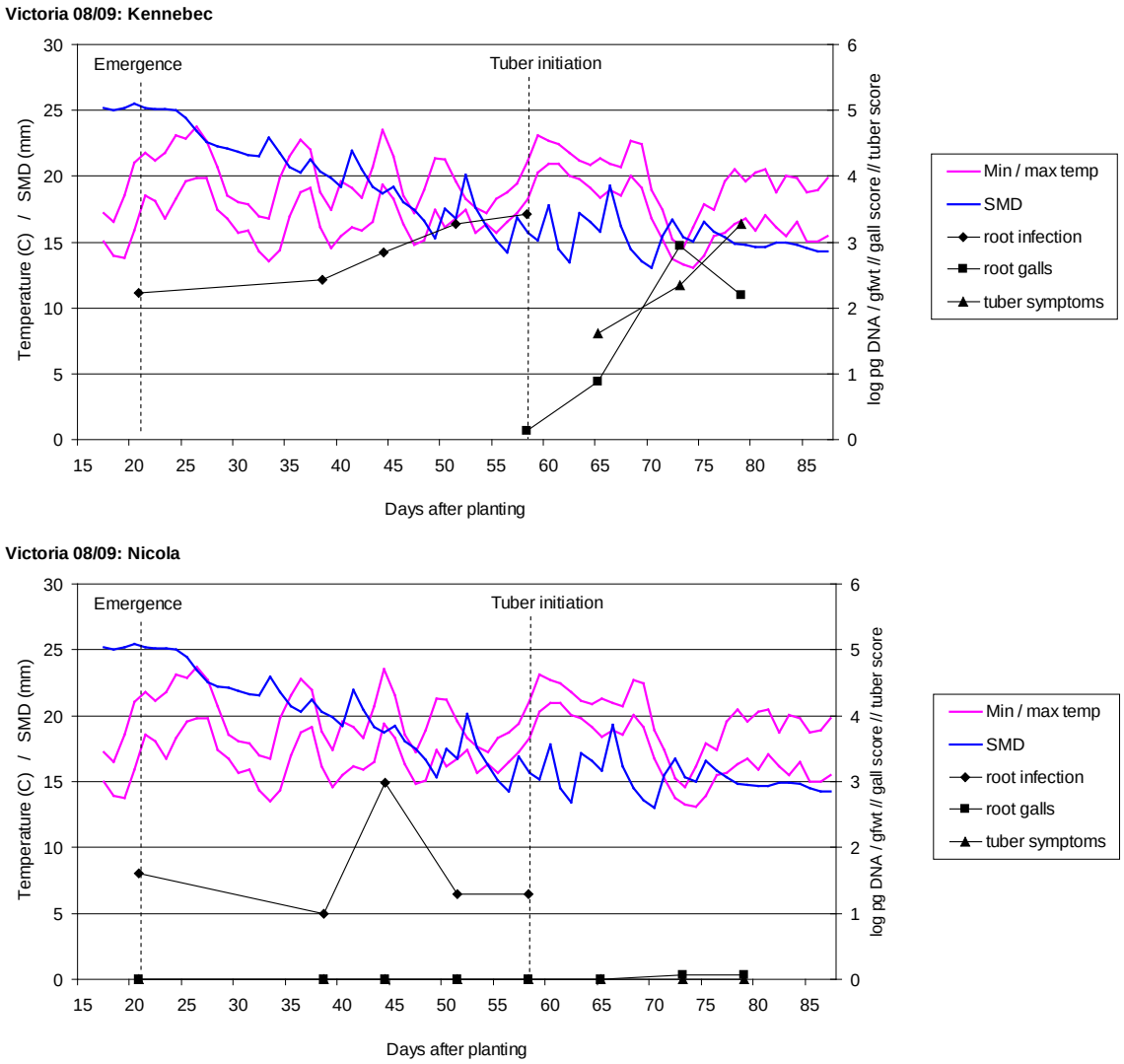


Figure 6. Minimum and maximum daily soil temperature (ºC) and mean soil moisture deficit (mm) values over the duration of the trial at Victoria 2008/9. The mean root gall disease severity on a 0-4 scale of increasing severity, and mean powdery scab severity on a 0-6 scale of increasing disease, and the amount of *S. subterranea* DNA (log pg DNA/g fresh weight root tissue) measured at weekly intervals at the Victoria site in 2008/9.

JHI- 2009

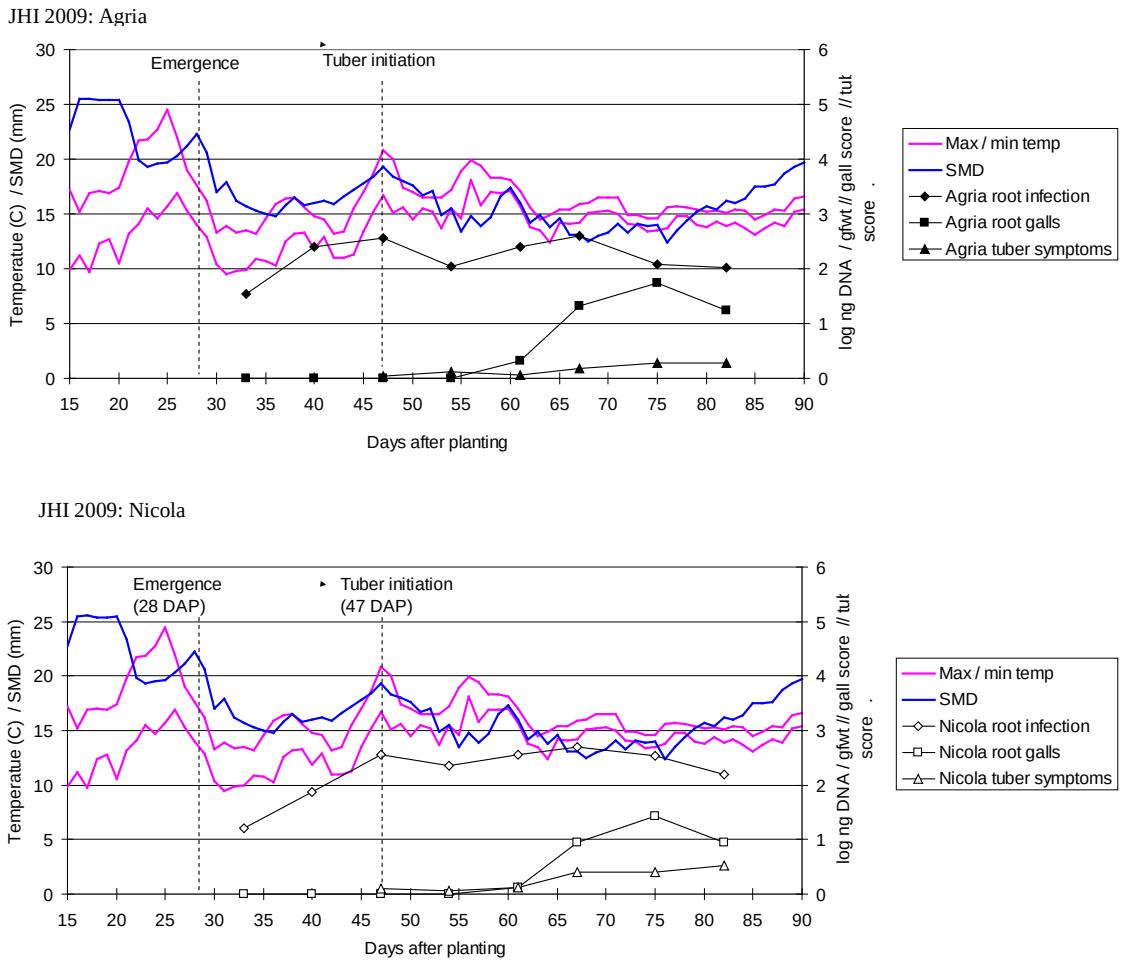


Figure 7. Minimum and maximum daily soil temperature (ºC) and mean soil moisture deficit (mm) values over the duration of the trial at JHI-2009. The mean root gall disease severity on a 0-4 scale of increasing severity, and mean powdery scab severity on a 0-6 scale of increasing disease, and the amount of *S. subterranea* DNA (log ng DNA/g fresh weight root tissue) measured at weekly intervals at the JHI site in 2009.

SAC- 2009

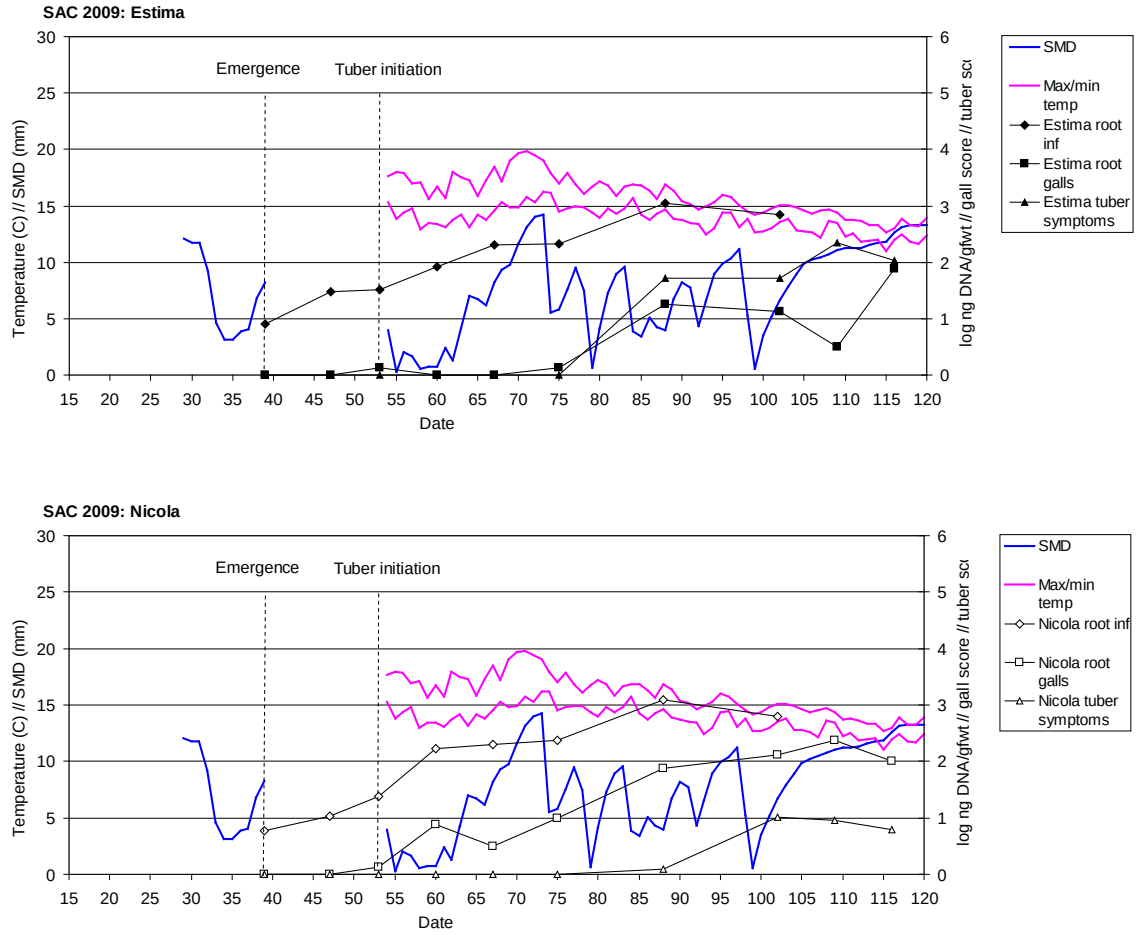
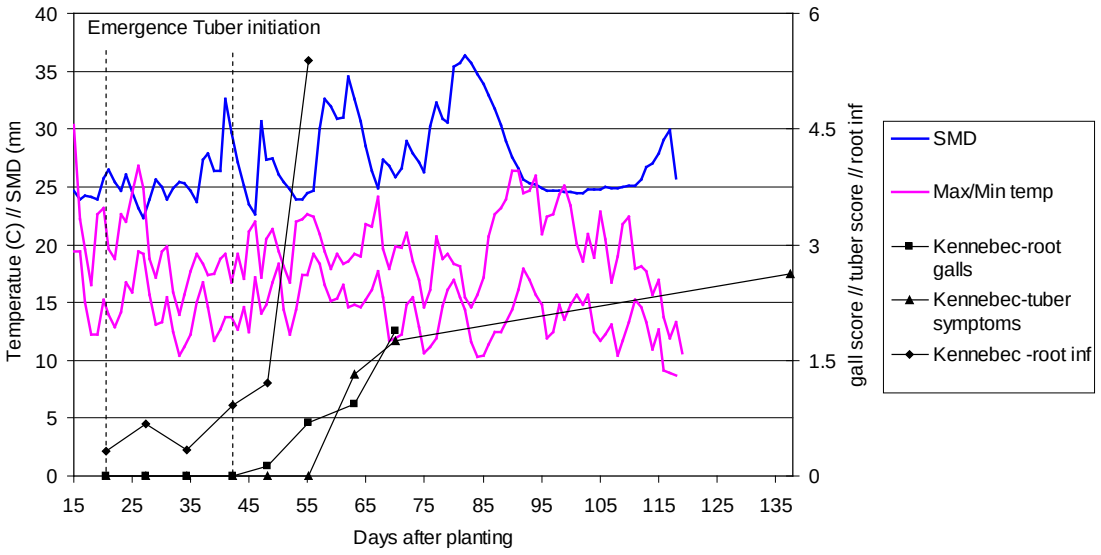


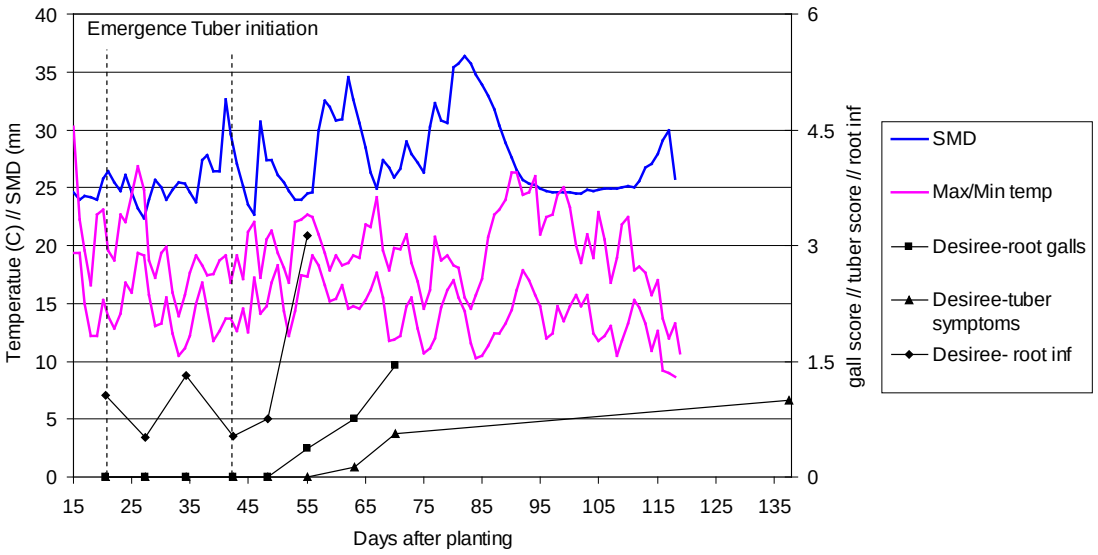
Figure 8. Minimum and maximum daily soil temperature (ºC) and mean soil moisture deficit (mm) values over the duration of the trials as recorded at SAC-2009. The mean root gall disease severity on a 0-4 scale of increasing severity, and mean powdery scab severity on a 0-6 scale of increasing disease, and the amount of *S. subterranea* DNA (log ng DNA/g fresh weight root tissue) measured at weekly intervals at the SAC site in 2009. SMD data is missing between 6 and 21 July but rainfall during this period was 97.8mm and it can be assumed that SMD was at or very close to 0.

Victoria 2009/10

Victoria-2009/10: Kennebec



Victoria-2009/10: Desiree



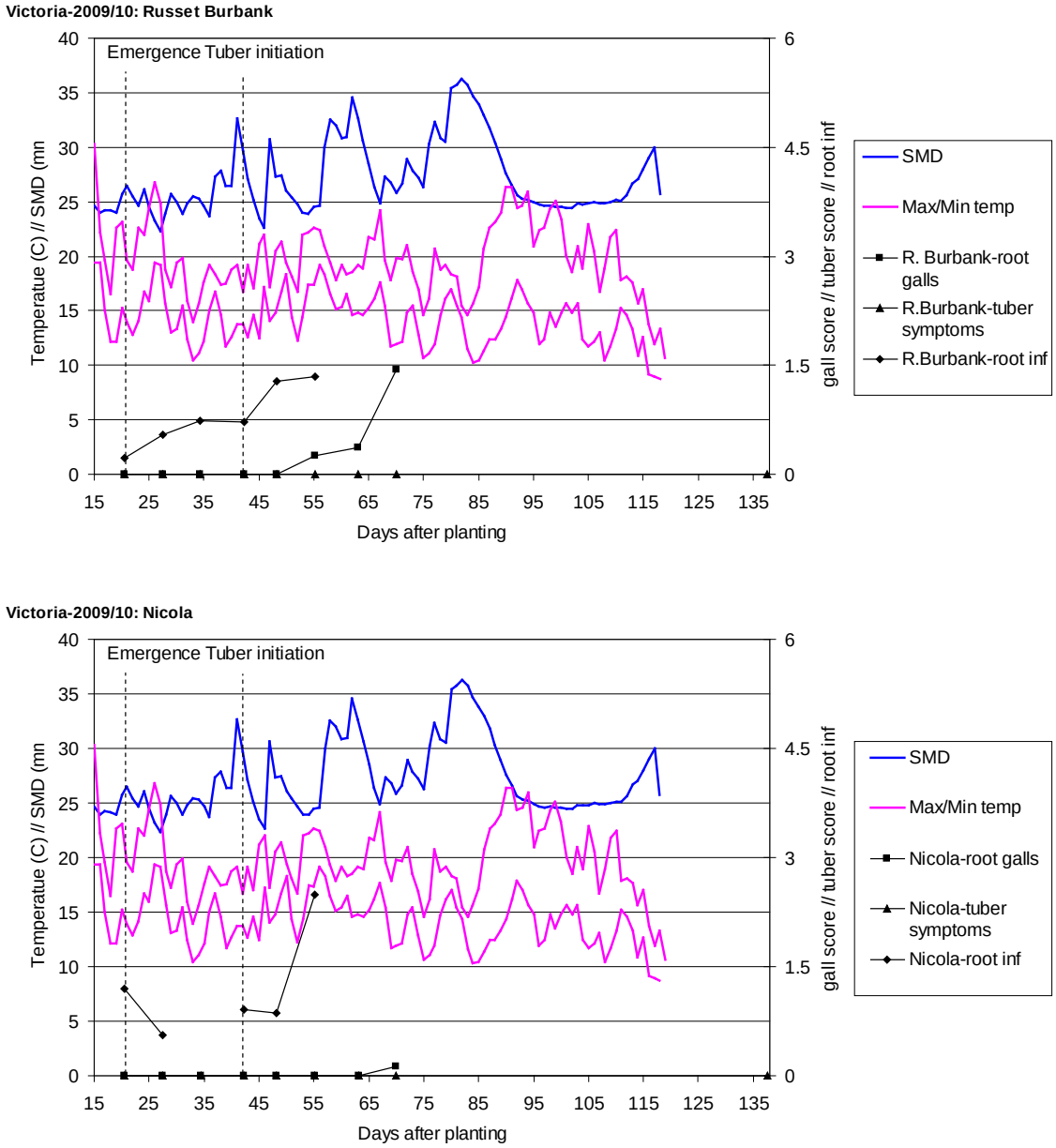


Figure 9. Minimum and maximum daily soil temperature (ºC) and mean soil moisture deficit (mm) values over the duration of the trial Victoria-2009/10. The mean root gall disease severity on a 0-4 scale of increasing severity, and mean powdery scab severity on a 0-6 scale of increasing disease, and the amount of *S. subterranea* DNA (log ng DNA/g fresh weight root tissue) measured at weekly intervals at the Victoria site in 2009/10.

JHI - 2010

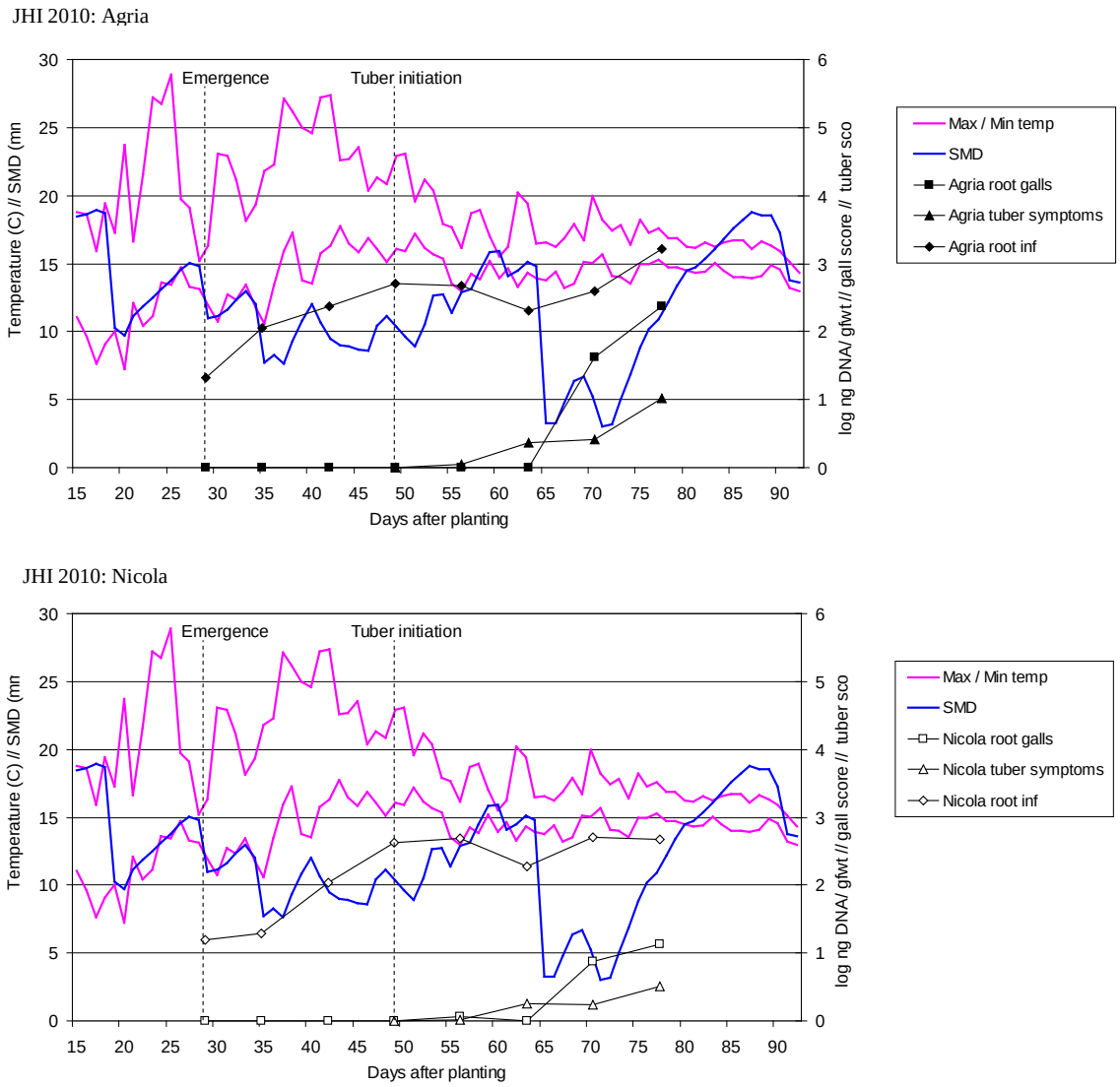


Figure 10. Minimum and maximum daily soil temperature (ºC) and mean soil moisture deficit (mm) values over the duration of the trial at JHI 2010. The mean root gall disease severity on a 0-4 scale of increasing severity, and mean powdery scab severity on a 0-6 scale of increasing disease, and the amount of *S. subterranea* DNA (log ng DNA/g fresh weight root tissue) measured at weekly intervals at the JHI site in 2010.

SAC - 2010

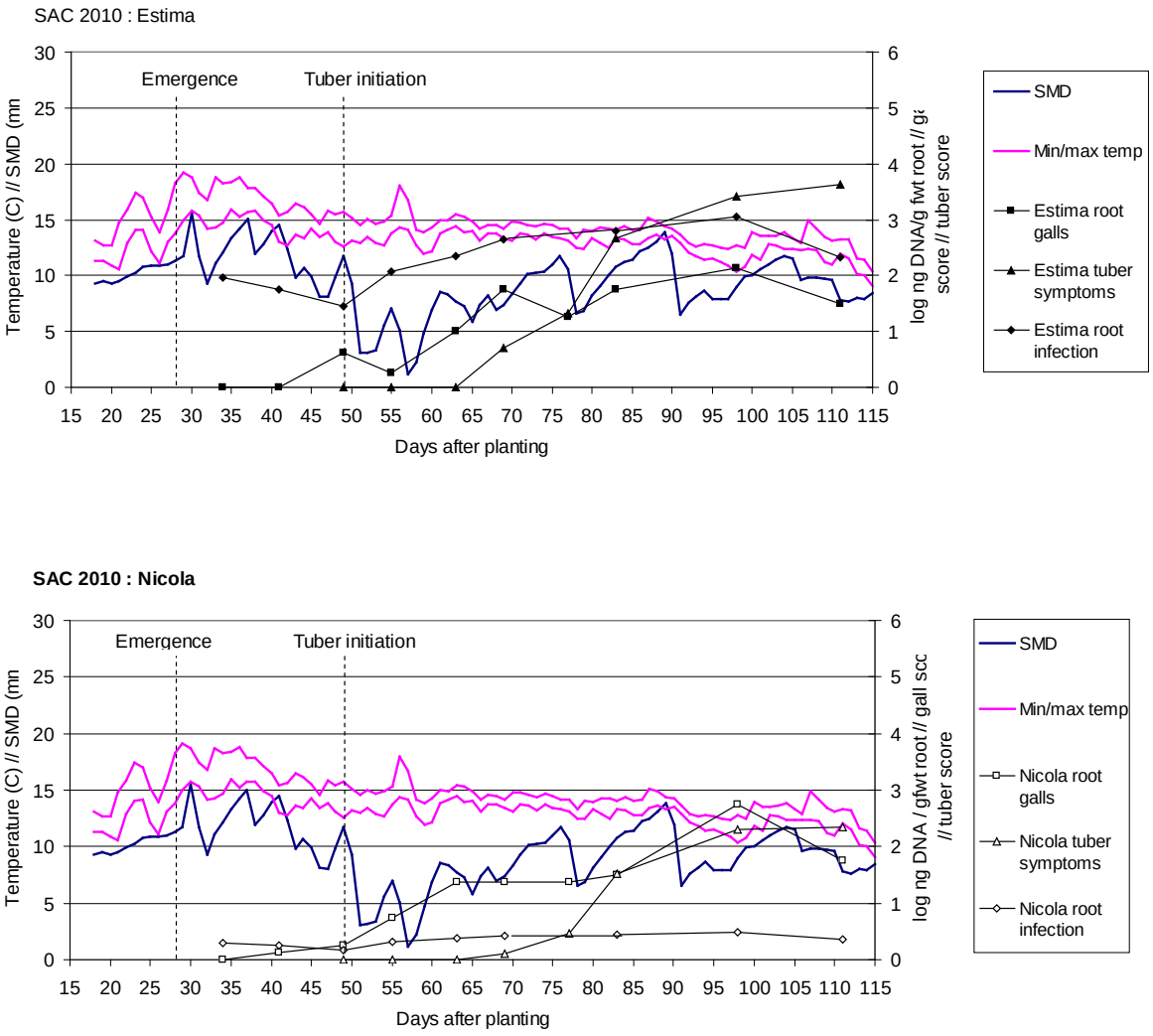


Figure 11. Minimum and maximum daily soil temperature (ºC) and mean soil moisture deficit (mm) values over the duration of the trial at SAC 2010. The mean root gall disease severity on a 0-4 scale of increasing severity, and mean powdery scab severity on a 0-6 scale of increasing disease, and the amount of *S. subterranea* DNA (log ng DNA/g fresh weight root tissue) measured at weekly intervals at the SAC site in 2010.

There was no clear relationship between the extent of root infection as determined by the mean root infection over sampling dates and mean root galling (Figure 12). For example, at JHI in 2008, Nicola had significantly higher levels of root infection but less root galling than Agria, whilst in 2009 there was no difference between either the level of root galling or root infection between the two cultivars. At SAC in 2008, root galling observations were similar for Estima and Nicola, yet Estima had significantly higher levels of infection (at the last sampling date) than Nicola. In

Tasmania and Victoria (2008/09) root infection was apparent in cultivar Nicola (albeit at a lower level than Kennebec) but little or no root galling was observed.

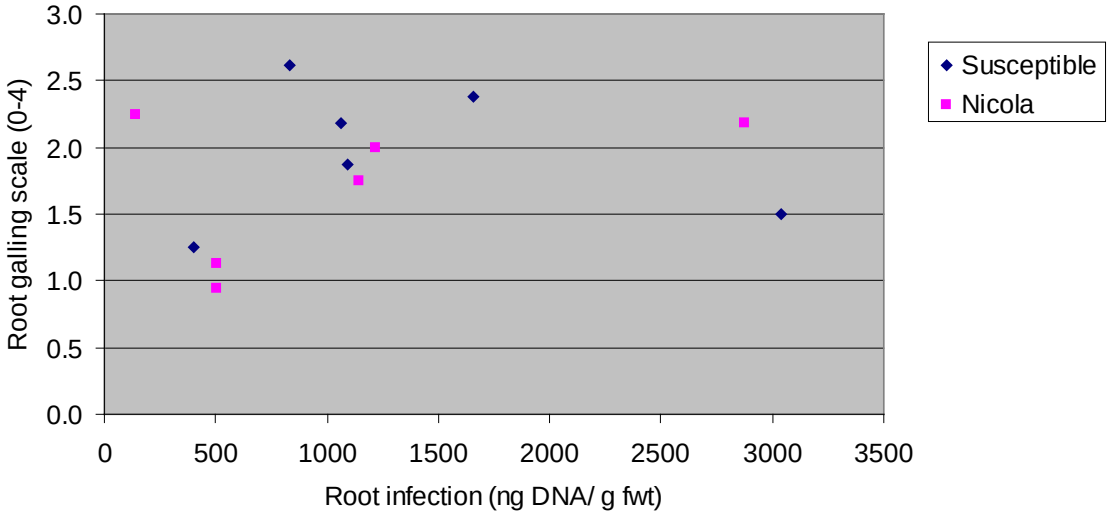


Figure 12. Mean root infection and the mean root galling score recorded at the final sample harvest at the Scottish sites (JHI and SAC in 2008, 2009 and 2010).

At all sites, tuber infection was observed at the first sampling time after tuber initiation (Table 5). In Tasmania, although tuber initiation occurred 7 WAP (similar to that of other sites), the first tubers to be assessed by real-time PCR were taken 2 weeks later (9 WAP), therefore the apparent later timing of tuber infection at this site compared to other sites is associated with the timing of samples rather than a real difference in the timing of infection.

The development of powdery scab symptoms on tubers was observed earlier in JHI in 2009 and 2010 than in 2008 and all years at SAC; this may be associated with the difficulty of detecting very small lesions on very small tubers, rather than reflecting real differences in symptom development. It wasn't until 9 WAP that the degree of symptom development was elevated above a negligible level. The longest recorded time from planting for powdery scab symptoms to be found was at SAC in 2009 and 2010 (11 WAP).

As with root infection and galling, there was little relationship between the extent of infection in symptomless tubers and powdery scab at final harvest (Figure 13). At JHI, in 2008, cultivar Agria had both more disease and its symptomless tubers had higher levels of *S. subterranea* DNA than Nicola. In 2009, Agria had more disease than Nicola at final harvest, but levels of infection in symptomless tubers were similar throughout the season. At SAC (2008 and 2009) Estima tubers had more disease than Nicola, but the extent of infection in symptomless tubers was similar in both cultivars. In both Tasmania and Victoria (2008/09 and 2009/10) tuber infection in Kennebec was much higher than in Nicola and this was reflected in the extent of powdery scab development, with Nicola having no symptoms.

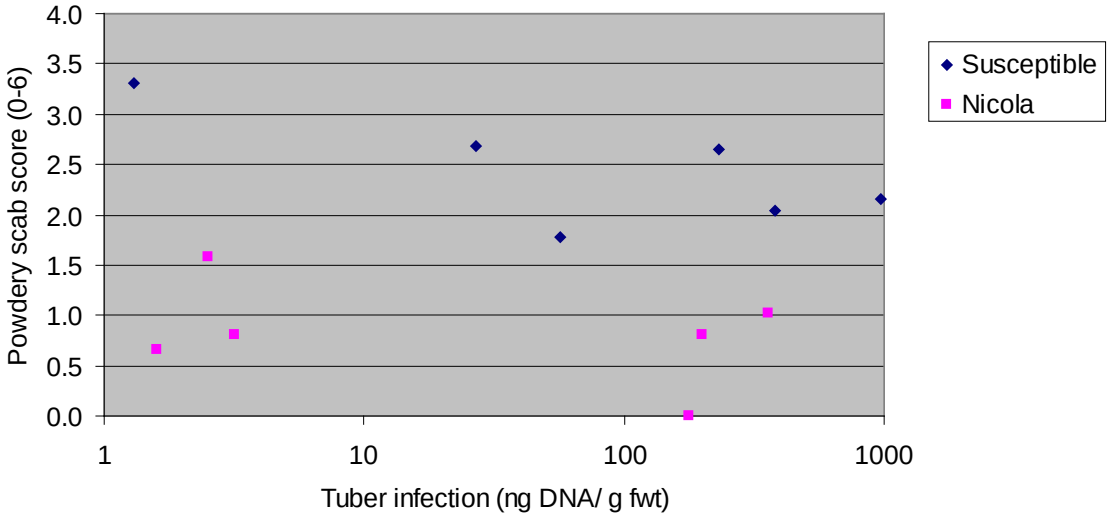


Figure 13. Mean tuber infection and the mean powdery scab score recorded at the final sample harvest at the Scottish sites (JHI and SAC in 2008, 2009 and 2010).

The extent of root galling and powdery scab symptoms

Root galling was found on the powdery scab susceptible cultivars at each site and on Nicola in Scotland, where it was often at comparable levels to the powdery scab susceptible cultivars. On a scale of 0 to 4, mean root galling was typically around 2 which corresponds to "several galls, mostly small (< 2 mm in diameter)". Very few root galls were observed on Nicola in Australian trials (Figure 14). There was significantly less root galling in 2009 at the JHI site than in other Scottish trials, but this year was neither warmer/colder nor wetter than other seasons.

There was no clear distinction between either years or sites with respect to the severity of powdery scab at final harvest in the susceptible cultivar at the Scottish sites (Figure 15); levels of powdery scab at harvest were higher than the across site average at SAC in 2008 and 2010, and lower than the across site average at SAC in 2009 and at JHI in 2008. However, whilst Nicola developed fewer powdery scab symptoms than either Agria or Estima in all of the Scottish trials, it still did develop some symptoms. At the three Australian sites no powdery scab symptoms were seen on Nicola.

The cultivar Nicola in Australia appears to be more resistant to powdery scab and root galling than it is in Scotland. The Nicola used in Australia has been verified by SASA to be the same cultivar as used in GB. Therefore differences in its resistance may be attributed to factors other than genetic ones, but analysis of the data did not identify a key factor that could explain this difference.

In Scotland, Nicola was less resistant to root than to tuber disease, a finding which was also reported by Merz *et al.,* (2012), whilst Agria and Estima were relatively susceptible to both. Merz *et al.,* (2012) also reported large differences in root galling for the same cultivar between years. However, they were unable to link differences to the average temperatures of August at two Swiss locations.

Soil inoculum levels

There was an elevation of soil inoculum levels during the trial (i.e. from pre-planting to final harvest) at the JHI trial site in 2009 and 2010 and to a lesser extent in Victoria 2009/2010, but not at other sites where data on soil inoculum levels at harvest were collected. There is no obvious link to production of root galling or powdery scab symptoms in the trials in which spore ball levels increased over and above that found in other trials. One factor which may influence the extent of spore ball contamination, is the maturity of the lesions at the time of harvest, i.e. if tubers are harvested before pustules burst releasing spore balls in to the soil, then build up of inoculum may be lessened. The final harvest at JHI in 2008 was earlier than in subsequent years, and there was little build up of inoculum in this trial.

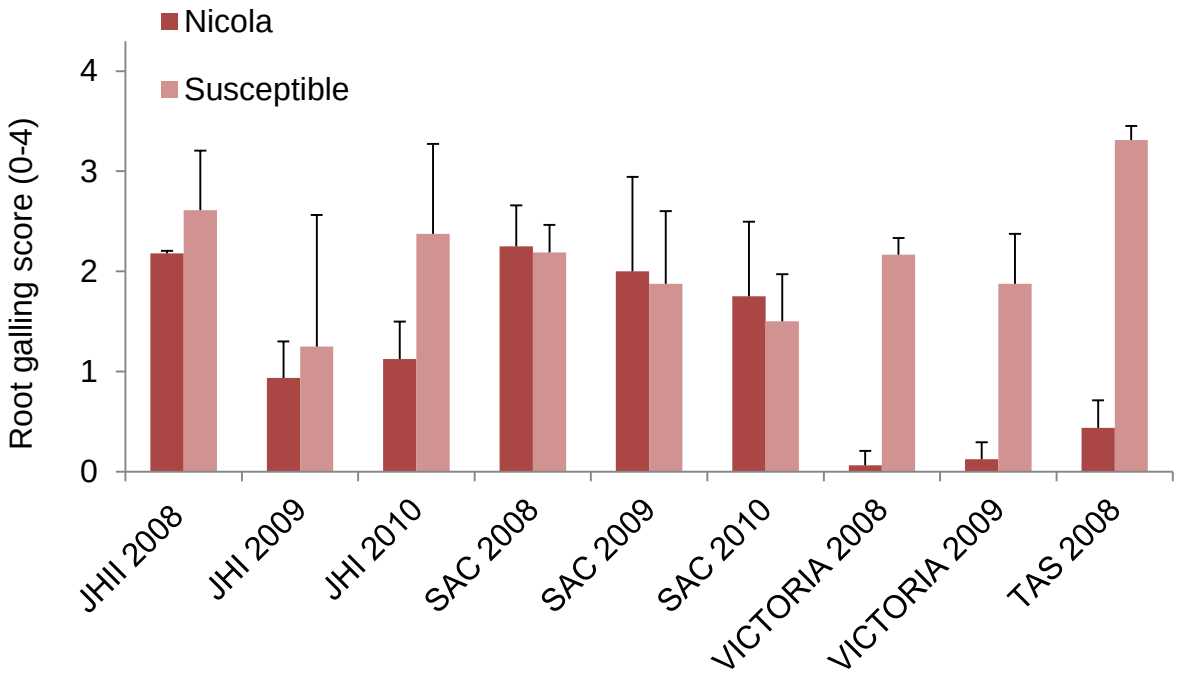


Figure 14. Mean root galling (score 0-4) observed in Nicola (the more powdery scab resistant cultivar) and the more powdery scab susceptible cultivar planted at each site (Agria at JHI, Estima at SAC and Kennebec at Victoria and Tasmania), at the final sample harvest.

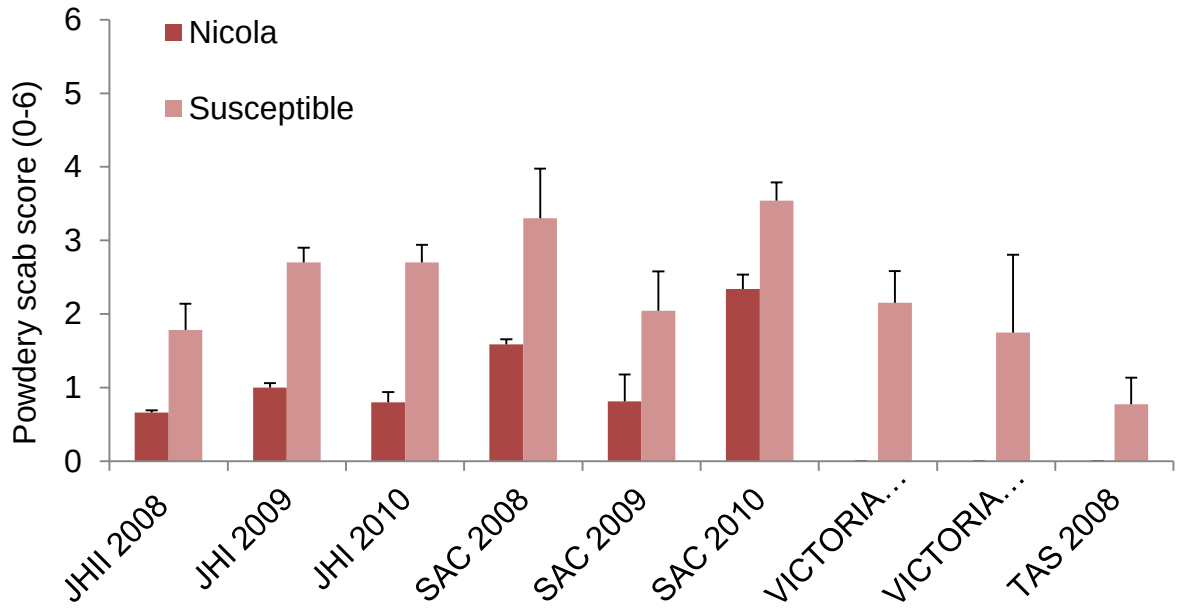


Figure 15. Mean powdery scab severity (score 0-6) observed in Nicola (the more powdery scab resistant cultivar) and the more powdery scab susceptible cultivar planted at each site (Agria at JHI, Estima at SAC and Kennebec at Victoria and Tasmania), at the final harvest.

4.1.3. Field trials to evaluate the effect of soil borne *S. subterranea* inoculum on disease development

Materials and Methods

In three years (2009, 2010 and 2011), four field trials were set up to investigate the effect of initial soil inoculum level on powdery scab development at final harvest. One trial was carried out at a site at the James Hutton Institute (JHI) in each year; and one trial was also carried out by SAC at Fingask, Aberdeenshire in 2011. In each of the three years the powdery scab susceptible potato cultivar Agria and the moderately susceptible cultivar Nicola (Merz *et al*. 2011) were used in the JHI trials. Cultivars Estima (susceptible) and Nicola were compared in the 2011 SAC trial. All seed was kept at 4°C in the dark until required.

At both JHI and SAC, trials were located on a field site on which potatoes had never knowingly been grown. The soil from each site was sampled and tested using real-time PCR and found to be negative for the presence of *S. subterranea*. In each of the trials four main plots (inoculum levels 1-4) were created. No inoculum was added to level 1 main-plots and each of plots 2-4 were amended with increasing amounts of dried tuber peel taken from tubers heavily infected with powdery scab. After the inoculum had been incorporated into the drills, a single soil sample was taken from each main plot (consisting of 25 x 10g cores bulked) and the level of *S. subterranea* (spore balls / g soil) determined by real time PCR.

Each main plot consisted of 4 rows of 12 plants, surrounded by guard rows and separated by at least 2 rows. Two replicates of each cultivar were planted in each

main plot. At JHI in 2009, 2010 and SAC in 2011 all plots were irrigated. In 2011 (JHI), a split plot design was created to compare irrigated with un-irrigated conditions. However, as no significant differences for disease incidence and severity were found between irrigated and un-irrigated treatments, the mean of both treatments are presented. At the time of final harvest, all progeny tubers from each plot were assessed for powdery scab incidence and severity (score 0 to 6), and results expressed as the mean incidence and severity score of powdery scab disease per plot.

Statistical Analysis:
All statistical analysis was carried out using GenStat 14th edition (VSN International Ltd, Hemel Hempstead UK). The effect of soil inoculum level (df=3) and cultivar (df=1) on progeny tuber disease in individual field trials was analysed using ANOVA.

Results and discussion

Visual assessment of seed stocks revealed that both cultivars used in the trials at JHI had powdery scab symptoms (Agria 10, 14 and 36%; Nicola 14, 44 and 52 % incidence) in 2009, 2010 and 2011, respectively. Mean powdery scab disease severity did not exceed 0.9 for either cultivar in any year. The Estima and Nicola stocks used in the 2011 SAC trial were visibly clear of powdery scab symptoms.

The amount of detectable *S. subterranea* in the 4 artificially created inoculum levels differed between years as would be expected as each were individually infested in the year of experimentation. In the JHI trials, the range of inoculum detected using real-time PCR in the main-plots ranged from close to zero to over 214 sporeballs / g soil (Table 6), which just exceeds the range found in fields used in commercial potato production (0 to 148 sporeballs / g soil recorded during Potato Council Project R253).

Table 6. *Spongospora subterranea* (sporeballs/ g soil) quantified in main-plots inoculated with varying levels of sporeballs.

	Soil inoculum (spore balls / g soil)			
	Level 1	Level 2	Level 3	Level 4
2009 (JHI)	1.2	0.1	3.7	57.2
2010 (JHI)	0.6	0.7	0.6	5.7
2011 (JHI)	1.3	17.2	20.3	214.5
2011 (SAC)	0.0	0.0	0.0	0.0

The amount of inoculum detected in Level 4 using real-time PCR was lower in 2010 than 2009 or 2011. The amount of inoculum detected in Level 3 (which received only one tenth of the dried potato peel added to Level 4), was approximately 10 fold lower than that detected in Level 4 in each year. However as inoculum levels below 1 sporeball / g soil may be detectable, but not accurately quantified, there was no discernable difference between detectable inoculum levels in Levels 1 and 2 in 2009, and between Levels 1, 2 and 3 in 2010, as all these soils had less than 1 sporeball/ g soil. In all years at JHI, some inoculum was detected in the Level 1 (un-infested) main-plots after the other main-plots had been artificially infested possibly as a result of wind-blown inoculum contaminating un-inoculated plots. The levels in the SAC 2011 trial were inoculated in the same way as those carried out at JHI, however, when soil was collected shortly after the incorporation of inoculum they all tested

negative for *S. subterranea*. The samples were re-analysed and no problem with either the extraction or real-time PCR was evident. The inoculum was incorporated prior to planting and it is not clear why no inoculum was detected as the sampling was done at sufficient depth and with an adequate number of cores.

In all four trials, different amounts of dried potato tuber peel incorporated into plots to give increasing levels of initial soil inoculum (Levels 1-4), significantly (ANOVA: $p<0.01$) affected both the incidence and severity of powdery scab on progeny tubers of both cultivars, although in 2011, the effect of inoculum level was greater in the more susceptible cultivar Agria than the more resistant cultivar Nicola ($p<0.01$). The results clearly demonstrate that the level of initial soil inoculum did affect both the incidence and severity of powdery scab on progeny tubers (Figure 17). Progeny tubers of Agria from plots to which no inoculum had been added (Level 1) had a mean disease severity score of less than 1, in contrast to progeny tubers from Level 4 plots which a mean disease severity score of 4 in 2009, 3 in 2010 and 2 in 2011. The difference in symptom coverage between tubers with a score of 1 and 4 is shown in Figure 16.

The more resistant cultivar, Nicola, reached a maximum of 72 % incidence of disease but with a mean disease severity score of just over 1. The more susceptible cultivar Agria reached a maximum of 99% incidence with a mean disease severity score up to 3.9. In all years, disease incidence and severity scores were significantly greater in Agria than Nicola ($P<0.01$) (Figure 17).

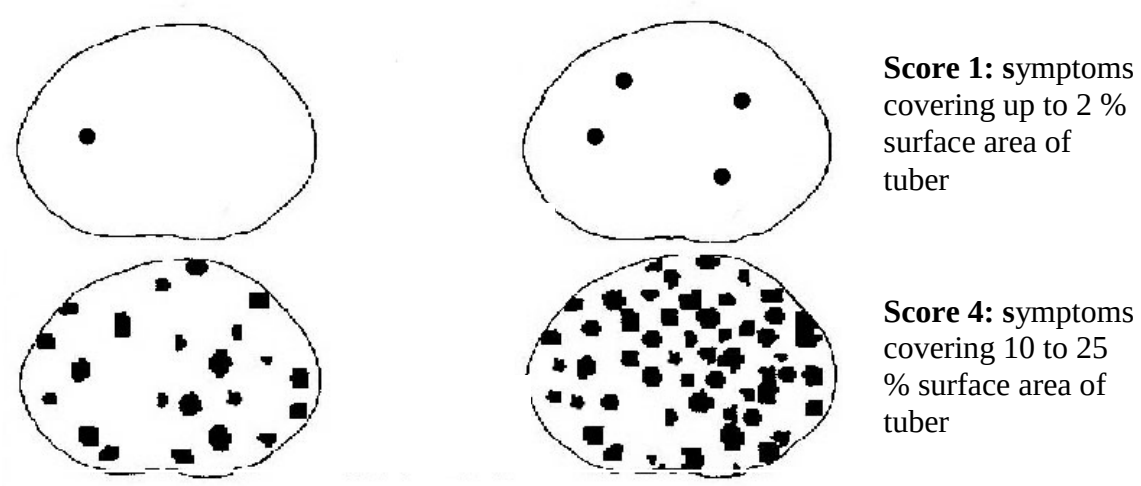


Figure 16. Disease severity scores, illustrating difference in surface area covered by symptoms on tubers with a score of 1 compared with those with a score of 4.

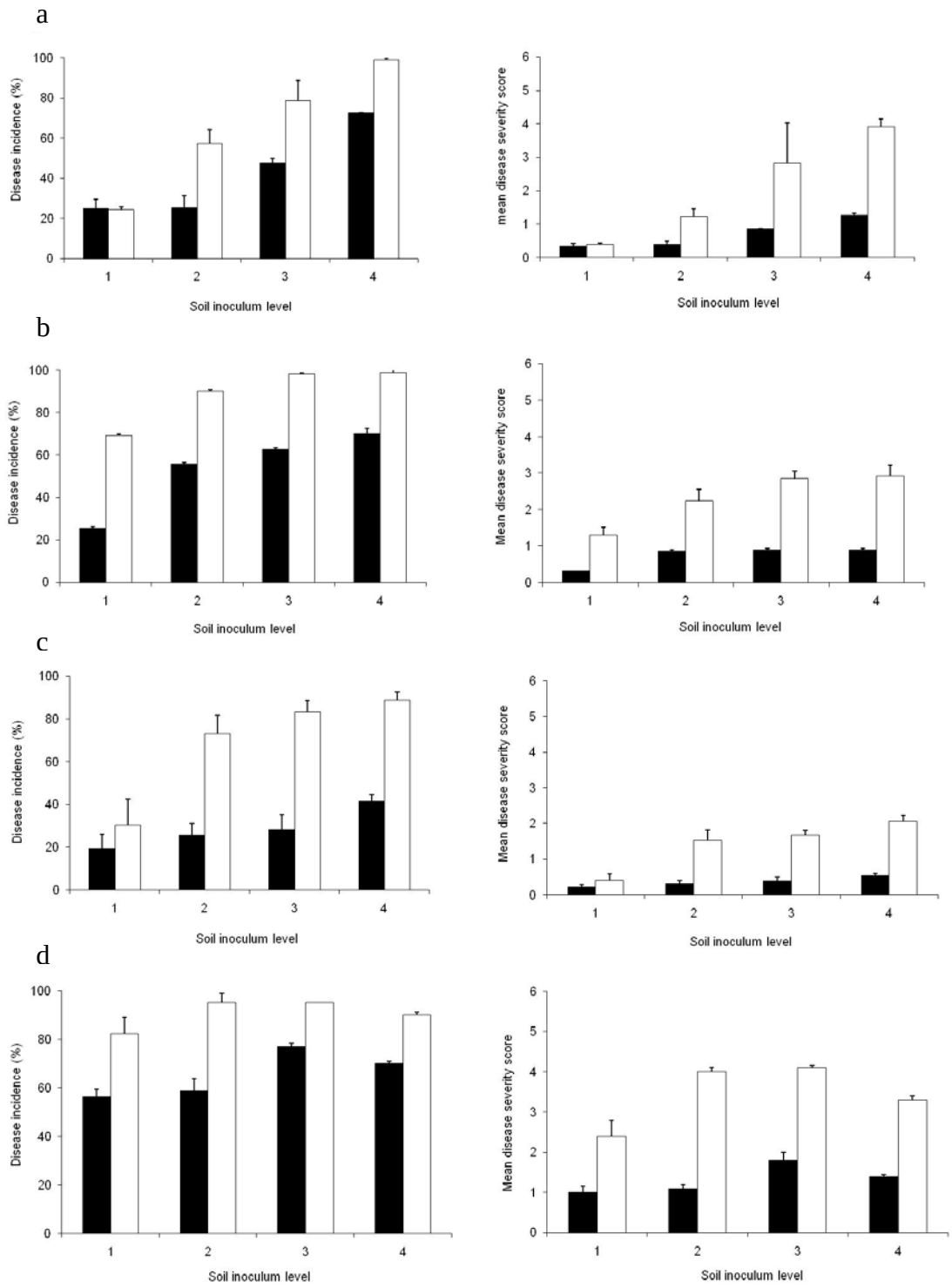


Figure 17. The effect of soil inoculum level (1-4 scale of increasing infestation) of *S. subterranea* on powdery scab incidence and severity in two cultivars, Nicola (■) and Agria (□), in field trials carried out in a) 2009 b) 2010 and c) 2011 (JHI) and d) 2011 (SAC). Mean of 2 replicate plots + SE in 2009, 2010 and 2011 (SAC) and 4 replicate plots + SE in 2011 (JHI).

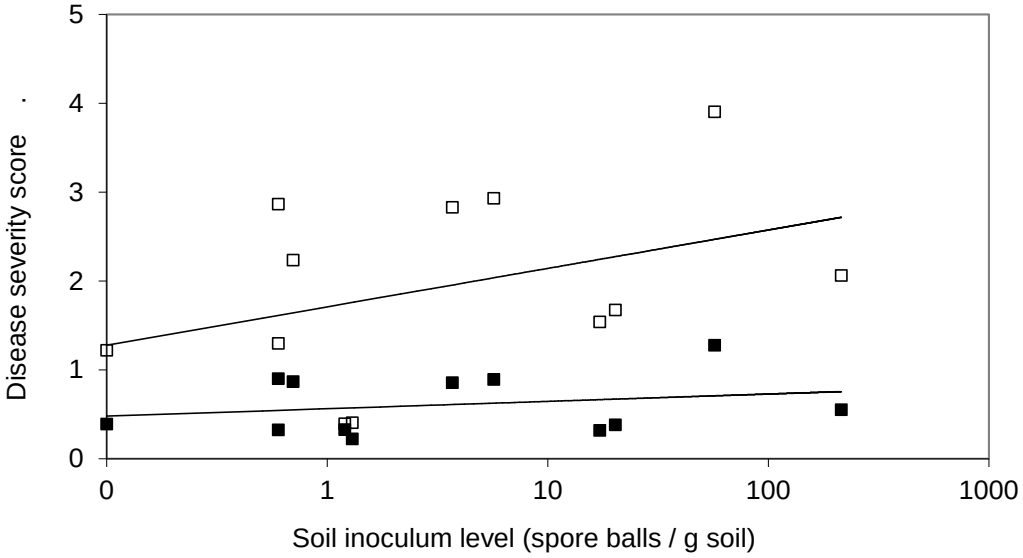


Figure 18. The effect of soil inoculum level of *S. subterranea* (spore balls / g soil) on powdery scab severity (disease score) in two cultivars, Nicola (■) and Agria (□), in field trials carried out in 2009, 2010 and 2011 at JHI.

4.1.4. Controlled environment trials to evaluate the effect of soil borne *S. subterranea* inoculum on disease development

The reasoning behind these experiments was to test the hypothesis that if seed is grown in conditions suitable for secondary infection, i.e. cool and damp soils, then as long as some inoculum is present in the soil, the initial inoculum level will be of less importance in causing disease than if conditions were less suitable for secondary infection. Therefore we would expect high levels of disease to result from all initial soil inoculum levels. The experiments were carried out twice (2009 and 2010) at JHI.

Materials and Methods

In 2009 at JHI, shortly after the incorporation of inoculum into the drill but before the field trial was planted, quantities of soil were collected from the main field trial plots. Two additional inoculum levels were created by mixing the soil from levels 2 and 3, to create 2.5 and from levels 3 and 4 to create 3.5. The level of soil inoculum (spore balls / g soil) in each soil treatment (1 x 60g subsample) was determined by real-time PCR.

Seed of the same stocks used in the field trials were used in the controlled environment trials. In 2009, visual assessment of seed stocks showed that both cultivars used the trials at JHI had powdery scab symptoms (Agria 10% and Nicola 14% incidence, respectively). Seed of both cultivars (Agria and Nicola) were planted in pots in the infested field soils and grown at 15ºC with five replicates. The controlled environment experiment was conducted at 70% relative humidity with a 16 hour light/8 hour dark regime. All pots (3 litre) were watered by hand to minimize cross contamination between pots. Soil was kept moist. Plants were grown for 144 days after which stems were cut off and watering ceased. The progeny tubers from each pot were harvested 16 days later and placed in a paper bag. The severity of powdery scab on each individual tuber was recorded on a disease scale of 0 to 6

and results expressed as the mean incidence and severity of powdery scab disease per pot.

In 2010 the experiment was repeated with some modifications. Agria seed was planted and grown under controlled environment conditions as described above. Visual assessment of the seed stock showed that it had powdery scab symptoms (14% incidence).To investigate relative levels of root infection under different levels of soil inoculum: four replicate plants from each inoculum level were harvested at weekly intervals for four weeks, the first being taken shortly after plants emerged (29 days after planting). At each sample harvest the roots were assessed for *S. subterranea* infection and visual symptoms of root galling. If tubers were present, they were peeled and assessed for *S. subterranea* infection. A final set of plants from each inoculum level were maintained for 112 days after which stems were cut off and watering ceased. The progeny tubers from each pot were harvested 14 days later and placed in a paper bag. The severity of powdery scab on each individual tuber was recorded on a disease scale of 0 to 6, and results expressed as the mean incidence and severity of powdery scab disease per pot.

Results and Discussion

The results from the 2009 controlled environment experiment are shown in Figure 19.

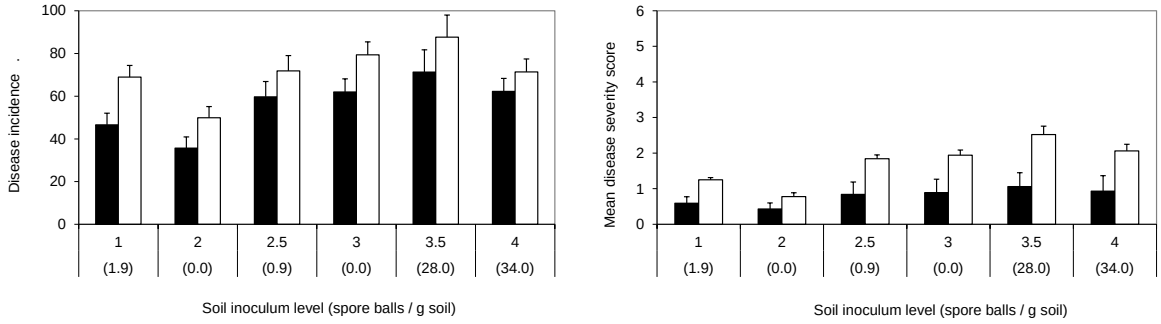


Figure 19. The effect of soil inoculum level on powdery scab severity (disease score) in Nicola (■) and Agria (□), under controlled environment conditions (2009), Mean of 5 replicates + SE.

Under the controlled conditions the more susceptible cultivar, Agria, developed relatively high levels of disease at low inoculum levels which contrasted with disease development under field conditions. However at the higher inoculum levels disease incidence and severity were lower in the controlled environment experiment than the corresponding field trial. Disease incidence and severity were significantly lower for the more resistant cultivar, Nicola, compared to Agria, and did not differ much between field and controlled environment conditions.

The results from the 2010 controlled environment experiment are shown in Figures 20A and B.

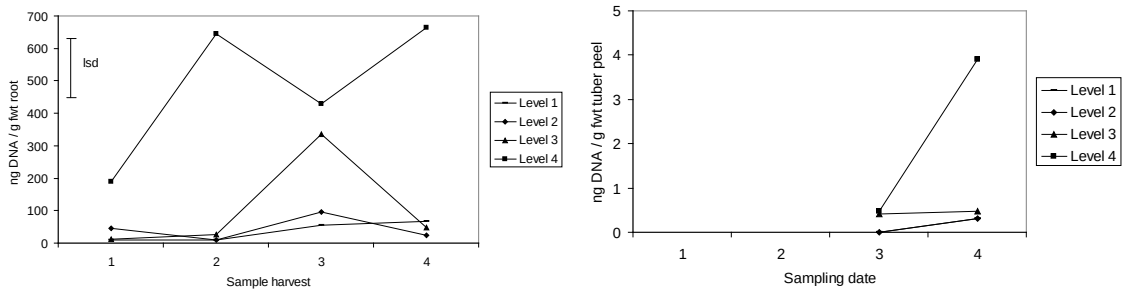


Figure 20A. *S. subterranea* DNA (ng / g fwt root tissue and ng / g fwt tuber peel) detected at weekly intervals from plant emergence onwards, when Agria seed grown in soil amended with different levels of soil inoculum (2010).

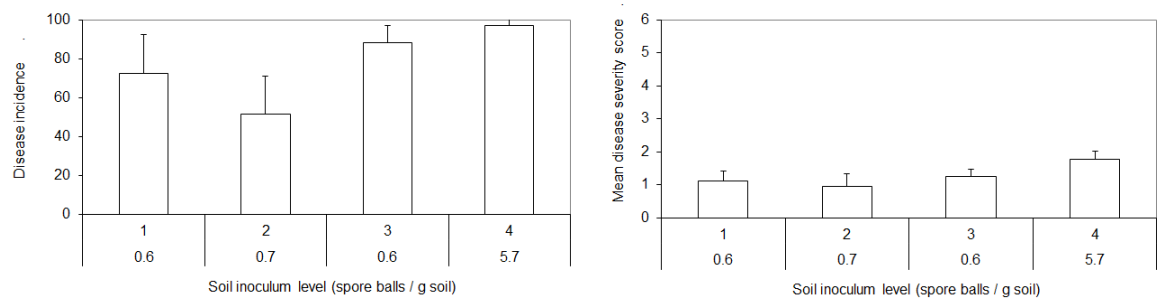


Figure 20B. The effect of soil inoculum level on powdery scab severity (disease score) in Agria, under controlled environment conditions (2010), mean of 4 replicates + SE.

Weekly sampling of the roots revealed that root infection was significantly (ANOVA: $p<0.01$) higher in the soil amended with the highest level of inoculum (level 4) compared to all lower levels. There was no significant difference between levels 1 to 3, which is not surprising considering that no difference was detected in the amount of soil inoculum (sporeballs / g soil) between levels 1-3. Root infection generally increased after the first sample harvest in level 4. No tubers were present until the third week of sampling. At the third sampling date, low levels of infection were detected in tubers grown in the level 3 and 4 soils. At the fourth sampling date, only tubers from level 4 soils had significantly higher levels of infection compared to both other levels and/or the previous sampling occasion. At final harvest, there was no difference between the incidence of disease across the soil inoculum levels in comparison to those occurring in the field trial, but the severity of disease in the controlled environment experiment was low irrespective of soil inoculum level.

The results from the controlled environment experiments were not consistent between years. The 2009 results indicated that under controlled environment conditions disease levels are less affected by the initial level of soil inoculum, and indeed quite high levels of disease occurred from very low levels of initial inoculum, but relatively low levels of disease were found at the higher soil inoculum levels. There was very little disease in the 2010 experiment across all soil inoculum levels within the controlled environment experiment. If conditions were truly “conducive” in the controlled environment experiments we would have expected higher levels of

disease, even if only in the highest inoculum level. This discrepancy may be due to conditions associated with plants grown in pots not being truly representative of field conditions. The plants were grown for longer in 2009 compared to 2010; 144 days cf. 112 days but this reflected the general health of the plants which had the stems cut off when they were beginning to senesce, and it is unlikely that this difference will have had a marked effect on the level of disease in the two years.

Conclusion

By growing plants in soils amended with different levels of soil inoculum we established a relationship between soil inoculum level and subsequent disease on progeny tubers.

- In four field trials carried out over three years, results show that the level of soil inoculum significantly affects the level of powdery scab on progeny tubers.
- We were unable to demonstrate experimentally that if conditions are suitable for secondary infection then high levels of disease consistently occur irrespective of the initial soil inoculum level.

4.2. *PMTV*

4.2.1. Introduction

Potato Mop Top Virus (PMTV) causes spraing, necrotic arcs or flecks in the flesh of tubers. *Spongospora subterranea* is the only vector of PMTV. Infection occurs soon after the vector has penetrated the host cell. PMTV is located inside the zoospores emerging form vegetative sporangia and it also resides in resting spores which may survive in soil for more than 15 years. Soils may, therefore, be infected by PMTV for a long period of time and PMTV can still be detected in soils 12 years after potatoes were grown (Jones and Harrison, 1972). A previous Potato Council-funded study investigated the extent of PMTV infection in Scottish seed potatoes; the effect on yield and quality; the transmission rate from seed to daughter tubers; and the relative importance of seed and soil inoculum on disease development (Carnegie *et al.,* 2007). This project has studied the timing of PMTV infection in the trial where soil was inoculated with *S. subterranea* infected with PMTV.

4.2.2. Field trials to investigate PMTV infection of potato in soils inoculated with *S. subterranea* infected with PMTV.

Materials and Methods

In field trials carried out at JHI in 2009 and 2010 primarily investigating *S. subterranea* infection and subsequent powdery scab development, experiments were inoculated with powdery scab spore balls infected with PMTV. This allowed the co-investigation of *S. subterranea* and PMTV in a number of experimental systems. Full details of the field experiments, plant sampling and sample processing are described in the sections outlined below:

- Standardised powdery scab field trials (Section 4.1.2). Root and tuber PMTV infection were investigated in 8 weekly samples commencing at plant emergence. Spraing symptoms and PMTV infection in progeny tubers were recorded.
- Field trials investigating the effect of powdery scab inoculum levels (Section 4.1.3). Spraing symptoms and PMTV infection in progeny tubers were recorded.

In root and tuber samples taken during the growing season, detection of PMTV using real-time PCR was performed using the assay of Mumford *et al.,* (2000). 2µl of neat NA was used in the PMTV assay. Results were expressed as the Ct value. All plates included a positive control in a dilution series and a negative control.

For progeny tubers, spraing symptoms were induced in infected tubers; all progeny were initially stored at 16°C for a 2 week period before being relocated to storage at 4°C for a further 2 weeks before being assessed. For spraing assessments, 25 progeny tubers per plot from the standard field trials, and 12 tubers per plot from the inoculum level field trials were cut stolon to rose and visually inspected for spraing symptoms. All 25 progeny tubers per plot from the standard field trials, but only 4 randomly selected tubers per plot from the inoculum level field trials were tested for PMTV infection using Elisa (Torrance *et al*., 1993).

Results and Discussion

Root and tuber PMTV infection

The results from the JHI 2009 and 2010 standard powdery scab field trials are very similar. In 2009, the roots of some, but not all, plants were infected with PMTV at the first two sampling dates, but all the plants of both cultivars were infected by the third date (49 DAP). With regard to tubers in 2009, tubers were first sampled at 49 DAP (3rd sampling date) but there was no PMTV detected in the tubers at this time, however subsequent samples had progressively higher levels of infection. In 2009, levels of PMTV in tubers were significantly higher in Nicola than Agria (Figure 21).

In 2010, when plants were sampled at the time of emergence (date 1; 28 DAP) no Agria roots were infected, but 3 out of the 4 Nicola plants sampled had roots infected with PMTV. By the second sampling date, 1 out of the 4 Agria plants had become infected with PMTV, and by the third sampling date (42 DAP) the roots of all plants of both cultivars were infected with PMTV. Tubers were not present until the fourth sampling date (49 DAP). At which time, all Nicola plants sampled had tubers infected with PMTV. The tubers of Agria had lower levels of infection than Nicola, and at all sample dates, only 3 of the 4 plants sampled had PMTV infected tubers (Figure 18).

In both years, there was no significant difference between the level of root infection between the two cultivars, but Nicola tubers had significantly higher levels of infection than Agria tubers. In both years, it was only tubers without any visual powdery scab symptoms that were tested; therefore PMTV infection occurred without any powdery scab symptoms present.

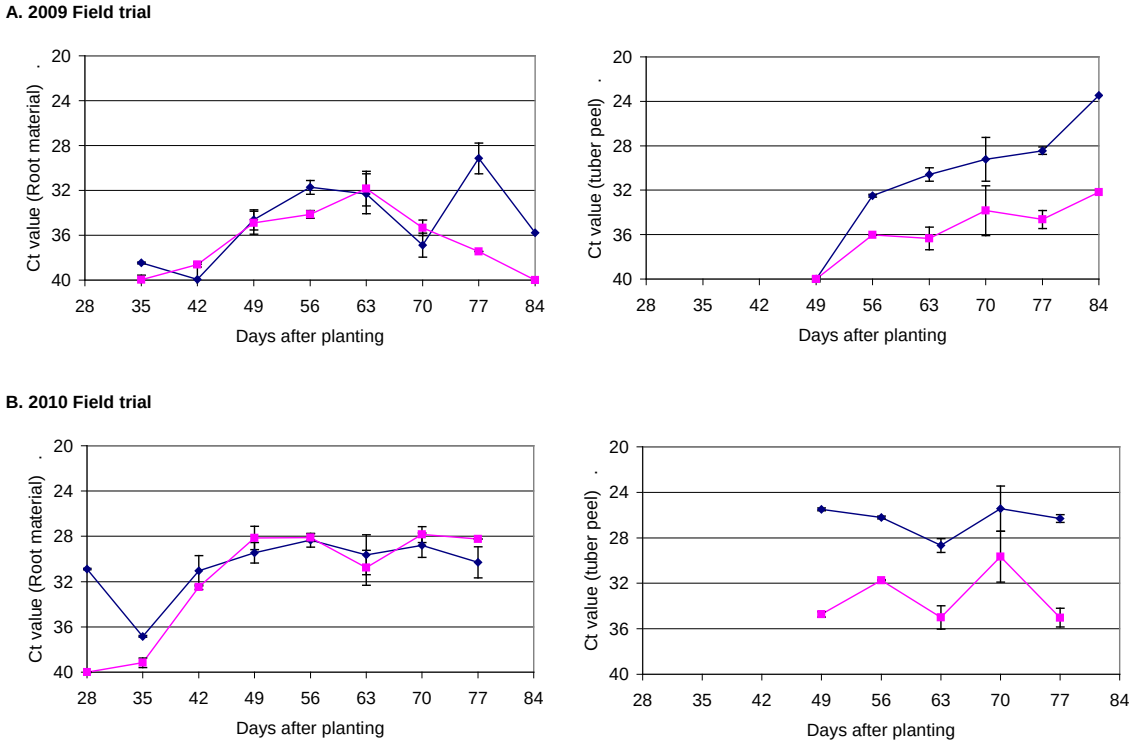


Figure 21. Mean Ct value of PMTV infection in roots and tubers measured at weekly intervals on two cultivars, Nicola (blue) and Agria (pink) at JHI in the standard powdery scab field trials in A. 2009 and B. 2010. Standard errors of means are shown. When Ct = 40, no PMTV has been detected.

Spraing and PMTV infection in progeny tubers

Progeny tubers from the final harvest of all four field trials were assessed for spraing and tested using ELISA for PMTV infection. In the standard field trials, few Agria tubers had visible disease symptoms (<4 % in 2009 and 2010), more Nicola tubers had spraing symptoms (6% in 2009 and 24 % in 2010). When tubers were tested with ELISA, the majority of both cultivars were infected with PMTV in 2010, with somewhat fewer Agria tubers testing positive in 2009 (Table 7).

In the inoculum level field trials, in both years, no difference in the level of spraing or PMTV infection was detected between inoculum levels; therefore the overall means are presented in Table 7. As with the standard field trials, overall, Nicola had a higher percentage of tubers with spraing symptoms than Agria (15% cf. 5% in 2009 and 42% cf. 6% in 2010). More Nicola tubers tested positive for PMTV infection than Agria tubers in both years (Table 7).

Nicola is known to express symptoms of PMTV (spraing) more than Agria or Estima However, for all cultivars, PMTV infection without spraing symptoms was common.

Table 7. Visual symptoms of spraing and results from Elisa testing on progeny tubers, at final harvest in 2009 and 2010 field trials.

		Visual spraing symptoms (% incidence)		ELISA testing (% positive)	
		mean	st. dev	mean	st.dev
Standard field trial					
2009	Agria	4	3.3	27	20.5
	Nicola	6	4.0	52	13.5
Standard field trial					
2010	Agria	1	2.0	65	10.0
	Nicola	24	26.7	65	25.2
Inoculum level field trial					
2009	Agria	5	4.0	9	12.9
	Nicola	15	5.4	18	32.7
Inoculum level field trial					
2010	Agria	6	4.2	42	49.9
	Nicola	42	9.6	75	17.0

4.3. *Rhizoctonia solani*

4.3.1. Introduction

Rhizoctonia solani is a fungus which exists as a number of Anastomosis Groups (AGs) that are known to infect different hosts. Several AGs (AGs 2-1, 2-2, 3, 4, 5, 8 & 9) of *R. solani* have been associated with diseases of potatoes. AG3 occurs as different subgroups (strains), including AG3-PT which occurs on potatoes. In this report AG3 or AG3-PT are referred to according to which real-time PCR assay was used to detect\quantify the presence of AG3: The assay used at Fera is specific for AG3-PT whilst the assay used at JHI detects all subgroups of AG3. During the project examples of symptoms associated with *R. solani* infection (and in some cases, infection with specific AGs) have been documented and are provided in a separate document. It should be noted that in the case of some symptoms (elephant hide, cracking and dry core) there may be other causes of the symptom and care is needed in deciding if a symptom is due to *R. solani.*

Previous Potato Council- funded research (project R253) established that soil-borne *R. solani* AG3 inoculum could be quantified accurately in artificially inoculated soils (Brierley *et. al*. 2009) using the real-time PCR assays mentioned above (Lees *et. al*. 2002). However, further work was required before it would be possible to develop disease risk assessments based on the results of diagnostic tests applied to samples of field soils. In order to establish disease risk based on diagnostic tests and to recommend appropriate control measures for *R. solani* an approach previously successfully adopted for *Colletotrichum coccodes* was initiated: integrated controlled environment and field studies that aim to unravel the epidemiology of the pathogen, whilst concurrently establishing the impact of control measures on the pathogen. The aspects that needed to be studied were:

- the relative importance of soil- and seed-borne inoculum levels in causing disease;
- the importance of soil type/soil treatments as a factor affecting disease development;
- whether the correlation between inoculum level and disease is influenced by AG;
- how *R. solani* exists in soil, with respect to propagule type and how this affects disease development and pathogen survival.

Relative importance of soil- and seed-borne inoculum levels in causing disease.

At the start of the project, the relative importance of seed and soil borne inoculum in disease development was still to be fully determined. Initially, seed-tuber borne inoculum of *R. solani* was often considered to be of primary importance compared to soil borne inoculum (Frank and Leach, 1980). Proximity of the seed-borne sclerotia to the emerging sprouts is thought to lead to consistently severe infections. However, more recent work has highlighted the importance of soil-borne infection (Kyritsis and Wale, 2004). Disease development may be affected by inoculum source, with seed-borne sources associated with severe stem cankers and soil-borne inoculum associated with severe infection of secondary stems, stolons and the development of black scurf. The relative importance of level of inoculum, for both seed borne inoculum and soil borne inoculum had not been extensively investigated. Gilligan (1997 a,b) found that the density of tuber-borne inoculum had a significant effect on the incidence and severity of disease. In contrast, there was no relationship between

level of seed inoculum (as determined by either visual assessment or real-time PCR) and disease when infected seed was planted into fields in which no soil inoculum was detected in the Potato Council project R253. It is possible that disease development from seed inoculum was prevented by use of seed treatments which are widely applied. (In 2010, 45.8% of seed was treated with a *R. solani* effective fungicide [pencycuron, flutolanil or tolclofos methyl]; 19% was treated in furrow with azoxystrobin and 7.1% of seed was treated with both). Alternatively, problems associated with soil inoculum quantification may have resulted in a failure to detect soil inoculum. Therefore, it was not known 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, or whether the relationship between seed-borne inoculum and disease is not straightforward.

The importance of soil type/soil treatments/soil moisture as a factor affecting disease development

The moisture content of soil can influence disease development. Kyritsis and Wale (2002a) in controlled environment studies, found that stem canker was most severe when the water holding capacity (WHC) of soil where 40% compared to soils at 20% or 60%. This is concordant with previous work by Hide and Firmager (1989) who found that stem canker severity was greater at 45% WHC compared to 75% or 90% in controlled environment conditions. Black scurf development on the mother tuber is also influenced by moisture: black scurf severity was greater at 20% or 40% WHC than at 60% (Kyritsis and Wale, 2002a). Observations from fieldwork support these conclusions, where irrigated plants displayed less stem canker (Simons and Gilligan, 1997b). Although there are few known studies on the affect of soil type on disease, it is expected that when high levels of soil inoculum are present soil type may have little impact on suppressing disease, but differences in soil type may become apparent under low disease pressure.

Whether the correlation between inoculum level and disease is influenced by AG.

Several AG (AGs 2-1, 2-2, 3, 4, 5, 8 and 9) of *R. solani* have been associated with diseases of potatoes. Knowledge of which AGs are present in the environment is important. For example, some AGs are limited in their ability to infect different parts of the potato plant; Hide and Firmager (1990) observed that AG8 was only capable of infecting potato roots. The AG could also influence disease management, certain AGs have different host ranges; AG2-1 is known to have a wide host range, whilst isolates belonging to the potato sub-group of AG3 are considered relatively host specific. This could affect survival through crop rotations. Also, some fungicides are known to be selective to certain AGs (Kataria and Gisi, 1999). The fungicide pencycuron is an example of such a selective chemical, with high activity against isolates of AG2-1 and AG3, with little or no activity against isolates of AG5 and AG8

Woodhall *et al*. (2007) found that AG 3-PT was by far the most important in the UK and consequently, previous work has concentrated on the detection and quantification of *R. solani* AG 3-PT. However, *R. solani* AG2-1, AG3 and AG5 can persist in soil as both sclerotia and saprophytic mycelium. 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, existing soil DNA samples (from R253) were retested in the Potato Council- funded project (R411) for the presence of other AGs to eliminate the possibility that other AGs confounded the relationship between detection and disease development. AG 2-1 was found in a

small number of the soils tested, 7 out of 108. There was no link between the presence of AG 2-1 and a previously unaccountable occurrence of disease. No AG 4 or AG 5 was detected in any of the soils tested. However, the importance of AG2-1 and perhaps other AG's on seed and in soil in causing disease was still unclear and warranted further investigation.

Previous studies determining the AGs in potatoes in the UK (eg Woodhall *et al.* 2007) or elsewhere (reviewed in Tsror, 2010) have relied on isolation and typically conventional hyphal fusion testing to determine AG. With the use of molecular techniques such as real-time PCR, direct testing of material, either seed or soil can be used to determine the prevalence of AGs in potato tubers and soil, which may previously have been undetected using traditional isolation techniques.

How *R. solani* exists in soil, with respect to propagule type and how this affects disease development and pathogen survival.

R. solani is thought to survive as many forms in the soil, in volunteer potatoes, in organic matter, as free-living hyphae, as sclerotia or on alternative hosts. Alternative hosts have proved to support the long term survival of AG3. For example AG3 has been isolated from barley roots (Murray, 1981), flax (Anderson, 1977) and sugar beet (Windels and Nabben 1989). In experimental conditions, many other crops were susceptible to AG-3 isolates, including buckwheat, carrot, cauliflower, lucerne, oats, radish, clover, tobacco, tomato, wheat, bean, lettuce, maize, onion, sweet clover and sunflower (Carling *et al.* 1986). In addition, *R. solani* AG-3 has been isolated from the roots and stems of many weeds present in potato fields in Spain (El Bakali *et al.* 2000) and Canada (Sturz *et al.* 1995). The relative frequency of the two types of inoculum (sclerotia and hyphae) 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 because: 1) the distribution of sclerotia and saprophytic hyphae in the soil is likely to be different; 2) DNA extraction efficiency may be lower for sclerotia than for hyphae; and 3) the amount of DNA in a propagule 'unit' (sclerotia or hyphal clump) is likely to vary markedly. Subsequently, the degree of correlation between DNA levels in sclerotia and hyphae with that of disease development is very likely to be different and therefore the epidemiological importance of each propagule type needed to be determined in order to establish the link between inoculum and disease.

Experimental approach

Previous studies (eg Kyritsis, 2003) have attempted to follow the development of visual disease progress on below ground parts of potatoes during crop development in the field. However, only since the availability of real-time PCR assays has the rapid, accurate quantification of inoculum in soil or potato tissue become possible. The field trials in this study were designed to provide a basic understanding of epidemiology and disease development so that a link could be made between time of infection and the development of disease symptoms caused by seed and soil-borne inoculum. The control measures evaluated in the field were: seed tuber fungicide treatment; crop duration (effect of delayed harvest); soil fungicide treatment, extra nitrogen and irrigation. In addition to field selection, these are the most practical disease control options currently available to growers.

To underpin the results from the field trials, a series of controlled environment and experimental trials were carried out to establish specific effects of inoculum on disease, such as seed and soil borne inoculum levels, inoculum type (sclerotia and

hyhae) and anastomosis groups. In addition the effect of factors such as soil type, soil organic matter and soil moisture content were investigated.

4.3.2. Field trials to evaluate *R. solani* control options

Materials and Methods

SAC Field trials (2009-2011)

Five field trials were established, one in 2009 and two in each of 2010 and 2011. In each year, one trial was carried out in the Woodlands field, Craibstone Estate, Aberdeen and in each of the latter two years one trial was carried out at West Fingask farm, Oldmeldrum, Aberdeenshire. The intention with each trial was to identify a site where either soil-borne *R. solani* (AG3) was confirmed as present or where its presence was likely as a result of cropping history, even if no *R. solani* was detected in soil using real-time PCR.

The Woodlands field comprises a number of beds in which short and long term trials have been carried out since 1920. The beds used for the trials in this series had been in grass for several years, before potatoes infected with various pathogens were planted in 2008. Each bed was tested for the presence of *R. solani* (AG3) up to 4 weeks prior to planting. Soil was taken from the top 5cm using a PCN spear from at least 100 locations across the proposed trial area. In total around 1 kg of soil was collected. The soil was thoroughly mixed and tested for *R. solani* (AG3) following the methods of Brierley *et al.* (2009) and Cullen *et al.* (2001). In 2009, inoculum was added to a single treatment in the Woodlands trial (see below).

The fields at West Fingask were tested for presence of *R. solani* (AG3) in March or April 2010 and 2011. Although potatoes had been grown within 3 or 2 years respectively, no *R. solani* was detected. In 2010, inoculum of *R. solani* (AG3) was added to the soil. In 2011, a mycelial solution was sprayed onto commercial seed tubers to produce 'commercial seed + seed inoculum' stock. The preparation and application of inoculum is described below.

Preparation and application of *R. solani* (AG3) inoculum: Woodlands 2009 (one treatment) and Fingask 2010 (all plots)
8-mm diameter mycelial discs, taken from the margin of a *R. solani AG3* culture (whose pathogenicity to potatoes was confirmed) were placed onto the centre of 9cm diameter Petri dishes containing potato dextrose agar. The plates were incubated at 25^{o}C for 7 days (the approximate time that the isolate took to grow to the edges of the Petri dish). Each plate of *R. solani* mycelial mass including agar was homogenised in 200ml sterile distilled water with a hand mixer emulsifier (Multiquick, Braun) for 1 min. Inoculum for the field trial was prepared by mixing the equivalent of mycelial mass from 1 Petri dish into 8kg sieved soil (Kyritsis, 2003). The soil used to produce inoculum was taken from the field in which the trial was to be grown. The soil and mycelium was thoroughly mixed for 5 minutes in a cement mixer. The soil moisture level at mixing was relatively dry (below 40% WHC).

In 2009, in the Woodlands field, inoculum was spread along a groove created in the top of each drill, after planting, at the rate of 1kg/4m. After placement of inoculum, the ridges were raked up to their former shape thus covering the inoculum. At Fingask in 2010, 2kg of inoculum soil was required to spread evenly onto each plot (1 kg/bed) just prior to planting. The planter was assumed to have incorporated the inoculum into the top layers of the ridges.

Preparation and application of *R. solani* (AG3) inoculum: Fingask 2011 (commercial seed + inoculum)
A solution of *R. solani* AG3 inoculum was prepared by homogenising one agar plate of inoculum, prepared as described above, in 1 litre of sterile water. The resulting mycelial solution was sprayed evenly onto the commercial seed tubers to produce the 'commercial seed + seed inoculum' stock. This is referred to as CS+ in the tables below.

Trial information
The details of the five trials carried out during the project are provided in Table 8(a)-(d). In 2009, the trial in the Woodlands field was a scoping trial to prepare for trials in subsequent years. The trial was carried out on two beds with high levels of soil-borne *R. solani* (AG3).

Table 8. Trial information for 5 trials evaluating control of *R. solani* (AG3)
(a) Variety, seed type and AG3 inoculum levels

Year	**Site**	**Main inoculum source**	**Soil test (pg *R. solani* AG3 DNA / g soil) ***	**Inoculum application**	**Seed type**	**Variety**
2009	Woodlands (Beds C&D)	Soil	11,308 & 1,386	1 treatment incorporated into ridge	Seed	Sante
2010	Woodlands (Bed B)	Soil	0		Mini-tubers (18-20mm)	Sante
2010	Fingask	Applied inoculum	0	Spread onto plot	Seed + Mini-tubers (18-20mm)	Sante
2011	Woodlands (Bed C)	Soil	4,206		Mini-tubers (20-25mm)	Markies
2011	Fingask	Applied inoculum	0	Mycelial solution sprayed onto commercial seed	Seed + Mini-tubers (20-25mm)	Markies

***** The soil tests were carried out before incorporation of the *R. solani* AG3 inoculum (Woodlands 2009, Fingask 2010).

(b) Planting, haulm destruction and harvest dates

Year	Site	Planting date	Early harvest date	Late harvest date	Haulm destruction dates	Year of last potato crop
2009	Woodlands	29-May-09	14-Oct-09	28-Oct-09		2008
2010	Woodlands	25-May-10	25-Sep-10	-	9-Sep-10 17-Sep-10	2008
2010	Fingask	03-Jun-10	05-Oct-10	26-Oct-10	10-Sep-10 17-Sep-10	2007
2011	Woodlands	02-May-11	19-Sep-11	20-Oct-11	16-Aug-11 22 Aug 11	2009
2011	Fingask	20-May-11	03-Oct-11	20-Oct-11	8-Sep-11 15-Sep-11	2009

(c) Trial layout

Year	Site	Trial design	Plot size	Seed spacing	Fertiliser		
					N	P	K
2009	Woodlands	Randomised block	4 drills x 18 tubers	20cm	200	50	200
2010	Woodlands	Randomised block	2 drills x 18 tubers	20cm	150	50	200
2010	Fingask	Split plot*	4 drills x 25 tubers	25cm	160	200	150
2011	Woodlands	Randomised block	2 drills x 23 tubers	20cm	150	50	200
2011	Fingask	Split plot*	4 drills x 25 tubers	25cm	120	151	151

*Seed stocks as main plots and treatments as sub-plots

(d) Trial location

Year	Site	Grid Reference	Soil type
2009	Woodlands	NJ872106	Sandy loam
2010	Woodlands	NJ872106	Sandy loam
2010	Fingask	NJ772283	Sandy clay loam
2011	Woodlands	NJ872106	Sandy loam
2011	Fingask	NJ773272	Sandy clay loam

In all field trials, the soil was prepared by ploughing, deep ridging and destoning. Planting was carried out using the SAC planter. Bed width for all trials was 1.8m and planting depth 13cm. Weed control, late blight control and aphid control were carried out as per standard practice. At Fingask, the crop protection applications were those applied to the adjacent farm field crop. Mocap 10G was applied at 100 kg/ha to the Woodlands beds prior to destoning in Woodlands field as low levels of PCN were present. Because of the limited number of mini-tubers available in each year, in the trials at Fingask only the centre two drills of mini-tubers plots were planted with mini-tubers. The outer two (guard) rows were planted with healthy commercial seed of the same variety.

In each trial there were four replicate blocks. The agronomy of each trial was appropriate for ware production. In the first two years, the variety Sante was used

for trials. This variety had shown susceptibility to *R. solani* (AG3) all growth stages in previous studies. However, in year 3, mini-tubers of Sante were unobtainable and the variety Markies was substituted.

After emergence in each trial, sub-plots were marked out in each plot. Where plots were 4 drills wide, sub-plots were marked out in only the inner two rows. For final harvesting a section of two drills was marked out at one end in each plot. In the Woodlands field this comprised 4 plants per drill (total 8 plants) and at Fingask 5 plants per drill (total 10 plants). The two trials at Fingask and the 2011 Woodlands field trial had two harvesting sub-plots marked out in each plot, one each for an 'early' and 'late' harvest. In the 2009 Woodlands field trial, separate plots were allocated for 'late' harvesting and in the 2010 Woodlands field trial there was a single harvest date.

From the remainder of each plot, further sub-plots were marked for sequential destructive sampling. These destructive sampling sub-plots comprised paired plants across two drills. In the scoping trial in Woodlands in 2009, the sub-plots comprised two pairs of plants but in subsequent years they comprised a single pair of plants. Each sub-plot (for harvest or destructive sampling) was bordered along each drill by a pair of guard plants. The destructive sampling sub-plots were randomly assigned for sampling at different stages of growth.

Timing of haulm destruction was pre-determined in each trial (Table 10) and was achieved using two applications of diquat (200g/litre as bromide - Reglone, Syngenta Crop Protection - 1.5 l product/ha and 2.5 l product/ha in 200 l/ha water) 5 days apart.

Treatments

Treatments changed slightly from year to year and are shown in Table 9. All trials contained an untreated control and Amistar (azoxystrobin 250 g/l; Syngenta Crop Protection) soil treatment. The soil treatment was applied in-furrow using backward and forward facing nozzles at the dose of 3 l product/ha in 100 l/ha solution. Monceren DS (pencycuron 12.5%, Bayer CropScience) seed treatment was applied just prior to planting by placing the seed to be treated in a paper bag, adding the appropriate dose of seed treatment and gently rolling the seed with the powder. Monceren DS was applied at 2 kg product/t in 3 of 5 trials and 1 kg/t in one trial.

Additional nitrogen was tested as a treatment in the 2010 Fingask trial. 50 kg/ha N was applied in addition to the standard fertiliser programme on 30 June.

Applying irrigation according to a weekly schedule was carried out for specific treatments in both Fingask trials. Rainfall and soil moisture were monitored on a weekly basis from emergence and water applied by trickle irrigation when soil moisture deficits were above 15mm for a 6 week period after tuber initiation and above 40mm subsequently. The irrigation applied is shown in Table 11.

Table 9. Summary of treatments applied in 5 trials evaluating control of *R. solani*

	2009 Woodlands	2010 Woodlands	2010 Fingask[2]	2011 Woodlands	2011 Fingask[3]
Untreated	+ (*)	+	+ (*)	+ (*)	+ (*)
Amistar soil treatment (3 l/ha)	+	+	+ (*)	+ (*)	+ (*)
Monceren seed treatment (2 kg/t)	+	+ (1)		+ (*)	+ (*)
Monceren seed (2 kg/t) treatment + Amistar soil treatment (3 l/ha)		+ (1)		+ (*)	
Added inoculum	+				
Extra nitrogen			+ (*)		
Irrigation			+ (*)		+ (*)

(*) indicates treatments where early and late harvests were made
(1) indicates seed treatment was applied at 1 kg/ha
[2] *R. solani* AG3 inoculum prepared by addition of 1 mycelial agar plate per 8kg sieved field soil – the inoculum was applied evenly to every plot at the rate of 2kg/plot.
[3] *R. solani* AG3 inoculum prepared as a mycelial solution and applied evenly onto commercial seed tubers- referred to as CS+ in the results tables.

Assessments on destructive samples from sub-plots
In each trial the programme of sampling was pre-determined. The sampling programme for each trial is shown in Table 10.

At each time of sampling in the trials in Woodlands field, the level of *R. solani* inoculum in soil around the developing daughter tubers was measured using PCR soil testing and the occurrence of *R. solani* symptoms assessed on roots, stems, stolons and daughter tubers. The symptoms assessed depended on the stage of crop growth. At the time of sampling, a minimum of 50g of soil was taken from region of the daughter tubers of each plant. Soil from each plant sampled across all plots of a treatment was aggregated and thoroughly mixed. This soil was tested for contamination by *R. solani* (AG3) using PCR. Where soil was not tested immediately, it was frozen until testing was possible. On two occasions in the 2011 Woodlands trial, soil was also tested for the presence of *R. solani* using a baiting technique described by Kyritsis (2003).

Sampling of plants in sub-plots was carried out carefully to preserve as much of the root and haulm tissue as possible. All tubers, irrespective of size were harvested on pre-harvest sampling occasions in each trial. Thereafter, only those greater than 25mm were retained for assessment at final harvests.

Table 10. Programme of pre-harvest destructive and harvest sampling for 5 trials evaluating control of *R. solani* (AG3)

Weeks after planting	Approximate growth stage	2009 Wood-lands	2010 Wood-lands	2010 Fingask	2011 Wood-lands	2011 Fingask
0	Planting date	29-May-09	25-May-10	03-Jun-10	02-May-11	20-May-11
4	50% emergence	3-Jul-09	21-Jun-10	29-Jun-10	7-Jun-11	28-Jun-11
6	50% emergence + 2 weeks				20-June-11	12-Jul-11
8	50% emergence + 4 weeks Early tuber bulking	30-Jul-09	20-Jul-10	27-Jul-10	4-Jul-11	26-Jul-11
10	50% emergence + 6 weeks		2-Aug-10	9-Aug-10	19-Jul-11	8-Aug-11
12	50% emergence + 8 weeks Flowering	26-Aug-09	17-Aug-10	23-Aug-10	1-Aug-11	23-Aug-11
14	50% emergence + 10 weeks		31-Aug-10	6-Sep-10		
16	50% emergence + 12 weeks Haulm destruction	16-Sep-09	13-Sep-10	22-Sep-10	15-Aug-11	5-Sep-11
20	Early Harvest - 4 weeks after Haulm destruction	14-Oct-09	25-Sep-10	05-Oct-10	19-Sep-11	03-Oct-11
22	Late harvest - 6 weeks after Haulm destruction	28-Oct-09		26-Oct-10	20-Oct-11	20-Oct-11

Emergence was monitored regularly in each trial to ascertain 50% emergence, when first destructive sampling took place in each trial. Thereafter crop vigour (on a 0-9 scale) and ground cover growth (as % ground cover) were assessed on the final harvesting area of plots.

Symptoms of *R. solani* infection of root, stems, stolons and daughter tubers were assessed visually. Normally, all sampled plant parts were carefully washed before assessment. Root browning was assessed as an index with browning categorised as 0, 1, 2 or 3 in severity (nil, slight, moderate and severe) and the index calculated using the formula below. Sometimes the extent of browning was also assessed as a percentage of the root system. Every stem and stolon on every plant was assessed for presence of canker symptoms. Again the infection was assessed into four categories and the index calculated using the formula below.

Disease Index (for root infection and stem or stolon canker) =

$$\frac{(\text{Total in category 1} \times 1) + (\text{Total in category 2} \times 2) + (\text{Total in category 3} \times 3)}{\text{Total number of roots, stems or stolons assessed}}$$

The percentage of stems or stolons exhibiting canker was also determined during assessment. The number of stolons showing pruning was counted on each plant

Every tuber harvested on every plant in pre-harvest destructive sampling was assessed for black scurf (% tubers infected and surface area infection) and for recognised symptoms accredited to infection by *R. solani* (elephant hide, dry core, cracking). Descriptions and photos of these symptoms used to assist assessment are shown in a separate document. Elephant hide was assessed as presence/absence and tubers with symptoms were visually placed into three categories. An index of Elephant hide symptoms was calculated using the formula above. Dry core and cracks were assessed as present or absent.

All tubers over 25mm in the harvest sub-plots were lifted. The yield and tuber numbers were determined as a total and in each of four grading fractions (<45mm, 45-65mm, 65-85mm and >85mm).

At each trial site meteorological data was recorded from after planting until harvest. Measurements recorded included precipitation (daily total), air temperature (daily maximum & minimum), soil temperature at tuber depth (continuous measurement) and, where facilities permitted, soil moisture content at tuber depth (continuous measurement)

Table 11. Timing and amount of irrigation applied to trials at Fingask

Fingask 2010		Fingask 2011	
Date	Amount applied (mm)	Date	Amount applied (mm)
5 July	25	13 June	25.2
13 July	17.5	21 June	20.4
14 July	8.3	29 June	14.4
19 July	19.2	12 July	25.2
		27 July	26.4

Results

Environmental conditions

Figures summarising the environmental data recorded during the 5 trials are provided in Figures 22 – 26 below.

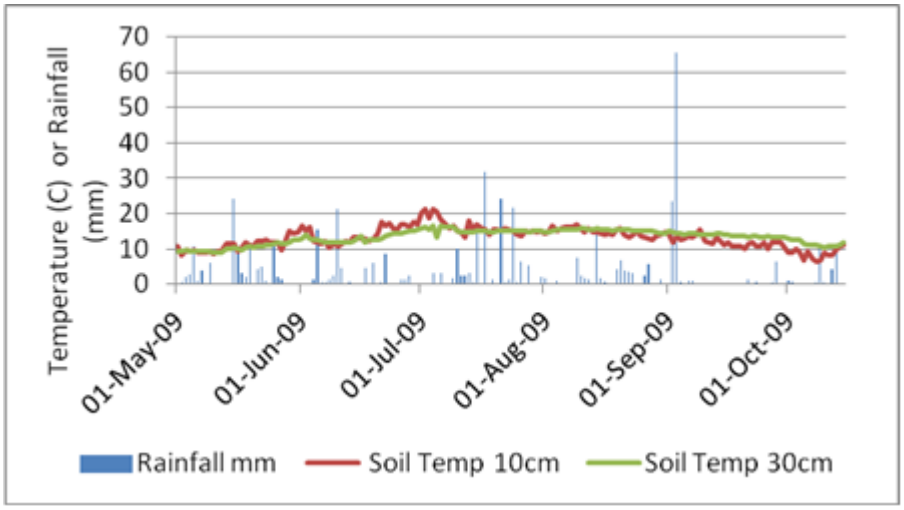


Figure 22. Soil temperatures and rainfall at the Woodlands site in 2009

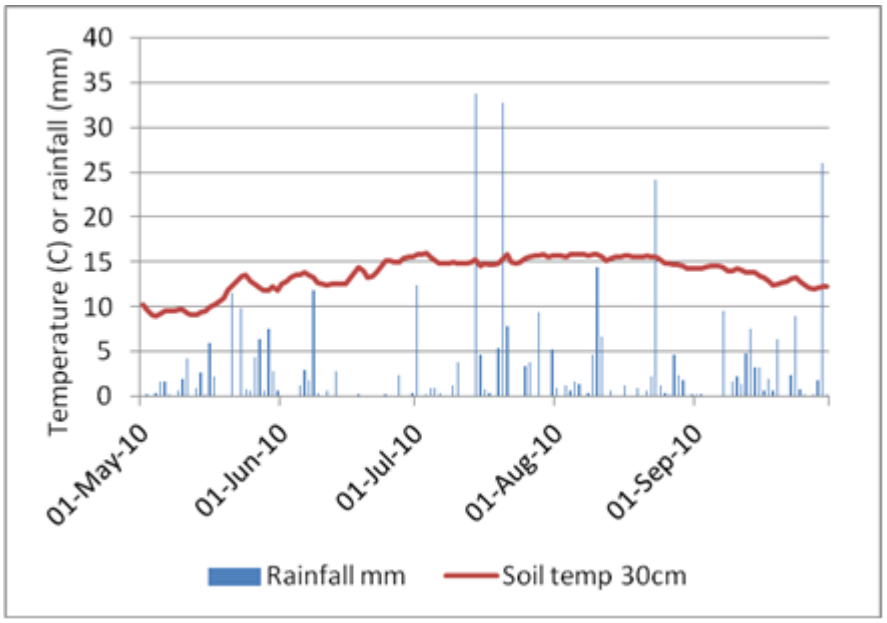


Figure 23. Soil temperature and rainfall at the Woodlands site in 2010

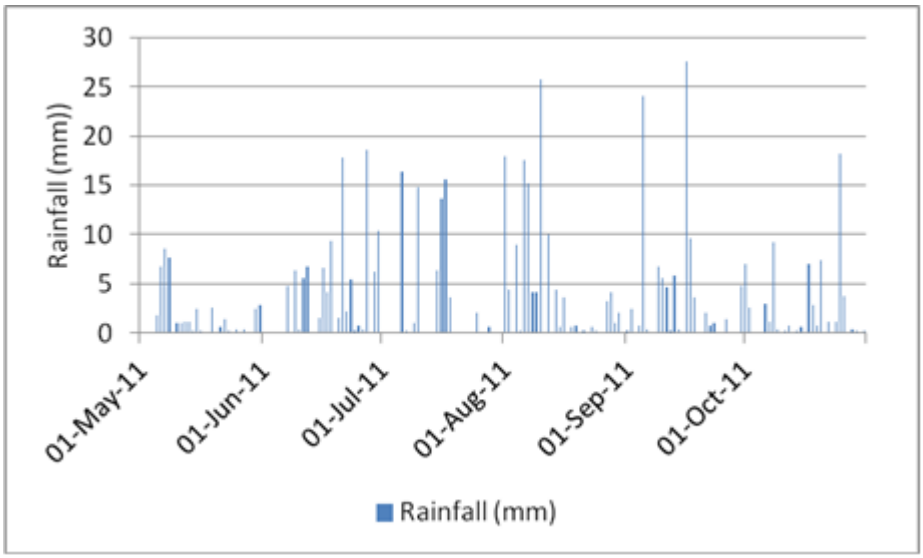


Figure 24. Rainfall at the Woodlands site in 2011

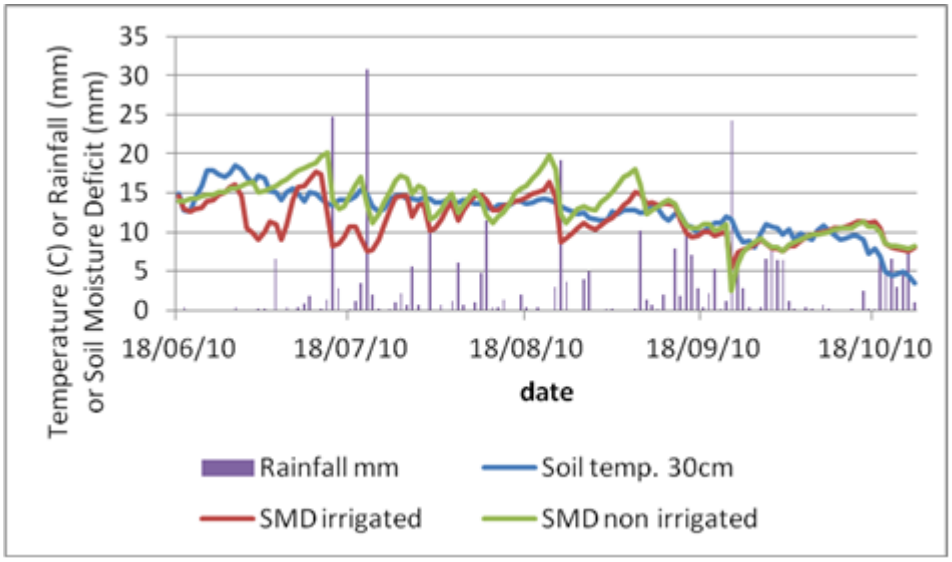


Figure 25. Soil temperature; rainfall; and SMD in irrigated and un-irrigated treatments at the Fingask site in 2010.

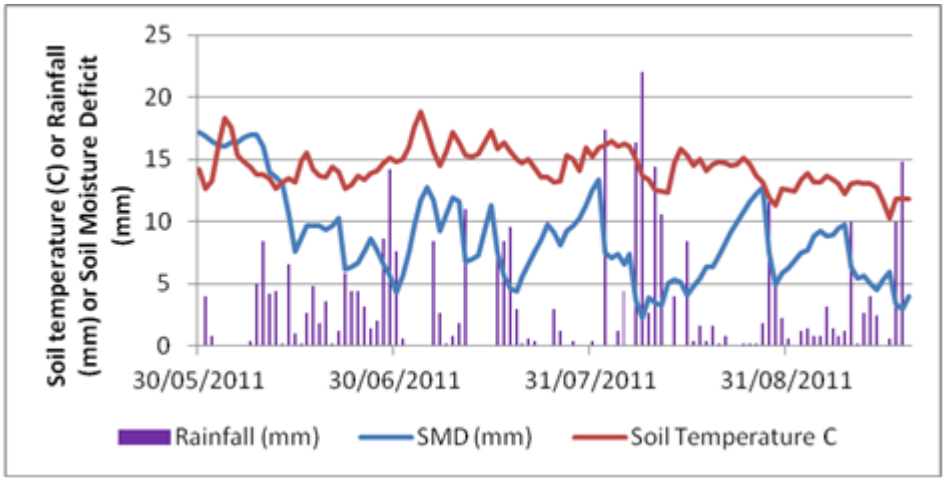


Figure 26. Soil temperature; rainfall; and SMD at the Fingask site in 2011.

In all three seasons, there was a period of weather where rainfall was above average. In each year, this period started in mid-summer (June or July) and continued until September (2009) or until after haulm destruction (2010 & 2011). The periodic saturated soils that resulted from heavy rainfall affected late development of *R. solani* (AG3) and symptoms caused by the pathogen were generally lower than anticipated. By contrast, the early stages of growth took place under generally good growing conditions, warm soil temperatures and sufficient rain to optimise crop growth.

Soil inoculum levels prior to planting

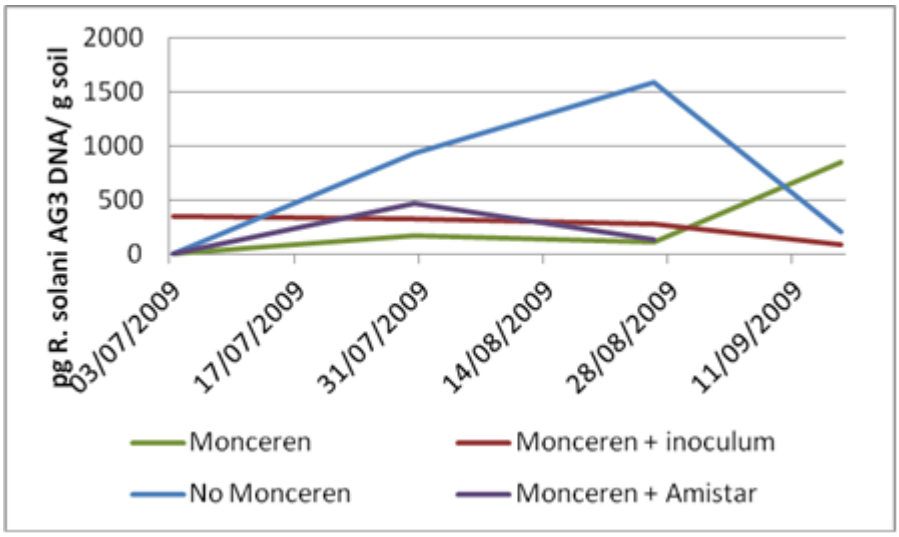
Inoculum of *R. solani* AG3 was detected prior to planting (and before incorporation of the *R. solani* AG3 inoculum) in just 2 out of 5 trial sites (Table 8). In the Woodlands trial in 2009 where 2 beds were used for the trial, soil inoculum was 11,308 and 1,386 pg DNA/g soil respectively. In the Woodlands 2011 trial, the inoculum was determined as 4,206 pg DNA/g soil. These levels of inoculum were considerably greater than all but one pre-planting levels detected elsewhere in this project in field soils (see Table 45).

Plant sampling
Whilst ground cover and vigour changed over time, in all five trials there were no significant differences detected between treatments (see Appendix 4).

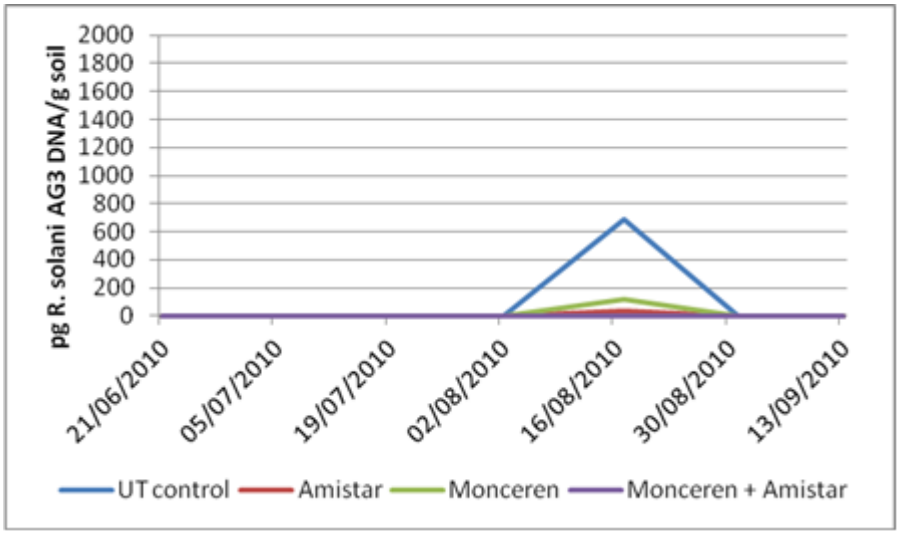
Destructive sampling for below ground disease assessments relied on 2 (or 4 in the Woodlands 2009 trial) plants per plot. It was found throughout that variability as a result of assessing a small number of plants was high. It was impractical in terms of time and space to assess more plants since a number of sampling occasions was required. In the tables below, the results for a particular character have been analysed across all sampling occasions (ie up to 9 sampling occasions per year. See table 10 for number and timing of sampling in each year). This was the best way to limit the impact of variability in individual assessments and to improve the chance of detecting statistical differences.

Pathogen levels during the growing season
The pattern of detection of *R. solani* AG3 in soil from around the developing progeny tubers with time differed in each of the three Woodlands trials (Figure 20). In 2009, the pathogen was detected in the one treatment where inoculum had been applied at the first sampling date (emergence) but not in the other treatments. However, in this treatment the level of inoculum remained relatively constant thereafter. At the second date of sampling the pathogen was detected in all treatments and was at the highest levels in the untreated. In the untreated control, the inoculum continued to rise at the third sampling date but declined thereafter. Inoculum levels in the Monceren; and Monceren + Amistar treatments were similar to that of the Monceren + inoculum treatments from the second sampling date onwards, rising above the other treatments at the last sampling date. By complete contrast in 2010, inoculum was only detected at one of 7 sampling dates, with the untreated control having the highest level of contamination. In 2011, inoculum was detected at the early stages of growth with, again the untreated control having the highest level of pathogen detected. By mid July, the pathogen was almost undetected in any treatment.

2009



2010



2011

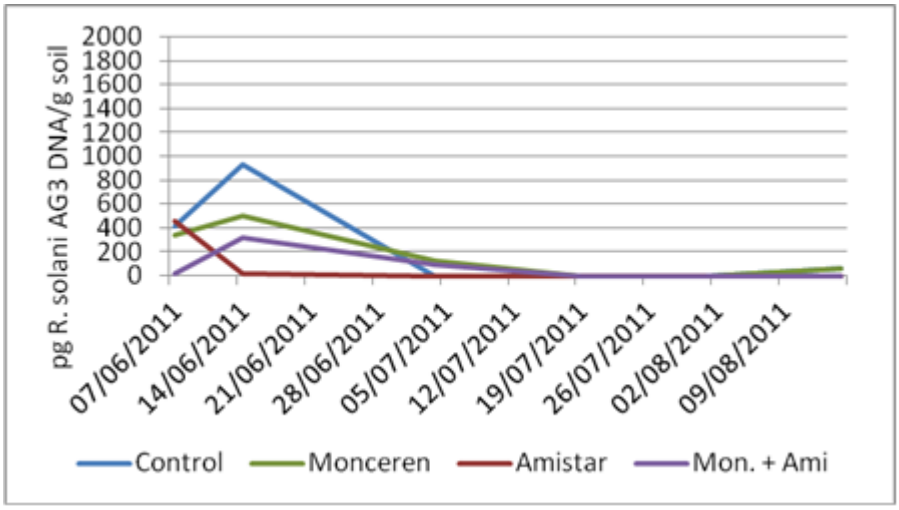


Figure 27. Detection of soil-borne *R. solani* (AG3) in soil in the root zone of plants from each treatment in the Woodlands field trial in 2009, 2010 and 2011, respectively.

Disease symptoms during the growing season

In the case of some tubers symptoms (elephant hide, cracking and dry core) there may only be slight differences in the appearance of symptoms caused by *R. solani* and those caused by other factors (*Streptomyces* spp. infection, irregular soil moisture, and wireworm infection, respectively). Therefore in the section below data on the levels of these symptoms in the five field trials are provided, however the Discussion section provides additional comment on whether the symptoms were likely to be due to *R. solani* infection or some other factor.

In all the tables below LSD values are reported at the 5% probability level. In each of the Fingask trials there were insufficient mini-tubers to apply all treatments planned. Thus, in each year one treatment was omitted from the mini-tuber stock. This meant that for the two trials, two statistical analyses were carried out; one including all stocks grown (commercial seed + mini-tubers) but across only those treatments common to all stocks; and a second where all treatments were included but only for those stocks in which all treatments occurred.

Stem canker

In all three years and in all trials, the period from planting to 50% emergence took between 4 and 5 weeks. This relatively rapid period to emergence restricted infection of stems and average stem canker levels were modest (Table 12).

Table 12. Average stem canker, expressed as an Index (0-3), across all dates of assessment in each of five field trials.

Site	Woodlands			Fingask					
Year	2009	2010	2011	2010			2011		
Soil inoculum level (pg *R. solani* AG3 DNA/g soil)	11,308 1,386	0	4,206	0			0		
Treatment/Seed stock				MT	CS	CSM	MT	CS	CS+
Untreated Control (No Monceren)	1.3	1.7	0.9	1.17	0.42	0.42	0.44	0.81	0.77
Amistar		1.0	0.5	1.25	0.33	0.68	0.43	0.71	0.81
Monceren	1.3	1.5	1.1				0.40	0.69	0.81
Monceren + inoculum	1.1								
Monceren + Amistar	1.2	1.3	0.8						
Extra N				-	0.78	0.77			
Irrigation				1.17	0.79	0.53	-	0.94	0.85
LSD Treatment	0.23	0.38***	0.41	0.225**/0.189			0.245/0.205		
LSD Date of assessment	0.21[3*]	0.42***	0.46*	0.251**/0.244***			0.300/0.205		
LSD Seed Source	-	-	-	0.159/0.189***			0.173/0.205		

MT=mini-tubers; CS=commercial seed; CSM=commercial seed + Monceren; CS+=commercial seed + inoculum.

LSD's for Fingask are for Commercial seed and all treatments / All seed sources but treatments without extra nitrogen

The data shown are the mean treatment values across a series of assessments made at different dates. In the ANOVA because there were replicates at each assessment, it was possible to analyse for significant differences in mean treatment value over time. The 3* significance value means that across the dates of assessment there were significant differences i.e. overall stem canker increased over time significantly – as suggested in Figure 28 (see below).

Stem canker index significantly increased with time in 4 out of 5 trials. An illustration of this is shown in Figure 28. This shows the fluctuation in incidence of stem canker index in the Woodlands 2011 trial across six assessment dates with a trend-line superimposed. The variation in incidence or index was ascribed to high variability between plants.

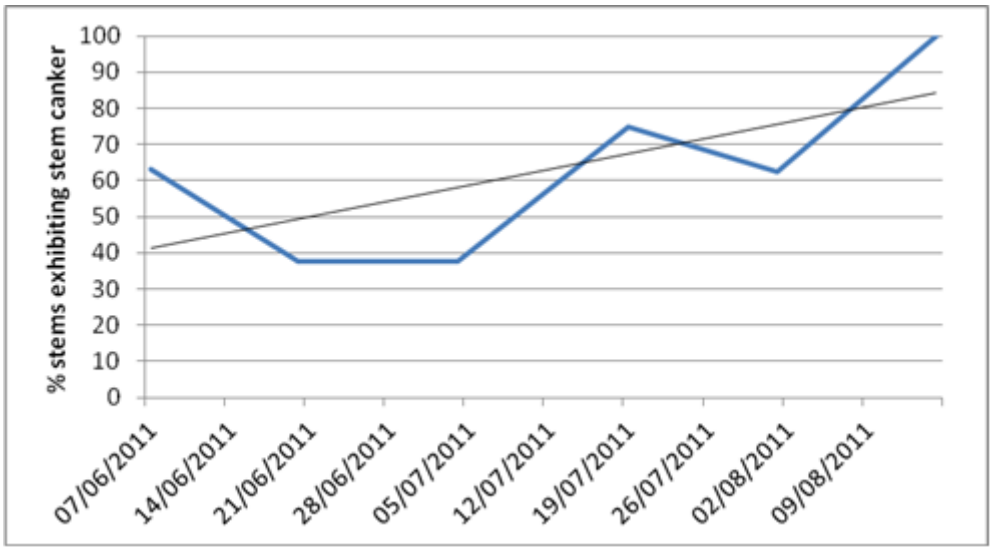


Figure 28. Fluctuation in stem canker incidence in the untreated control over six assessments with time. A trendline is superimposed. Woodlands 2011.

Differences in stem canker index between treatments were only recorded in two trials (Table 12: Woodlands 2010 and Fingask 2010). In the Woodlands trial, Amistar treatments significantly reduced stem canker. However, in the Fingask trial, the irrigation and extra nitrogen treatments resulted in increased stem canker within the commercial seed stocks. There was also a significant increase in stem canker where mini-tubers were the seed source compared to commercial seed.

Stolon canker
There was a significant increase in stolon canker over time in all trials (Table 13).

Table 13. Average stolon canker, expressed as an Index (0-3), across all dates of assessment in each of five field trials.

Site	Woodlands			Fingask					
Year	2009	2010	2011	2010			2011		
Soil inoculum level (pg *R. solani* AG3 DNA/g soil	11308 1386	0	4206	0			0		
Treatment/Seed stock				MT	CS	CSM	MT	CS	CS+
Untreated Control (No Monceren)	2.0	0.6	0.73	0.14	0.22	0.04	0.23	0.71	0.71
Amistar		0.2	0.45	0.13	0.00	0.30	0.27	0.50	0.54
Monceren	2.0	0.5	0.93				0.31	0.60	0.48
Monceren + inoculum	1.4								
Monceren + Amistar	1.3	0.2	0.53						
Extra N				-	0.08	0.20			
Irrigation				0.19	0.38	0.14	-	0.85	0.94
LSD Treatment	0.35***	0.29**	0.33*	0.149/0.116			0.211**/0.218		
LSD Date of assessment	0.27*	0.33**	0.36***	0.166/0.149**			0.258***/0.154***		
LSD Seed Source	-	-	-	0.105/0.116			0.149/0.154		

MT=mini-tubers; CS=commercial seed, CSM=commercial seed + Monceren; CS+=commercial seed + inoculum. LSD's for Fingask are for Commercial seed and all treatments / All seed sources but treatments without extra nitrogen

The stolon canker index was greatest in the Woodlands 2009 trial, where the soil inoculum levels as measured by real-time PCR were also highest. In this trial the Monceren + inoculum and Monceren + Amistar treatments resulted in significantly less stolon canker (Table 13). Although by comparison stolon canker was more modest in the other trials, significant treatment effects were also evident in the Woodlands 2010 & 2011 trials where those treatments in which Amistar was applied had lower stolon canker indices. A significant treatment effect was also observed in the Fingask 2011 trial where the irrigation treatment significantly increased stolon canker over the Amistar and Monceren treatments in the commercial seed stocks.

Dry core
Of the symptoms on tubers considered to be caused by *R. solani* (AG3), dry core occurred at more than trace levels in three trials (Table 14). In these, the level of dry core significantly increased over time. There were almost no differences between treatments in any trial, the only significant difference being the irrigation treatment at Fingask 2010 which resulted in less dry core than the untreated control on commercial seed stocks. Overall fewer tubers, from both Fingask and Woodlands, appeared to be affected by dry core in 2011 than 2010. This may be due to differences in susceptibility between Sante (2010 trials) and Markies (2011 trials).

Table 14. Average dry core, expressed as % tubers affected, across all dates of assessment in each of five field trials.

Site	Woodlands			Fingask					
Year	2009	2010	2011	2010			2011		
Soil inoculum level (pg *R. solani* AG3 DNA/g soil	11308 1386	0	4206	0			0		
Treatment/Seed stock				MT	CS	CSM	MT	CS	CS+
Untreated Control (No Monceren)	8	15	0	18	19	25	0.0	0.4	0.5
Amistar		15	0	21	19	17	0.0	0.7	0.2
Monceren	10	13	0				0.0	0.7	0.4
Monceren + inoculum	13								
Monceren + Amistar	10	10	0						
Extra N				-	17	18			
Irrigation				14	14	16	-	0.0	0.0
LSD Treatment	2.7	6.93	-	4.1*/3.5			0.68/0.69		
LSD Date of assessment	2.1***	7.74***	-	4.6***/4.6***			0.76**/0.53**		
LSD Seed Source	-	-	-	2.9/3.5			0.48/0.53		

MT=mini-tubers; CS=commercial seed, CSM=commercial seed + Monceren; CS+=commercial seed + inoculum. LSD's for Fingask are for Commercial seed and all treatments / All seed sources but treatments without extra nitrogen

Cracking

The degree of cracking as a result of *R. solani* (AG3) infection varied between trials (Table 15). There was evidence that cracking increased with crop development. In some trials, control treatments reduced cracking but this was not consistent. In the Woodlands 2010 trial, Amistar reduced cracking and in the 2010 Fingask trial, irrigation significantly reduced cracking.

Table 15. Average cracks, expressed as % tubers affected, across all dates of assessment in each of five field trials.

Site	Woodlands			Fingask					
Year	2009	2010	2011	2010			2011		
Soil inoculum level (pg *R. solani* AG3 DNA/g soil	11308 1386	0	4206	0			0		
Treatment/Seed stock				MT	CS	CSM	MT	CS	CS+
Untreated Control (No Monceren)	2.5	13.5	2.6	6.5	2.6	4.8	1.25	0.3	1
Amistar		6.5	2.0	4.3	6.4	6.9	1.83	1.1	0.4
Monceren	1.4	12.0	4.6				1.54	1.4	1.8
Monceren + inoculum	1.3								
Monceren + Amistar	1.8	5.5	1.5						
Extra N				-	2.4	2.9			
Irrigation				1.8	2.0	2.6	-	1.2	1.4
LSD Treatment	1.82	6.31*	1.62	2.29***/2.04***			1.35/1.76		
LSD Date of assessment	1.41	7.06***	1.62(*)	2.56***/2.64***			1.51/1.36**		
LSD Seed Source	-	-	-	1.62/2.04			0.95/1.36		

MT=mini-tubers; CS=commercial seed, CSM=commercial seed + Monceren; CS+=commercial seed + inoculum. LSD's for Fingask are for Commercial seed and all treatments / All seed sources but treatments without extra nitrogen

Elephant Hide

Elephant hide symptoms increased with time (usually significantly) in all trials (Table 16).

Table 16. Average elephant hide, expressed as % tubers affected, across all dates of assessment in each of five field trials.

Site	Woodlands			Fingask					
Year	2009	2010	2011	2010			2011		
Soil inoculum level (pg *R. solani* AG3 DNA/g soil	11308 1386	0	4206	0			0		
Treatment/Seed stock				MT	CS	CSM	MT	CS	CS+
Untreated Control (No Monceren)	49	51	14	47	42	45	17	19	14
Amistar		46	12	35	41	40	12	17	17
Monceren	46	48	20				15	14	12
Monceren + inoculum	39								
Monceren + Amistar	41	46	14						
Extra N				-	42	37			
Irrigation				32	33	36	-	18	14
LSD Treatment	7.6*	9.44	5.31	5.2*/4.4***			5.0/6.0		
LSD Date of assessment	5.9***	10.56***	5.31**	5.8***/5.7***			5.6***/4.7***		
LSD Seed Source	-	-	-	3.7/4.4			3.5/4.7		

MT=mini-tubers; CS=commercial seed, CSM=commercial seed + Monceren; CS+=commercial seed + inoculum. LSD's for Fingask are for Commercial seed and all treatments / All seed sources but treatments without extra nitrogen

Whilst differences were recorded (e.g. a reduction over the untreated control where inoculum was added at Woodlands 2009, and a reduction as a result of irrigation at Fingask 2010), there were no consistent effects of any treatment where percentage tubers affected by elephant hide were assessed. When severity was considered, significant reductions over the untreated control were present in the 2009 Woodlands trial by the Monceren + inoculum and Monceren + Amistar treatments. In other trials, whilst control treatments reduced severity, the reduction was not significant.

Stolon Pruning

Stolon pruning increased with time in all trials, mostly significantly (Table 17). Treatment effects were rarely significant, although Amistar treatment consistently reduced stolon pruning.

Table 17. Average stolon pruning, expressed as % tubers, across all dates of assessment in each of five field trials.

Site	Woodlands			Fingask					
Year	2009	2010	2011	2010			2011		
Soil inoculum level (pg *R. solani* AG3 DNA/g soil	11308 1386	0	4206	0			0		
Treatment/ Seed stock				MT	CS	CSM	MT	CS	CS+
Untreated Control (No Monceren)	1.6	1.1	1.3	0.1	0.3	0.5	0.3	1.4	1.7
Amistar		0.2	0.9	0.3	0.2	0.7	0.1	1.1	0.9
Monceren	1.6	0.7	2.5				0.6	1.5	1.5
Monceren + inoculum	1.0								
Monceren + Amistar	1.2	0.4	0.9						
Extra N				-	0.6	0.5			
Irrigation				0.2	0.4	0.4	-	2.2	1.7
LSD Treatment	0.80	0.41	1.33	0.22/0.17			0.67*/0.58*		
LSD Date of assessment	1.03***	0.46	1.49**	0.25***/0.22***			0.75***/0.45***		
LSD Seed Source	-	-	-	0.16/0.17***			0.47/0.5		

MT=mini-tubers; CS=commercial seed, CSM=commercial seed + Monceren; CS+=commercial seed + inoculum. LSD's for Fingask are for Commercial seed and all treatments / All seed sources but treatments without extra nitrogen

Root Browning

Root browning (Table 18) was present in all trials but at generally low levels. The level of browning increased with time, often significantly. Significant differences between treatments were evident in the Fingask trials. Here nitrogen (2010) and irrigation (2011) increased root browning over other treatments.

Table 18. Average root browning, expressed as % root browning, across all dates of assessment in each of five field trials.

Site	Woodlands			Fingask					
Year	2009	2010	2011	2010			2011		
Soil inoculum level (pg *R. solani* AG3 DNA/g soil	11308 1386	0	4206	0			0		
Treatment/ Seed stock				MT	CS	CSM	MT	CS	CS+
Untreated Control (No Monceren)	0.3	0.13	0.15	-	0.2	0.2	0.06	0.23	0.19
Amistar		0.15	0.2	-	0.1	0.1	0.06	0.13	0.21
Monceren	0.5	0.15	0.15				0.07	0.04	0.17
Monceren + inoculum	0.3								
Monceren + Amistar	0.3	0.03	0.08						
Extra N				-	0.4	0.3			
Irrigation				-	0.1	0.2	-	0.27	0.33
LSD Treatment	0.26	0.140	0.086	0.13**/-			0.109**/0.112		
LSD Date of assessment	0.20[3*]	0.157	0.097[3*]	0.14***/-			0.133[3*]/0.080[3*]		
LSD Seed Source	-	-	-	0.09/-			0.077/0.080		

Black Scurf

Black scurf was rarely recorded on progeny tubers before haulm destruction. However, in the Woodlands 2009 trial it was present 8 weeks after emergence in mid-August. In this trial, where infected seed was planted, the incidence and severity of black scurf were significantly reduced by Monceren + inoculum and Monceren + Amistar treatments over the No Monceren or Monceren only treatments (Table 19).

Table 19. Woodlands 2009: Black scurf assessments during crop growth

Treatment	Black scurf incidence % tubers	Black scurf severity Index
Monceren	14	0.42
Monceren + inoculum	1	0.02
Monceren + Amistar	4	0.12
Untreated control (No Monceren)	19	0.52
LSD Treatment	6.6***	0.238***
LSD Date of assessment	5.1***	0.184***
LSD Treat x Date	11.4***	0.411**

Disease symptoms at harvest

Elephant Hide

The symptoms ascribed to *R. solani* at harvest were at broadly similar levels to those assessed during growth. With elephant hide (Table 20 v Table 16) there was considerable variability in results and no consistent effect of treatment. In general, elephant hide increased slightly with delayed harvest. This effect was only significant in the Fingask 2011 trial.

Table 20. Average elephant hide at harvest (first /second harvest), expressed as % tubers, across all dates of assessment in each of five field trials.

Site	Woodlands			Fingask					
Year	2009	2010	2011	2010			2011		
Soil inoculum level (pg *R. solani* AG3 DNA/g soil	11308 1386	0	4206	0			0		
Treatment/ Seed stock				MT	CS	CSM	MT	CS	CS+
Untreated Control (No Monceren)	48/-	68/-	24/39	38/44	23/24	23/24	52/75	29/40	45/47
Amistar		53/-	31/34	27/32	18/25	22/27	48/47	31/48	46/48
Monceren	45/-	69/-	30/37				47/38	30/43	34/43
Monceren + inoculum	51/-								
Monceren + Amistar	66/-	59/-	15/42						
Extra N				-/-	23/29	20/30			
Irrigation				34/30	22/19	21/23	-	35	27
LSD Treatment	17.9/-	14.3/-	28.1/31.1	6.1/5.3			8.1/7.8		
LSD Date of assessment				4.3/4.4			5.7**/6.3*		
LSD Seed Source	-	-	-	4.3/5.3			5.7/7.8**		

MT=mini-tubers; CS=commercial seed, CSM=commercial seed + Monceren; CS+=commercial seed + inoculum. LSD's for Fingask are for Commercial seed and all treatments / All seed sources but treatments without extra nitrogen

Dry Core

There was a suggestion in the dry core assessments at harvest that delaying harvest reduced symptoms. The only treatment effect on dry core was a significant reduction as a result of irrigation in the Fingask 2010 trial .(Table 21)

Table 21. Average dry core at harvest (first /second harvest), expressed as % tubers,across all dates of assessment in each of five field trials.

Site	Woodlands			Fingask					
Year	2009	2010	2011	2010			2011		
Soil inoculum level	11308 1386	0	4206	0			0		
Treatment/ Seed stock				MT	CS	CSM	MT	CS	CS+
Untreated Control (No Monceren)	10/-	1.3/-	1.2/1.0	14.7/ 5.7	9.8/ 8.9	6.0/ 9.5	9.6/ 0.8	8.2/ 1.0	4.9/ 1.9
Amistar		1.1/-	1.0/1.0	16.0/ 8.7	6.3/ 6.4	8.6/ 9.5	9.7/ 0.6	8.7/ 2.6	4.9/ 1.7
Monceren	10/-	1.5/-	0.0/1.8				7.2/ 4.9	2.6/ 3.4	5.5/ 3.0
Monceren + inoculum	10/-								
Monceren + Amistar	9/-	3.2/-	0.0/0.0						
Extra N				-/-	8.0/ 5.2	9.5/ 8.4			
Irrigation				6.3/ 4.5	3.9/ 5.4	5.2/ 7.6	-/-	5.6/ 1.9	4.7/ 3.8
LSD Treatment	12.5/-	5.01/-	2.9/5.2	2.96/2.60**			2.11/2.95		
LSD Date of assessment	-	-	-	2.10/2.12			1.49***/2.41***		
LSD Seed Source	-	-	-	2.10/2.60			1.49/2.95		

Cracking

Cracking was at generally low levels throughout all trials (Table 22). As a result of considerable variability, there was an inconsistent effect of delayed harvest on levels of cracking.

Table 22. Average cracking at harvest (first /second harvest), expressed as % tubers, across all dates of assessment in each of five field trials.

Site	Woodlands			Fingask					
Year	2009	2010	2011	2010			2011		
Soil inoculum level	11308 1386	0	4206	0			0		
Treatment/ Seed stock				MT	CS	CSM	MT	CS	CS+
Untreated Control (No Monceren)	4/-	4.5/-	0.0/5.1	0.1/ 0.6	0.0/ 9.1	0.7/ 1.4	0.0/ 0.7	1.0/ 1.5	1.3/ 3.9
Amistar		0.6/-	1.0/1.0	0.9/ 0.0	1.1/ 0.4	1.6/ 0.0	11.8/ 3.8	0.5/ 3.2	2.9/ 2.3
Monceren	1/-	1.7/-	2.7/2.0				1.0/ 2.1	3.4/ 2.8	2.9/ 1.4
Monceren + inoculum	5/-								
Monceren + Amistar	5/-	1.9/-	1.0.2.1						
Extra N				-/-	0.0/ 0.0	1.1/ 0.4			
Irrigation				0.5/ 0.0	0.0/ 0.0	0.0/ 0.5	-/-	4.4/ 4.1	3.6/ 1.3
LSD Treatment	7.6/-	3.88/-	5.6/5.6 7	2.54/2.01			1.97/2.00*		
LSD Date of assessment	-	-	-	1.80/1.64			1.39/1,64		
LSD Seed Source	-	-	-	1.8/2.01			1.39/2.00		

MT=mini-tubers; CS=commercial seed, CSM=commercial seed + Monceren; CS+=commercial seed + inoculum. LSD's for Fingask are for Commercial seed and all treatments / All seed sources but treatments without extra nitrogen

Black Scurf

Overall, there were low levels of black scurf at harvest, except in Woodlands 2009 (Tables 23 and 24). Here black scurf incidence was significantly reduced by the Monceren + inoculum and Monceren + Amistar treatments. The pattern was similar with severity but reductions just failed to reach significance.

Table 23. Average black scurf incidence on progeny tubers at harvest (first /second harvest), expressed as % tubers infected, across all dates of assessment in each of five field trials**.**

Site	Woodlands			Fingask					
Year	2009	2010	2011	2010			2011		
Soil inoculum level (pg *R. solani* AG3 DNA/g soil	11308 1386	0	4206	0			0		
Treatment/ Seed stock				MT	CS	CS M	MT	CS	CS+
Untreated Control (No Monceren)	88/-	7/-	58/52	0/0	1/14	0/0	24/44	19/14	22/26
Amistar		4/-	58/48	0/0	0/0	0/0	6/4	0/0	0/0
Monceren	77/76	9/-	39/61				23/25	0.5/0	18/18
Monceren + inoculum	32/-								
Monceren + Amistar	26/-	3/-	25/35						
Extra N				-/-	0/0	0/0			
Irrigation				0/0	0/0	1/0	-/-	1.8/13	1/20
LSD Treatment	36.3**/ -	13.0/ -	61.8 /71.7	5.11 /4.01			16.1 /17.0		
LSD Date of assessment	-	-	-	3.61 /3.28			11.4 /13.9		
LSD Seed Source	-	-	-	3.61 /4.01			11.4/ 17.0		

MT=mini-tubers; CS=commercial seed, CSM=commercial seed + Monceren; CS+=commercial seed + inoculum. LSD's for Fingask are for Commercial seed and all treatments / All seed sources but treatments without extra nitrogen

Table 24. Average black scurf on progeny tubers at harvest (first /second harvest), expressed as an Index (0-3, % surface area in 2009), across all dates of assessment in each of five field trials.

Site	Woodlands			Fingask					
Year	2009	2010	2011	2010			2011		
Soil inoculum level (pg *R. solani* AG3 DNA/g soil	11308 1386	0	4206	0			0		
Treatment/ Seed stock				MT	CS	CSM	MT	CS	CS+
Untreated Control (No Monceren)	6.2/-	0.4/-	0.6/0.6	0/0	0/1.9	0/0	0.3/ 0.8	0.2/ 0.2	0.3/ 0.4
Amistar		0.3/-	0.6/0.5	0/0	0/0	0/0	0.1/ 0.0	0.0/ 0.0	0.0/ 0.0
Monceren	4.5/5.4	0.6/-	0.4/0.7				0.4/ 0.5	0.0/ 0.0	0.2/ 0.3
Monceren + inoculum	3.0/-								
Monceren + Amistar	1.1/-	0/-	0.3/0.4						
Extra N				-	0/0	0/0			
Irrigation				0/0	0/0	0/0	-	0.0/ 0.2	0.0/ 0.2
LSD Treatment	3.64/-	1.0/-	0.62/0.82	0.66 /0.52			0.24 /0.28*		
LSD Date of assessment	-	-	-	0.47 /0.42			0.17 /0.23		
LSD Seed Source	-	-	-	0.47 /0.52			0.17 /0.28		

MT=mini-tubers; CS=commercial seed, CSM=commercial seed + Monceren; CS+=commercial seed + inoculum. LSD's for Fingask are for Commercial seed and all treatments / All seed sources but treatments without extra nitrogen

Yield

There were few differences in total yield and total tuber number between treatments (Table 25). In the Woodlands 2010 trial, Monceren alone reduced yield significantly over the untreated control. In this trial all control treatments reduced tuber number over the untreated control (Table 26). In the 2010 Fingask trial, irrigation reduced yield and tuber number significantly over the untreated control and Amistar treatments.

Table 25. Total yield, expressed as t/ha, across two harvest dates (one harvest date for Woodlands 2009 & 2010) in each of five field trials.

Site	Woodlands			Fingask					
Year	2009	2010	2011	2010			2011		
Soil inoculum level	11308 1386	0	4206	0			0		
Treatment/ Seed stock				MT	CS	CSM	MT	CS	CS+
UT Control (No Monceren)	62.3	36.2	26.5	35.1	33.3	33.1	29.8	37.9	38.7
Amistar		34.7	26.5	34.1	34.6	36.6	27.4	37.9	38.9
Monceren	60.4	25.6	25.2				27.1	37.0	36.5
Monceren + inoculum	79.3								
Monceren + Amistar	61.8	28.2	26.4						
Extra N				-	32.6	33.2			
Irrigation				26.3	29.1	29.6	-	37.5	41.0
LSD Treatment	25.86	8.29*	5.50	3.59*/2.84***			3.14/3.65		
LSD Date of assessment	-	-	-	2.54***/2.32***			2.21/2.98		
LSD Seed Source	-	-	-	2.54/2.84			2.21/3.65		

Table 26. Tuber numbers, expressed as '000s/ha, across two harvest dates (one harvest date for Woodlands 2009 & 2010) in each of five field trials.

Site	Woodlands			Fingask					
Year	2009	2010	2011	2010			2011		
Soil inoculum level	11308 1386	0	4206	0			0		
Treatment/ Seed stock				MT	CS	CSM	MT	CS	CS+
UT Control (No Monceren)	639	267	223	271	325	333	227	387	438
Amistar		188	216	265	329	369	234	381	435
Monceren	623	160	227				245	381	410
Monceren + inoculum	613								
Monceren + Amistar	524	174	220						
Extra N				-	334	331			
Irrigation				212	314	309	-	393	421
LSD Treatment	119.8	70.7*	43.2	31.2/24.1**			37.5/26.4		
LSD Date of assessment	-	-	-	22.1***/19.7***			26.5/21.5		
LSD Seed Source	-	-	-	22.1/24.1***			26.5***/26.4		

MT=mini-tubers; CS=commercial seed, CSM=commercial seed + Monceren; CS+=commercial seed + inoculum. LSD's for Fingask are for Commercial seed and all treatments / All seed sources but treatments without extra nitrogen

Discussion

Although soil-borne *R. solani* (AG3) was detected in just two of the five trial sites pre-planting, disease developed in each trial. The sites were chosen either because soil-borne inoculum was detected or because its presence was suspected from the cropping history. The fact that disease developed in every trial in which mini tubers were used confirms that the pathogen was present in the soil and clearly either the sampling or the testing procedure were insufficient to confirm the pathogen's presence in three instances.

In each year of the trials, the rainfall during the cropping period was above average, particularly during the second half of crop growth. The impact of soil-borne *R. solani* (AG3) in causing disease is affected by environmental conditions. The pathogen is favoured by relatively dry and warm conditions and the wet seasons impacted on pathogen and disease development, particularly at the end of the season.

In the three Woodlands trials (2009, 2010, 2011), the changes in the level of soil inoculum during crop growth were followed by sampling soil around the developing tubers and using the real-time PCR test to determine the level of *R. solani* AG3. In each of the three trials the pattern of development of soil-borne inoculum was different. Only in the years when it was detected pre-planting (2009, 2011) was it present at the first sampling date. In the 2010 trial in Woodlands field, prior to which no inoculum was detected, the pathogen was only detected in the soil around developing tubers on one occasion (in mid August, 83 days after planting). In the last two years, the inoculum level was not detected after about 8 weeks post emergence. Kyritsis (2003) also followed levels of *R. solani* in soil during the cropping season using a non-AG-specific method and recorded a rise after haulm destruction. This suggested that development of the pathogen was favoured when plant tissue was

dying or when skins are setting and sclerotia are developing. Prior to that, levels of soil-borne inoculum were detectable and relatively stable. The results suggest that there was an interaction between the soil-inoculum and the weather conditions that influenced whether *R. solani* persisted, increased or decreased. In Kyritsis's study, soil conditions for both his field trials at and after haulm destruction were relatively dry and warm.

Kyritsis (2003) studied the impact of soil conditions (temperature and moisture) on infection on stem canker infection from soil-borne inoculum under controlled conditions. His studies, suggested that as soil became wetter, irrespective of temperature, stem canker declined. The work did not include a saturated soil treatment but by extrapolation it can be concluded that very wet conditions are not favourable to the pathogen. Ritchie (2006) studied the impact of environmental factors on the biology of *R. solani*. Mycelial growth, sclerotial biomass production and germination declined with decreasing osmotic, matric and soil water potential, with mycelial growth prevented between -3.5 MPa and -4. OMPa on osmotically adjusted media, at -2.0 MPa on matrically adjusted media and -6.3 MPa in soil. Commercial experience of low levels of black scurf in wet seasons also supports the contention that wet soil conditions limit the development of the pathogen.

The small sampling size on each sampling occasion, 2-4 plants per plot, resulted in considerable variability in assessments. This is, perhaps, unsurprising for a pathogen that in mycelial form survives on organic matter in soil and is thus un-evenly dispersed. Such variability means that significant differences are difficult to detect. Larger sampling units would be advisable in future field work, however, there were practical limitations to sampling size in this study given the limited bed size in the Woodlands field and how many plants could be assessed in the laboratory within the time allocated. In an attempt to detect significant differences, the data for disease symptoms were analysed across a number of assessments. The average level of infection is presented for each of the symptoms in the results. It should be noted that averages do not reveal the final extent of infection at the last date of assessment.

Many of the symptoms of *R. solani* (AG3) infection such as stem canker, stolon canker, dry core, cracking, elephant hide increased significantly during crop development. This increase may reflect continued infection of new tissue or expansion of already established lesions. Thus the increase in stem canker over time recorded in four trials could be ascribed to expansion of infected tissue as the haulm grows. Conventional wisdom is that stem canker infection effectively stops once stems emerge from the soil. However, as the stem grows the cells expand in size and any lesions are likely to expand similarly.

In contrast, a significant increase in stolon canker over time probably reflects continued infection. Where stolons are killed by *R. solani*, the remaining tissue often rots away or is re-absorbed into the plant. The steady increase in stolon infection up to haulm destruction suggests that the pathogen continued to be active throughout the cropping period. Stolon pruning was at low levels throughout the growth of each trial but because tissue of pruned stolons disappears, the amount of stolon pruning might be better measured by accumulating the assessment scores.

Of the tuber symptoms believed to be caused by *R. solani* (AG3), (ie elephant hide, cracking and dry core), elephant hide and dry core have been confirmed by Koch's

postulates elsewhere but not using material collected from the five field trials (see results from Fera in this report for elephant hide for AG3 and BNR). There may be other causes of each symptom. Thus cracking can be a result of irregular soil moisture during tuber development, continuously wet soil conditions or virus infection, dry core has been attributed to wireworm damage (Keiser, 2007) and symptoms described as elephant hide have been ascribed to *Streptomyces* spp. infection (see Fiers, 2010 for comparable symptoms described as netted scab, russet scab or polygonal lesions). Evidence that *R. solani* is implicated would come from a reduction in symptoms where a control measure is applied. In the five trials carried out in this project, there was not strong evidence that the symptoms were the result of *R. solani* infection as few symptoms were significantly or consistently reduced as a result of control measures being applied. Only cracking was reduced by Amistar treatment in the Woodlands trials.

All three tuber symptoms, dry core, cracking/distortion and elephant hide increased over time. Assuming the symptoms were caused by *R. solani*, this may have been due to expansion of lesions as tuber swelling took place or continued infection.

In three trials, inoculum of a pathogenic strain of *R. solani* was added to enhance soil inoculum. In the scoping trial in Woodlands field 2009, surprisingly, rather than increasing disease on developing plant parts, the added inoculum tended to reduce symptoms. Thus stolon canker and black scurf incidence were significantly reduced where inoculum was added (Monceren + inoculum v. Monceren) and added inoculum reduced (but not significantly) stem canker, elephant hide and stolon pruning. Similar effects were not apparent in the Fingask trials where comparisons were made of commercial seed with or without added inoculum, although there was a consistent but non-significant increase in total tuber number. However, in these trials soil inoculum levels were not detected by PCR analysis compared to that in the Woodlands field 2009 trial. The possibility of *R. solani* strains acting antagonistically has been raised previously, and it is conceivable that the inoculum, placed above the seed tuber in the zone where stem, stolon and tuber development would occur, acted antagonistically to the natural soil inoculum.

In this trial series, there was no significant or consistent effect of Amistar on total yield. Similarly, seed fungicide treatment with Monceren had no significant effect on total yield, except in the Woodlands Field trial in 2010 where it significantly reduced yield. In the Woodlands field trials in 2010 and 2011 where healthy mini-tubers were used as seed, there was no benefit in combining a seed tuber fungicide treatment with the use of Amistar on total yield. Where tested, neither extra nitrogen or irrigation had positive effects on yield. In fact, at Fingask in 2010, the irrigation treatment reduced yield over the untreated control significantly.

Apart from the Woodlands field trial in 2010 where both fungicide treatments (alone or in combination) reduced total tuber numbers significantly, neither Monceren seed tuber treatment or Amistar had a significant or consistent effect on total tuber number.

No single control measure evaluated consistently reduced disease on haulm (stem or stolons) or tubers. Of the options compared, Amistar most frequently reduced disease significantly, but depending on the symptom assessed, significant reductions were confined to one or two trials. It might have been anticipated that given the risk of infection from soil-borne *R. solani,* the fungicide treatments, especially, Amistar,

would have resulted in large effects in reducing disease levels. This should have been most notable in the Woodlands field trials in 2009 and 2011 where soil-borne inoculum was detected at very high levels. However, the effect of fungicide treatment was not dramatic in these trials. This might be explained by the extreme inoculum pressure present at planting proving too much to control. In the Woodlands field trial in 2010 where no soil-borne inoculum was detected and the pressure was much lower at planting, fungicide treatments resulted in significant reductions in disease. This may suggest that fungicide treatments work best in locations where inoculum pressure is not high – at least initially.

The greatest level of black scurf incidence and severity in any trial occurred in the Woodlands field in 2009. This was the site where the highest level of soil-borne inoculum was also recorded. The second highest incidence of black scurf occurred in the Woodlands field 2011 trial. This trial also had a high level of soil inoculum prior to planting. It is well understood that black scurf is reduced by harvesting as early as possible. However, throughout the trials in this series, no such effect of harvest date was observed. This could be accounted for by the wet summers which had a dampening effect on development of *R. solani*.

4.3.3. Controlled environment experiments to study *R. solani* inoculum types, their detection and impact on disease development

A programme of work, consisting of 3 controlled environment experiments has been carried out to investigate the relative contribution of inoculum types (seed vs soil inoculum; sclerotia vs hyphae) and factors affecting inoculum detection and disease development.

Materials and Methods

Some of the methods were common to the different controlled environment experiments carried out by Fera and JHI during 2009 to 2011. These are described below. The methods specific to individual experiments are described separately in each relevant section.

Inoculum preparation

Inoculum preparation was common to all experiments, except JHI 2009 (details described separately). An isolate of AG3-PT, Rs08, was removed from long term storage at -20°C and grown on Potato Dextrose Agar (PDA). A hyphal tip sub-culture was then taken and checked for purity and culture identity was then confirmed as AG3-PT by real-time PCR. The culture was used to inoculate PDA plates (90 mm petri dishes) or potato dextrose broth (50 ml PDB in 100 ml disposable plastic containers). Both types of potato dextrose media were incubated in the dark at 20°C for 6 weeks (plates) or 15 days (broth). Sclerotia or hyphae were then purified from the media. Sclerotia were removed from plates using a scalpel blade, and then left to air dry at room temperature for two days. Hyphae were separated using Miracloth (Calbiochem) and washed several times using sterile distilled water. The hyphae were left to air dry over night. Sub-samples of the sclerotia and hyphae were taken and plated out onto 2% tap water agar to confirm viability.

Controlled environment conditions

Conditions were set at 18˚C, with a 16hr light/8hr dark photoperiod in all experiments. Relative humidity was 50 % in Fera experiments and 70 % in JHI. Plants were watered as required.

Real-time PCR: quantification of soil and tuber inoculum

At Fera, soil samples were stored at -20˚C until DNA was extracted using Fera's standard method from 250 g of soil (Woodhall *et al*., 2012). DNA was quantified using Fera's in house real-time PCR assay for AG3-PT against known DNA standards.

At JHI, tuber and soil DNA extractions were carried out according to the methods of Cullen *et al.,* (2001) and Brierley *et al.,* (2009) respectively and the amount of *R. solani* (AG3) DNA detected using the assay of Lees *et al.,* (2002) was expressed as ng DNA /ml tuber sap or pg DNA /g soil.

Disease assessments

Stems were assessed using the disease keys for stems from Carling and Leiner (1990): 0 = no damage or lesions present; 1 = minor damage, one to several lesions less than 5 mm in size; 2 = moderate damage, lesions larger than 5 mm and some girdling present; 3 = major damage, large lesions and girdling or death present on most stems; 4 = all stems killed. The relative surface area of black scurf on the seed tuber was also recorded using the black scurf severity key from Woodhall *et al*.,

(2008): 0 = no sclerotia present; 1 = less than 1% of the tuber surface area covered in sclerotia; 2 = 1 to 10%; 3 = 11 to 20%; 4 = 21 to 50%; and 5 = 51% or more.

Effect of seed vs soil inoculum

Two controlled environment experiments were carried out to determine the relative importance of seed- and soil-borne R. solani *AG3 inoculum on disease. These used different varieties and tested different soil and seed inoculum levels. By experimentally manipulating the level of soil inoculum it was intended that the threshold level (under controlled environment conditions) for causing disease could be ascertained. In addition, the interaction between seed and soil inoculum is also described. A third experiment was carried out to study the impact of propagules type (hyphae vs scleortia) on disease development.*

Controlled Environment Study 1: JHI Experiment 2009

Materials and Methods

Seed inoculum

The black scurf susceptible potato cultivar Sante (resistance rating 3 on a 1-9 scale of increasing resistance) was used in this experiment. A commercial seed stock of Sante with black scurf was sorted visually into three disease categories based on the percentage surface area covered with symptoms: < 1 %; 1 – 10 % and > 10 % surface area. Un-contaminated seed were in the form of mini-tubers, however, these were found to have some black scurf symptoms. An additional seed category was created by treating mini-tubers with Monceren DS (pencycuron) at the full dose (2 kg/tonne). All seed was kept at 4 °C in the dark until required. For each seed inoculum category used in the controlled environment experiment 10 seed tubers were peeled and assessed individually for *R. solani* contamination using real-time PCR.

Soil inoculum

Inoculum was prepared using three UK isolates of *R. solani* (AG3) isolated from potato (JHI isolates R3, R10 and R37). The cultures were used to inoculate Potato Dextrose Agar plates (90 mm petri dishes) and were incubated in the dark at 18°C in the dark for 19 days. An inoculum suspension containing all three isolates was then made by scraping the fungal colonies from 19 individual cultures into 1 litre of sterile distilled water (SDW). The inoculum contained sclerotia and hyphae. The inoculum was diluted to give a range of concentrations, undiluted, 1:10, 1:50 and 1:100 and added to batches of JHI compost (JHI, Invergowrie, Dundee). Inoculated compost was mixed thoroughly by hand before filling 7 litre pots. Control treatments had an equivalent volume of water added. The level of soil inoculum (pg DNA / g soil) in each treatment was determined by real-time PCR.

Experiment

Tubers from each of the five seed categories were planted in the control and infested compost with four replicates. Plants were grown for 104 days after which stems were cut off and watering ceased. At this time the number of stems and the incidence of stem canker per plant were recorded. The progeny tubers from each pot were harvested 14 days later and placed in a paper bag. The severity of black scurf on each individual tuber was recorded, and results expressed as the mean incidence and severity of black scurf disease per pot. At final harvest, soil was taken from treatments to which no soil inoculum had been added but which had been planted with seed of different inoculum loads, to determine the level of soil inoculum post harvest using real-time PCR.

Results and discussion

Seed and soil inoculum levels

Despite apparent visible traces of black scurf on mini-tubers, no *R. solani* DNA (AG 3, AG 2-1, AG 4 or AG 5) was detected. The three disease categories which were sorted, from a seed stock with black scurf, according to the percentage tuber surface covered in black scurf, were found to have increasing levels of *R. solani* AG3 DNA with increasing level of visual symptoms (Table 27). Due to the large variation in DNA recorded in each category, tubers with <1 % black scurf were not significantly different from those with between 1-10% disease coverage.

Table 27. *R. solani* AG 3 DNA (ng DNA / ml tuber sap) on seed tubers categorised according to the % surface area with black scurf symptoms. Values represent the mean of 10 individual tubers and values suffixed with different letters are significantly different (Mann-Whitney p< 0.001).

	Seed categories (based on visual black scurf symptoms)			
	Mini-tubers	< 1 %	1-10 %	> 10%
R. solani DNA ng/ml tuber sap	0[a]	46[b]	322[b]	10,640[c]
Standard error	0	13	227	2,930

No *R. solani* was detected in the control soil treatment, but neither was it detected in the two lowest levels of infestation (Table 28). Inoculum was detected in the two highest levels of infestation, but there was no significant difference between the levels. Based on previous results from artificially inoculated field soils, we would have expected the levels of inoculum in the lower dilutions to have been detected. It appears that the compost may have inhibited *R. solani* detection at lower levels.

Table 28. *R. solani* DNA (pg DNA / g soil) in soil inoculated with different concentrations of inoculum. Values represent the mean of 4 individual batches of compost and values suffixed with different letters are significantly different (Mann-Whitney p< 0.05).

	Soil inoculum level				
	Control (1)	1: 100 (2)	1:50 (3)	1:10 (4)	Neat (5)
R. solani DNA pg/g soil	0	0	0	2,235 [a]	5,107 [a]
Standard error				2,235	2,046

Number of stems per plant

The mean number of stems per plant was 4.5 with a minimum of 1 and a maximum of 10. The level of seed and soil inoculum had no affect on the number of stems per plant. Mini-tubers (whether treated or untreated) had fewer stems than the contaminated seed categories. However, this difference cannot be attributed to the presence of black scurf on the contaminated tubers, indeed, the number of stems did

not increase with an increase in the level of seed contamination, but rather to the two different stocks.

Diseased stems

The occurrence of stem canker was recorded when the stems were cut-off near the end of the experiment. As some of the plants were already senescing, stem canker could not always be positively identified, and some diseased stems may have had black dot, black leg or have simply senesced. The untreated mini-tubers produced significantly fewer diseased stems than the contaminated seed (31.4 % cf. > 51.2%; $p<0.05$), but there was no increase in stem disease with increasing level of seed contamination. Treated mini-tubers had significantly fewer diseased stems than untreated minitubers (5.7% cf. 31.4%; $p<0.05$). Strangely, there were significantly fewer diseased stems in the highest level of soil inoculum (level 5) compared to all other levels of soil inoculum ($p<0.05$).

Number of tubers per plant

On average, each plant produced 15.9 tubers (greater than 10 mm diameter). There were fewer tubers per plant at the highest level of seed contamination compared to lower levels ($p<0.05$). Untreated mini-tubers produced similar numbers of tubers per plant to the low levels of seed inoculum, however, treated mini-tubers produced significantly fewer tubers than untreated mini-tubers and contaminated seed. There was no significant difference in the number of tubers produced per plant with the level of soil inoculum.

Incidence and severity of black scurf

The un-inoculated soil treatments produced significantly lower levels of disease (incidence and severity) compared to all subsequent levels of soil contamination (Figure 29). There was a significant seed/soil interaction ($p<0.05$), with seed inoculum having a significant affect on disease incidence and severity in the absence of soil inoculum, i.e. mini-tubers (both treated and untreated) and low levels of seed inoculum (<1 and 1-10%) resulted in less disease than the highest level of seed inoculum (> 10 %). Whilst the level of seed inoculum did not have any affect on disease incidence in the presence of soil inoculum, treated mini-tubers had significantly less disease (incidence and severity) at all levels of soil inoculum compared to other seed treatments.

Soil inoculum post harvest

As expected, where no inoculum was added to the soil or introduced by the seed (mini-tubers) no *R. solani* was detected in the pot compost at final harvest (Table 29). When contaminated seed was grown in un-inoculated compost, the level of soil inoculum was relatively high at the time of final harvest, irrespective of the seed inoculum level (Table 29).

Table 29. The level of inoculum in compost taken at final harvest from pots in which contaminated seed was planted into un-inoculated soil treatments.

	Seed categories (based on visual black scurf symptoms)			
	Mini-tubers	< 1 %	1-10 %	> 10%
R. solani DNA				
pg/g soil	0	1464	728	1601
Standard error	0	1690	530	951

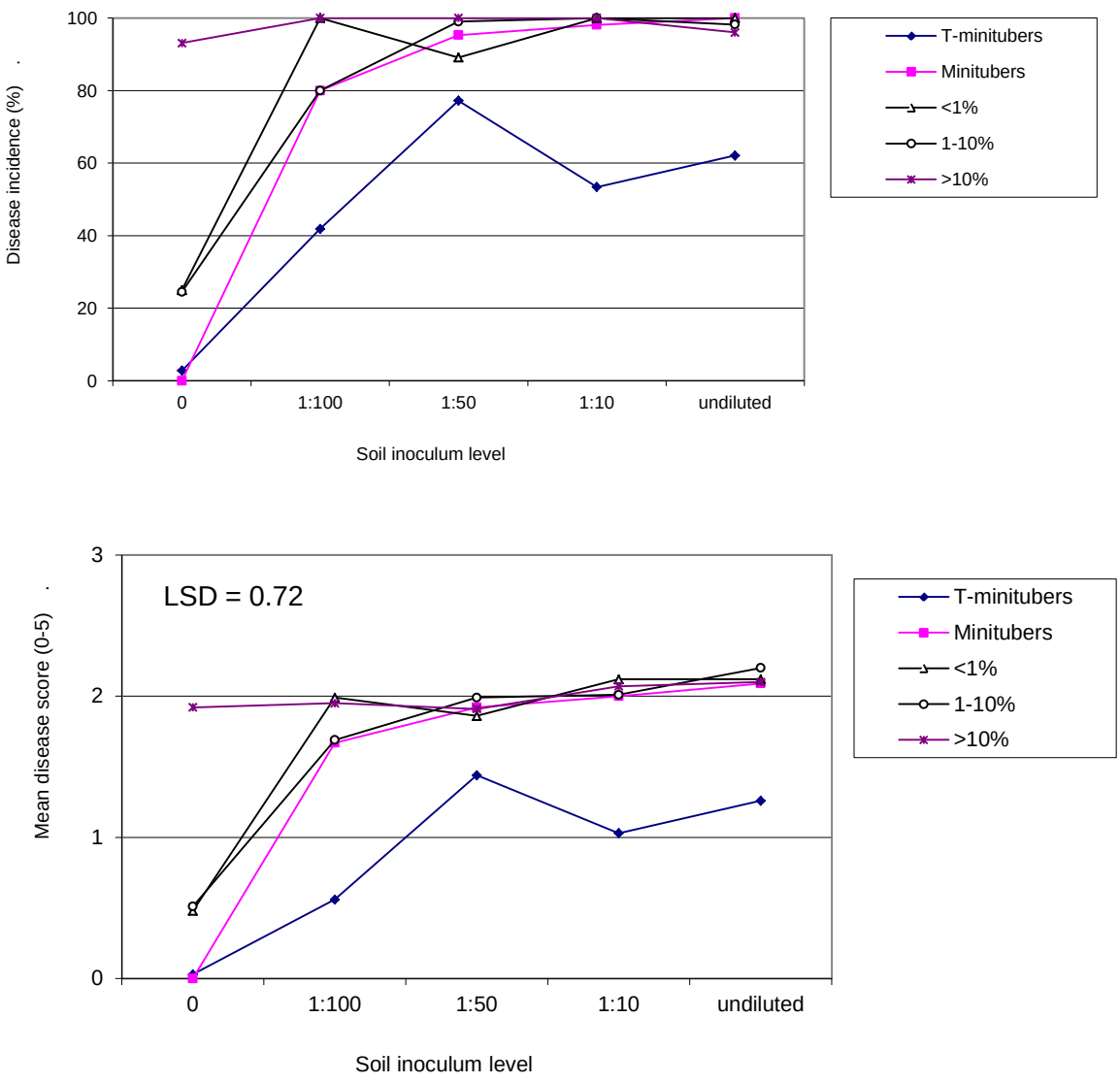


Figure 29. The incidence and severity (scale 0 to 5) of black scurf on progeny tubers with different levels of seed and soil inoculum (T-minitubers = mini tubers treated with Monceren DS).

Effect of seed vs soil inoculum
Controlled Environment Study 2: Fera Experiment 2010

Materials and methods

Seed and Soil inoculum levels

Soil-borne inoculum consisted of pure sclerotia mixed with compost using a cement mixer to give sclerotia at the following levels (w/w) 0.00005, 0.0001, 0.0002 and 0.0005. This is equivalent to 0.05 mg, 0.1 mg, 0.2 mg and 0.5 mg per g of soil. Seed-borne inoculum was prepared the previous year. Healthy tubers (cv. Desiree) were inoculated with isolate Rs08 and grown in controlled environment conditions. The harvested seed was washed, then separated into three batches: asymptomatic seed; infested seed with visual black scurf levels approximately 5% ('low' seed inoculum); and infested seed levels at approximately 15% ('high' seed inoculum). Asymptomatic seed consisted of seed displaying no black scurf or any blemishes associated with *R. solani*. Presence of *R. solani* in the asymptomatic seed was confirmed through real-time PCR for AG3-PT on DNA extracted from tuber peel. Clean seed was also prepared at the same time in un-inoculated soil and used in the control and soil inoculum treatments accordingly. Pots (height 22 cm, diameter 25 cm) were used in the inoculum level/source experiment and each contained 3.75 kg of compost. Tubers for this experiment were in the size range of 35-45 cm.

Each treatment consisted of eight replicate plants, in separate pots containing John Innes No. 3 compost as the growing medium. Pots were placed in a controlled environment room. After four weeks, four replicates were harvested and stems assessed using the disease key for stems from Carling and Leiner (1990). Black scurf severity on the seed tuber was also recorded using the key from Woodhall *et al.,* (2008). After a further 15 weeks, the haulms of the plants were removed and after an additional 3 weeks, the tubers were harvested and assessed for scurf severity. Incidence and severity of elephant hide was also recorded. At planting, 4 weeks and at 18 weeks, a 50 g soil sample was taken for subsequent DNA extraction and AG3-PT quantification by real-time PCR.

All statistical analyses were carried out using Genstat release 13 (VSN International Ltd). Analysis of the data from the controlled environment experiment was performed by ANOVA. Where data were skewed, an arcsine transformation was performed. The contrasts directive within Genstat was used to compare treatment levels where many comparisons were possible.

Results and discussion

Disease assessments for plants inoculated with different levels of soil-borne or tuber inocula are given in Table 30. No disease was present in the non-infested soil or asymptomatic seed treatments. Stem infection and stem death were observed in all other treatments. No significant difference ($P>0.05$) can be seen between plants inoculated at different levels of soil inoculum. The number of stems killed was highest in the plants with high seed inoculum treatment and this was significantly higher than the low seed inoculum plants ($P=0.45$). In almost all the AG3-PT inoculated plants, sclerotia developed on the mother tubers irrespective of whether the inoculum originated from seed or soil. The surface area of black scurf on mother tubers was higher where the seed inoculum level was high, compared with the low seed inoculum treatment ($P=0.045$).

Table 30. Effect of inoculum source and inoculum level on severity of stem canker, stem death, root disease and development of black scurf of mother tuber

Soil inoculum (g/ g of soil)	Seed inoculum	Stem disease key (0-4)	% Stems killed	Mother tuber black scurf severity key (0-5)
0.00005	0	3.0	33.3 (31.3)[b]	2.0
0.0001	0	2.9	27.1 (27.6)	2.0
0.0002	0	2.2	27.1 (27.6)	2.0
0.0005	0	2.8	39.2 (38.7)	1.5
0	Asymptomatic	0.0	0.0 (0.0)	0.0
0	Low	1.1	16.7 (17.6)	2.0
0	High	2.6	52.0 (50.1)	4.0
0	0	0.0	0.0 (0.0)	0.0
LSD (P<0.05; df 31)		1.44	(28.11)	0.52

[b]Arcsine transformed values for non-normal data given in parenthesis.

Table 31. Levels of black scurf and elephant hide (% incidence) and *R. solani* AG3-PT DNA in treatments at planting, 4 weeks and at 18 weeks

Soil inoculum (g/ g of soil)	Seed inoculum	Black scurf %	Elephant hide %	DNA at planting (pg/g soil)	DNA at 4 weeks (pg/g soil)	DNA at 18 weeks (pg/g soil)
0.00005	0	100	33	1,130	650	10,071
0.0001	0	100	50	986	3,458	560
0.0002	0	30	45	1,103	525	2,273
0.0005	0	29	63	24,662	1,103	45
0	Asymptomatic	0	0	0	0	0
0	Low	36	0	0	17	328
0	High	40	0	0	4	13
0	0	0	0	0	0	0
LSD (P<0.05; df 31)		47.4	46.2	6,181.2	1,737.2	6,702.5

Elephant hide was observed in all soil-borne inoculum treatments (Table 31). The highest levels of black scurf were observed in two of the soil-borne inoculum treatments, although not in the treatment with the highest level of soil-borne inoculum. In all treatments, DNA levels in soil changed over time. However, in the highest level of soil-borne inoculum (0.0005), the level of AG3-PT DNA in the soil reduced significantly ($p<0.05$) between planting and harvest.

Progeny tubers from the asymptomatic seed tuber treatment tested positive for *R. solani* in eyeplug tests at harvest. No visible *Rhizoctonia* could be detected in seed from the same batch tested 8 months later. This may suggest that hyphae on the tuber are not as able to survive as well as sclerotia.

In this study, infested soil from as little as 0.00005 (w/w sclerotia) and sclerotia infested seed both caused significant levels of stem lesions and stem death. Considerable disease developed at the lowest soil borne inoculum level (0.00005) suggesting the inoculum threshold for disease to occur is somewhat lower. Asymptomatic tubers which were PCR-positive failed to cause stem disease. Therefore the presence of a positive PCR from tuber DNA may not always be an accurate indicator of disease risk.

Interestingly, elephant hide was only observed in the soil inoculated plants and was highest in the highest level of soil borne inoculum. Previously Koch's postulates have been difficult to obtain for this symptom. However, high levels of soil inoculum could help in this instance.

Also of interest with the quantitative PCR data, the inoculum level at the high soil inoculum level decreased rapidly after planting. This level was associated with high stem canker levels and low levels of black scurf. This could explain the lack of relationship between levels of stem canker, black scurf and inoculum level.

Conclusions

The results from the two controlled environment studies are consistent – in both studies very low levels of seed inoculum did not cause high levels of disease whereas high levels of seed inoculum (> 10 % surface area with black scurf) and even very low levels of soil inoculum resulted in high levels of black scurf on progeny tubers. In the Fera study, elephant hide symptoms were recorded and it was observed that high levels of soil borne inoculum were associated with increased incidence of the symptoms.

Effect of propagule types

An experiment was undertaken to investigate the effect of different propagule types (sclerotia or hyphae) of R. solani *(AG-3PT) on the amount of stem canker and black scurf that developed under controlled environment conditions. The relationship between inoculum level (as detected by real-time PCR) and disease development was also investigated.*

Controlled Environment Study 3: Fera Experiment 2009

Materials and methods

Purified sclerotia or hyphae were added to 'JHI compost' to produce the following inoculum levels (w/w): for hyphae 0.00005, 0.0001, 0.00025, 0.0005 and 0.001; for sclerotia 0.0001, 0.0002, 0.0005, 0.001 and 0.004. In addition one treatment consisted of no inoculum (control) and another consisted of a 0.01 (w/w) inoculum level placed in the top 200g above the seed.

Inoculum was mixed with compost thoroughly by hand and there were 8 replicates for each inoculum level. Mini tubers (Desiree SE1), grade 35 - 45 mm, which had been previously visually inspected for black scurf and hyphae using a microscope were planted in the centre of the pot at approximately 120 mm depth. Pots (in saucers) were assembled in a randomised block in a controlled environment room.

After 6 weeks, four of the replicates were removed from the trial, washed and assessed for disease on stems, stolons and roots. The haulms of the remaining four replicates were removed 14 weeks post planting to encourage skin finish and the tubers were harvested three weeks afterwards. Tubers were washed, graded, weighed and counted and incidence and severity of black scurf assessed.

50 g soil samples were taken at planting and when plants were harvested at 6 weeks and 17 weeks. Soil samples were stored at -20˚C prior to quantification of *R. solani* AG3-PT using real-time PCR.

Statistical analysis

Analysis of the data was done by Generalised Linear Modelling using Genstat 12 edition (VSN International Ltd).

Results and discussion

Summary results for the experiment are shown in Table 32. No disease was observed in the non-inoculated controls. Low levels of stem and stolon canker were observed in the first level of hyphal inoculum (0.00005g/ g soil) but no black scurf was observed. Relatively high levels of stem and stolon disease were observed in the other inoculum levels for both sclerotia and hyphal inoculum. A good relationship (r^2=0.89) was observed when hyphal inoculum concentration was plotted against stem canker severity score. No such relationship was observed for sclerotia inoculum, possibly because the disease levels observed was already very high.

Plants grown in compost inoculated with hyphae at ≥0.00025g/ g soil produced significantly fewer tubers (all below 7 on average, compared to above 12.8 in the non-inoculated, and 0.00005 and 0.0001 inoculated pots) (p=0.002); Figure 30. In addition, increasing the amount of hyphal inoculum reduced the total weight of tubers

(*p*=0.008). There was no correlation between sclerotia inoculum level and tuber weight (*p*=0.247) or tuber numbers (p=0.229).

A 100% incidence of black scurf on tubers was observed in all treatments except the non-inoculated control and the two lowest levels of hyphal inoculum (0.00005 and 0.0001g / g soil). There were good correlations between increasing inoculum levels of hyphae and sclerotia with increasing surface area affected by black scurf (*p*<0.001).

At equivalent levels of inoculum in compost (w/w), sclerotia produced more severe black scurf than hyphae (*P*=0.002). In general, stem canker and black scurf incidence and severity were higher in sclerotia-inoculated pots than in compost inoculated with hyphae. In the pots inoculated with concentrated inoculum above the tuber, high stem disease was observed but lower levels of stolon infection; however, black scurf incidence and severity was high (Table 32).

The amount of DNA detected in soil generally increased with inoculum concentration, although DNA could not be detected in compost inoculated with hyphae at 0.00005 and sclerotia at, or below, 0.0005.

This experiment showed that there were differences between inoculum types in terms of detection and the ability to produce disease. Hyphal inoculum was detected at lower levels than sclerotia. This is likely to be because sclerotia were not as uniformly distributed in soil at low inoculum levels (<0.0005g / g soil) as hyphae. Hyphal inoculum at the lowest level (0.00005g / g soil) caused disease in stems but did not produce black scurf. This could be because at low levels, inoculum did not survive long enough in compost to initiate disease on stolons or tubers. Black scurf severity and incidence was similar for all other inoculum levels. This suggests that although disease could be low in stems, in the situation of using compost and controlled environment conditions, it could build up to critical levels over the season to cause significant black scurf later in the season.

The inoculation of the soil in this experiment has provided information on the level of inoculum (expressed by weight) of *R. solani* required to initiate disease, and the disease incidence data will enable experiments in future to better determine the threshold of inoculum required for disease. Previous experiments for *R. solani* either used sand-maize meal inoculum or macerated agars plates. From this type of inoculum, one cannot easily determine the quantities of *R. solani* present. Sclerotia, harvested from plates and dried and then weighed, would appear from this experiment, to be the ideal source of inoculum. This is because the weight of them can be easily determined; sclerotia are also persistent and can cause significant disease. Future work should utilise this type of inoculum and determine the threshold for disease development. The sclerotia inoculum level used in this study appears quite high and it would have been better, with hind sight, to use a lower level of inoculum. This is perhaps why no relationship between sclerotia inoculum level and stem canker severity was observed as maximum disease severity had already been reached in these treatments. Future work should be undertaken at lower inoculum levels for sclerotia.

The detection of AG3-PT in soil was poor in this experiment. There may be two reasons behind this, firstly, the compost used may have contained PCR inhibitory

compounds which reduced assay sensitivity. Compost is known to contain higher levels of humic acids which interfere with PCR reactions. Secondly, the structure of the compost used is much more bulky than field soil. This interfered with DNA extraction also and would explain why DNA yields were lower than in some other experiments. Future experiments should use a soil or compost more representative of potato field soil.

Table 32. Summary of percentage stem and stolon infection, tuber weight and number, black scurf incidence and severity with DNA detected in soil at planting and at harvest (H=Hyphal inoculum, S= Sclerotia inoculum). *, **, *** following the means represent differences within either hyphal or sclerotial treatments compared with the zero control at $p<0.05$, $p<0.01$, and $p<0.001$, respectively.

	(g/g)	Stems	Stolons	Tubers		Black Scurf		DNA detected in soil (pg/g soil)	
Type	spike	% with lesions	% with lesions	weight	number	incidence (%)	Severity	planting	harvest
Zero	0	0	0	343	12.8	0	0	0	0
H	0.00005	38	23	368	13.3	0	0	0	0
H	0.00010	75**	41	396	14.0	51	1.0*	4.7	0
H	0.00025	100***	71**	330	3.5 **	100	2.5***	2	0
H	0.00050	100***	47*	262	8.3	100	2.7***	67	36.8
H	0.00100	100***	83**	219**	6.0	100	2.5***	13.6	3.1
S	0.00010	100***	45**	233*	5.0 *	100	2.4***	0	0
S	0.00020	88***	85***	270	8.5	100	2.5***	0	0
S	0.00050	90***	70***	257	6.5	100	2.1***	0	0
S	0.00100	100***	25	348	7.5	100	3.0***	12.68	5.16
S	0.00400	90***	70***	334	10.7	100	3.3***	75.64	18.5
S	0.0001 located in top soil	92***	32*	262	7.7	100	3.3***	0	4.16

Figure 30. Tubers from a control (un-inoculated) pot above and tubers from a pot inoculated with 0.004 w/w *Rhizoctonia solani* AG3-PT sclerotia or hyphae below. In inoculated pots, less tubers were present although all these tubers were greater in size and individual weight than controls. Inoculated tubers displayed high levels of black scurf.

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4.3.4. Controlled environment experiment to study the impact of soil type on the detection of *R. solani* inoculum and disease development

The aim of this controlled environment experiment was to investigate the effect of soil type on the development of black scurf after inoculation with R. solani *(AG 3) sclerotia, and to determine if soil type affects inoculum detection.*

JHI Experiment 2010:

Materials and Methods

Seed

Mini-tubers of the black scurf susceptible potato cultivar Sante (resistance rating 3 on a 1-9 scale of increasing resistance) were used in this experiment. The mini-tubers were visually free of disease, 10 tubers were peeled and assessed individually for *R. solani* AG3 contamination using real-time PCR (all tested negative). All seed was kept at 4 °C in the dark until 4 days before planting when it was transferred to a glasshouse to allow it to sprout.

Soil/composts

Eleven different soils/composts were compared as listed in Table 33.

Soil inoculum

Sclerotia (0.03g) and sufficient soil/compost to fill a 7 litre pot were put together in a plastic bag and mixed thoroughly before filling the pot. Therefore each inoculated pot received the same quantity of inoculum based on soil volume, but the % weight/weight varied depending on soil type, ranging from 0.00004 w/w for the lightest peat to 0.000005 w/w for the heaviest sandy loam soil (see Table 33).

Control treatments had no inoculum added, and each was tested by real-time PCR for the presence of *R. solani* AG3 and AG2-1. For inoculated soils, as the amount added to each pot was so small and added as sclerotia, a subsample was not taken from the pots for determination of soil inoculum as the likelihood of either collecting a clump or missing inoculum altogether was high. Instead, two separate sets of inoculations were constructed, using smaller samples of soil/compost (240g). The first set (volume), replicated the inoculation treatments, therefore the equivalent concentration of 0.03g sclerotia/pot. In the second set (weight), each soil type was inoculated with the equivalent of 0.00003g sclerotia/ g soil. Both these sets of inoculated soils were tested using real-time PCR to compare the detection of inoculum levels in the different soils/composts.

Experiment

Single tubers were planted in the control and infested soils/composts, with four replicates. Field soils and peat, but not composts, were fed with 1:1:1 NPK containing magnesium and trace elements, weekly. Plants were grown for 126 days after which stems were cut-off and watering ceased. At this time the number of stems and the incidence of stem canker per plant were recorded. The progeny tubers from each pot were harvested 14 days later. The severity of black scurf on each individual tuber was

recorded, and results expressed as the mean incidence and severity of black scurf disease per pot. Soil from each inoculated pot was sampled after harvest and the level of inoculum determined by real-time PCR.

Table 33. The eleven different soil/compost types used in the comparison; the mean weight (kg) of each when filling a 7litre pot; their textural analysis and class; the mean g/g sclerotia/pot when 0.03g sclerotia added to each pot.

Soil ID	Sand (% w/w)	Silt (% w/w)	Clay (% w/w)	Textual class	Mean pot weight kg	g/g sclerotia/ soil
1. SCRI comp.	-	-	-	-	0.88	0.000034
2. J.I. No. 3	-	-	-	-	2.29	0.000013
3. Peat	-	-	-	-	0.69	0.000043
4. Fen topsoil	39	39	22	Clay Loam	2.74	0.000011
5. Fen farm soil	35	40	25	Clay Loam	3.03	0.000010
6. Fera	27	53	20	Clay Loam	3.92	0.000008
7. N. Yorkshire	36	43	21	Clay Loam	3.13	0.000010
8. Balrownie	45	38	17	Sandy silt loam	6.69	0.000004
9. Airntully	58	30	12	Sandy loam	6.79	0.000004
10. Carpow	39	36	25	Clay loam	6.56	0.000005
11. Sandy	92	4	4	Sand	6.52	0.000005

Results and Discussion

Number of stems per plant and stem canker

The mean number of stems per plant was 1.4. There was no significant difference between the different soil types or with the introduction of soil inoculum. Stems were inspected for the presence of stem canker when the stems were cut-off near the end of the experiment, however there were no positive instances of stem canker found.

Number of tubers per plant

There were fewer tubers per plant in inoculated soils compared to un-inoculated soils (7.7 *cf.* 10.7 tubers per plant; p=<0.001). There was no difference in the number of tubers per plant between soil types.

Incidence and severity of black scurf

No *R. solani* DNA was detected on the mini-tubers, or in any of the un-inoculated soils/composts. There was no black scurf recorded on progeny tubers when plants were grown in un-inoculated soil of any type. In the inoculated treatment there appeared to be some differences in the extent of disease on progeny tubers when grown in different soil types (Figure 31A). JHI compost (1) resulted in the highest incidence of disease, whilst Fen top soil (4), Fera (6) and Carpow (10), all clay loams, had low

incidence of disease compared to JHI compost (1), however, between replicate variance was high as indicated by the large standard errors, and this resulted in these apparent differences being non-significant (Mann Whitney; p=0.057). The severity of black scurf across all soil types was relatively low (less than 1 on a scale 0 to 5) and showed a similar pattern to disease incidence with different soil types (Figure 31B).

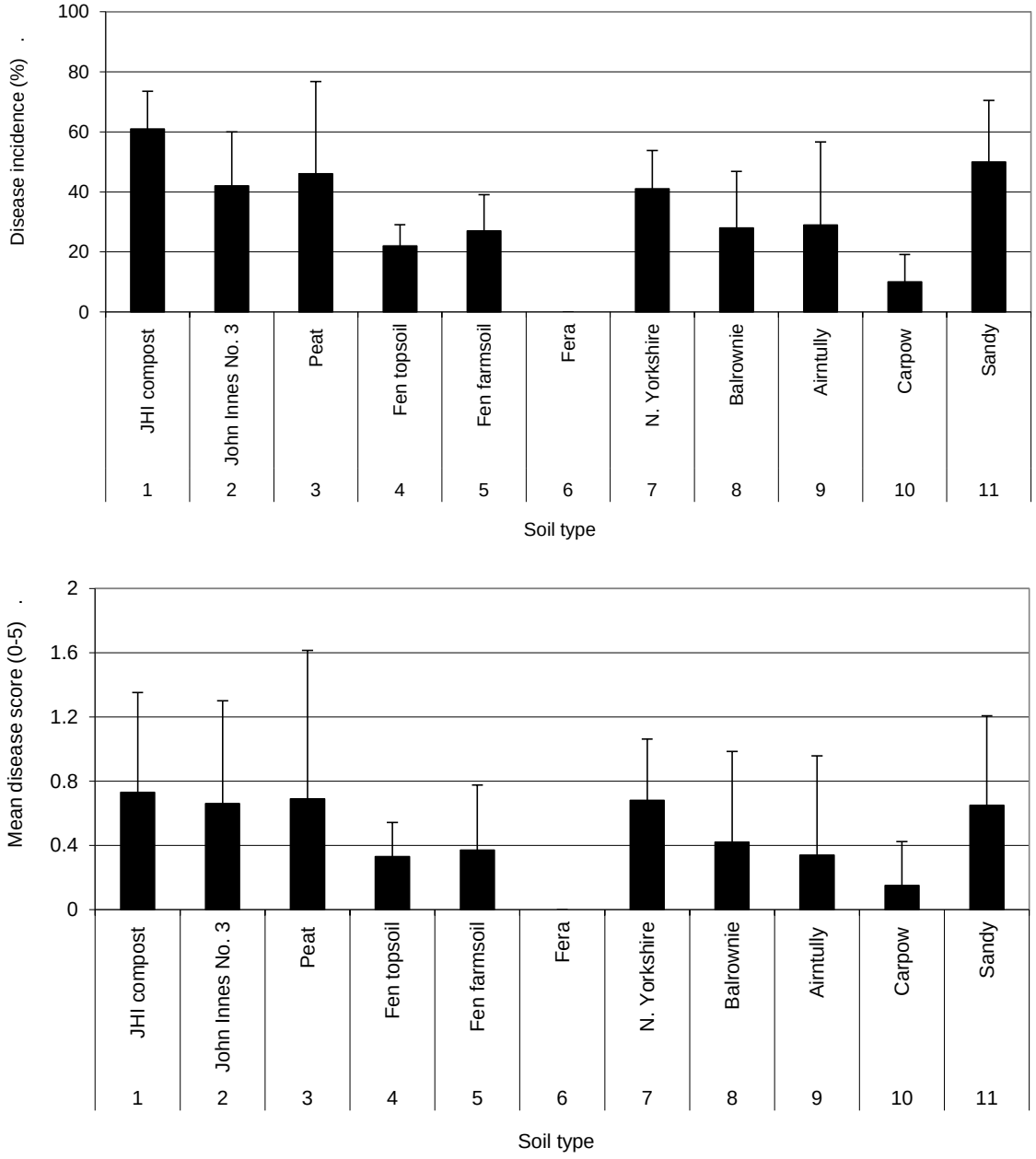


Figure 31. A. Incidence and B. Severity of black scurf on progeny tubers from mini-tubers grown in a range of soil types inoculated with 0.03g sclerotia/pot. Mean of four replicates + standard error.

Whilst each inoculated pot received the same quantity of inoculum based on soil volume (0.03g/pot), the weight/weight of inoculum/soil varied depending on soil type, ranging from 0.00004 w/w for the lightest peat (3) to 0.000005 w/w for the heaviest soils (8, 9,

10 & 11). There was no apparent relationship between the mean disease incidence for each soil type and the inoculum level based on weight/weight inoculum received (Figure 32). Although the two lightest soils (JHI compost and peat, 1 and 3 respectively) had the highest w/w and a high incidence of disease, there was no pattern between the remaining 9 soils.

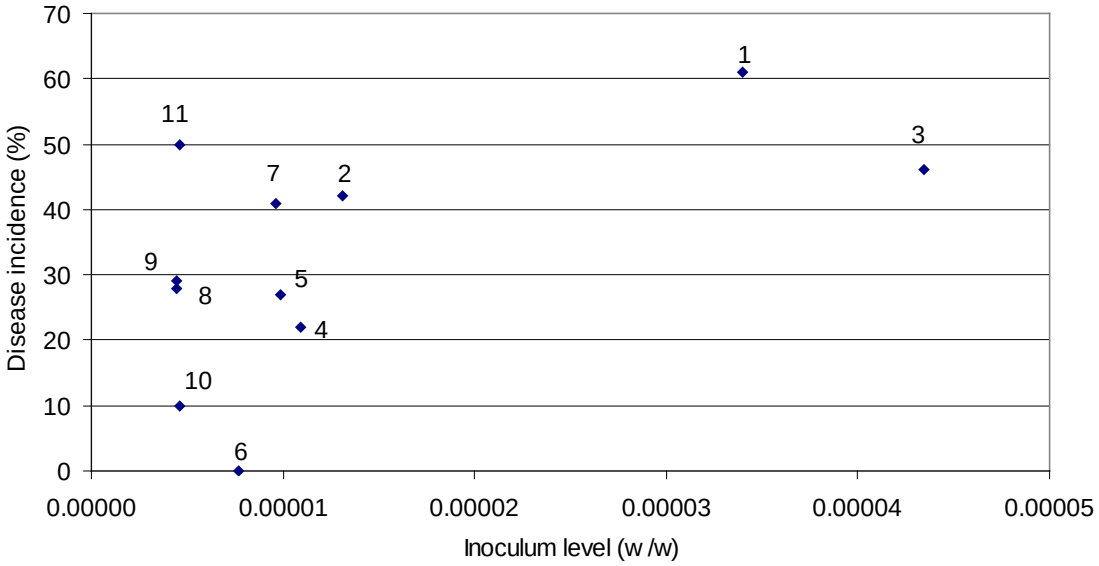


Figure 32. The incidence of black scurf on progeny tubers from mini-tubers grown in a range of soil types in relation to the w/w of sclerotia/pot.

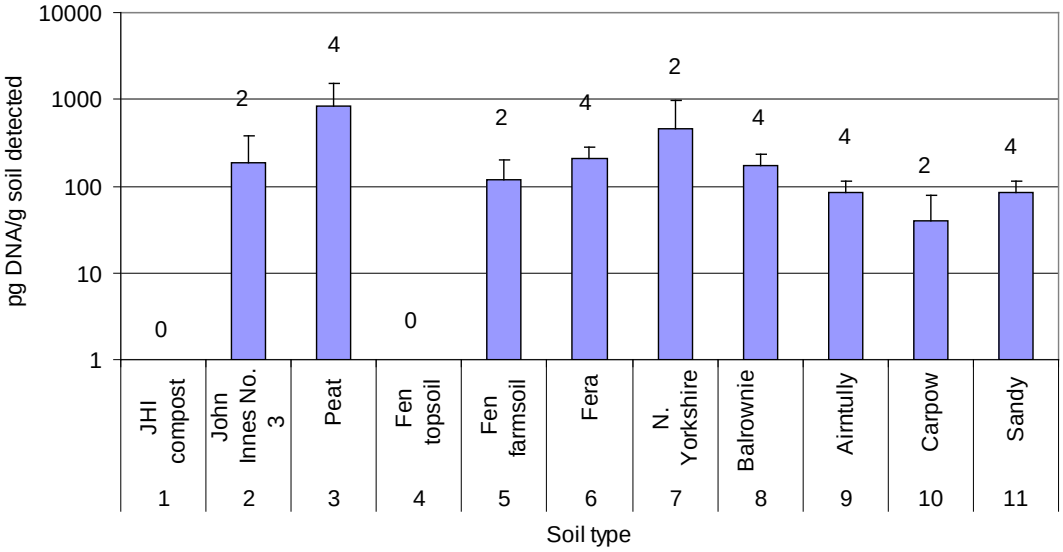
Detection of soil inoculum in different soil types

When soil/compost samples were inoculated with very low levels of inoculum (equivalent to 0.03g sclerotia per pot), the level of inoculum detected using real-time PCR was around 100 pg DNA / g soil, but for the JHI compost and one of the Fenland soils no inoculum was detected (Figure 33). The variation in detection was quite high and for four out of the nine soils in which inoculum was detected, this was only in two of the four replicates (Figure 33A).

As the w/w sclerotia per pot varied between the different soil types and this would account for some of the variation in pg DNA detected per g soil, detection of inoculum in the different soil types was also compared in inoculations standardised on a weight/weight basis- 0.00003 g sclerotia per g soil (Figure 33B). No inoculum was detected in four of the eleven soils.

The levels of inoculum added to the soils in these experiments were intentionally low, and were at the threshold of detection. However, these low levels were sufficient to cause disease.

A. Soil inoculations (soil volume)- 0.03g sclerotia/pot



B. Soil inoculations: 0.00003 g sclerotia per g soil

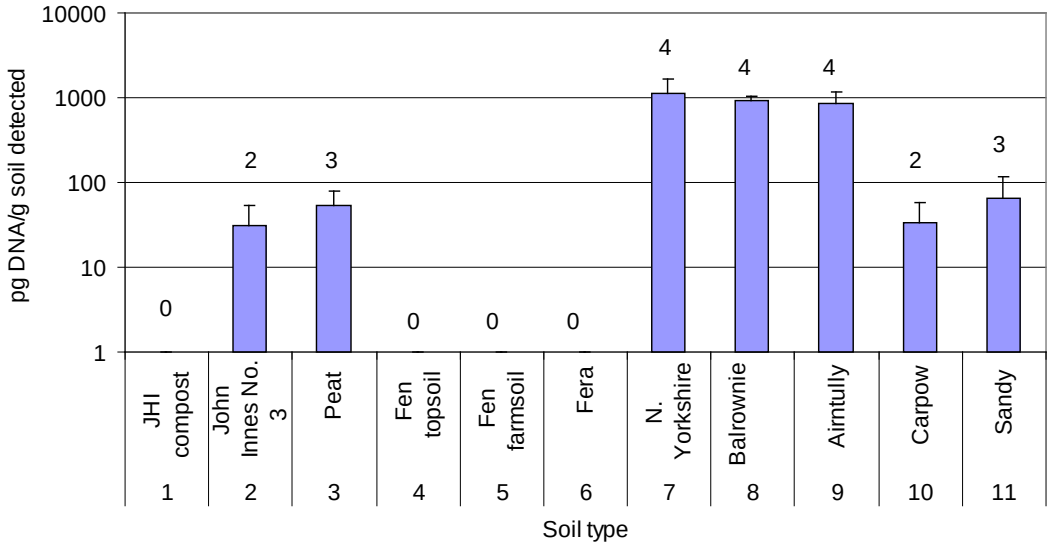


Figure 33. *R. solani* DNA (pg DNA / g soil) detected in soils inoculated with A. the equivalent of 0.03g sclerotia per pot, and with B. the equivalent of 0.00003g sclerotia per g soil; Error bars indicate standard error of mean, the number above each column indicates the number of replicates out of 4 which were positive.

Levels of soil inoculum in different soil types – post harvest

Relatively high levels of *R. solani* were detected in the two composts (JHI and John Innes No. 3) and the N. Yorkshire (7) and Airntully (9) soils after the progeny tubers were harvested (over 7,000 pg DNA/ g soil). Significantly less (compared to JHI compost: Mann Whitney $p<0.05$) was detected in other soil types, with detection in the two fen soils being particularly low (0 and 79 pg DNA/g soil in soil type 4 and 5, respectively), see Figure 34. There was no relationship between the level of disease on progeny tubers and the level of inoculum detected post harvest.

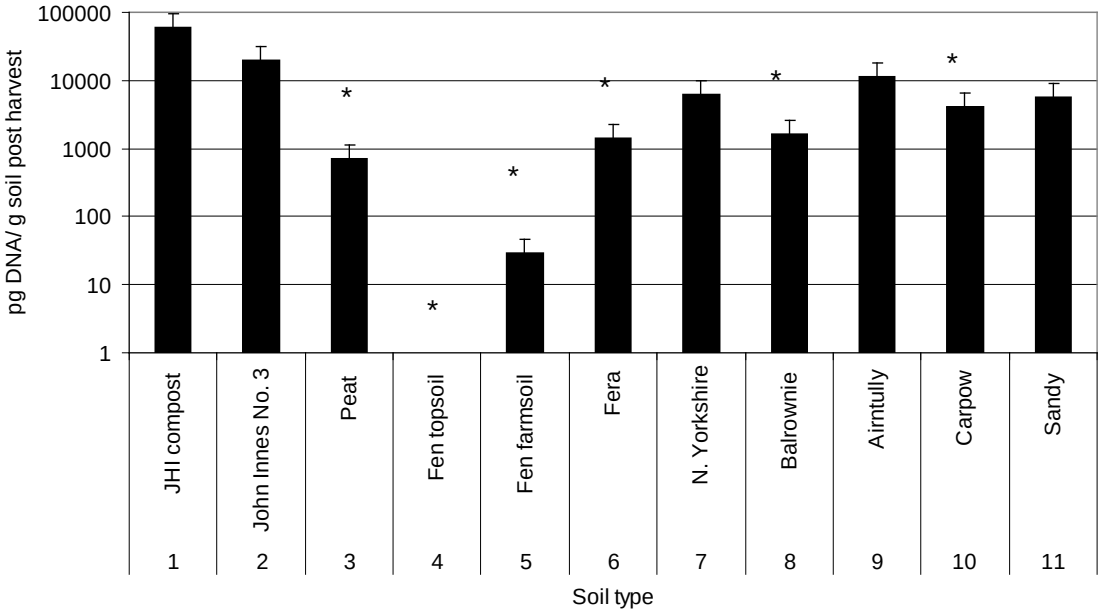


Figure 34. *R. solani* DNA (pg DNA / g soil) detected in soils sampled post harvest. Soils marked with an asterisk are significantly different to the JHI compost (Mann Whitney; $p<0.05$).

Inoculum was consistently un-detectable from the Fen top soil (5), although inoculated treatments did develop black scurf. This Clay Loam soil may have contained more humic compounds than other soils, which may have had adverse effects on the real-time PCR assay. Although low amounts of inoculum could not be detected in the JHI compost, post harvest very large amounts of inoculum were detected.

Interactions between soil micro-flora which will be absent in peat and composts but may suppress disease in other soils, for example the Fen soil, may account for some of the differences in inoculum detection and disease development.

4.3.5. Controlled environment experiment to study the impact of soil moisture on disease development

The aim of this controlled environment experiment was to investigate the influence of soil moisture level on stem disease and black scurf.

Fera experiment 2011

Materials and Methods

Infested soil was prepared by mixing pure sclerotia into compost using a cement mixer to give sclerotia at 0.00013 (w/w) (equivalent to 0.13 mg *R. solani* sclerotia per g of soil). Minitubers (Cv. Markies) were planted at approximately 100 mm depth in pots (height 22 cm, diameter 25 cm) each contained approximately 3 kg compost (John Innes No. 3). Uninoculated pots did not contain sclerotia of *R. solani*. Each treatment was replicated 10 times. Treatments are shown in Table 34. Treatments consisted of five watering regimes: at 20% (low), 30% (medium) or 40% (high) volumetric water content; an initial regime of 20% to tuber initiation (TI) followed by 40% to harvest; and an initial 40% regime to TI followed by 20% to harvest. A preliminary experiment was undertaken to determine approximate volumes of water required to adjust the moisture content to the desired level. All moisture readings were undertaken with a HH2 moisture metre connected to an SM300 sensor (Delta T Devices, Cambridge, UK). Moisture levels were measured 2-3 three times a week and adjusted as appropriate.

Pots were placed in a controlled environment room. After seven weeks, treatments 1 to 4 were harvested and stems assessed for disease. Black scurf on the mother tuber was also recorded. At this time the watering regime was reversed for treatments 9 and 10.

Table 33. Treatments in the effect of moisture on *R. solani* AG3-PT experiment

Treatment No.	Inoculated	Plants harvested & assessed	Moisture level
1	No	Tuber initiation	Medium
2	Yes	Tuber initiation	Low
3	Yes	Tuber initiation	Medium
4	Yes	Tuber initiation	High
5	No	End of season	Medium
6	Yes	End of season	Low
7	Yes	End of season	Medium
8	Yes	End of season	High
9	Yes	End of season	Low for 7 weeks then to high
10	Yes	End of season	High for 7 weeks then to low

At 15 weeks from planting, haulm of plants were removed and after an additional 3 weeks, the tubers were harvested and assessed for black scurf severity. Incidence and severity of elephant hide was also recorded. At planting, 7 weeks and at 18 weeks, a 50 g soil sample was taken from each treatment for subsequent DNA extraction and AG3-PT quantification by real-time PCR.

All statistical analyses were carried out using Genstat release 13 (VSN International Ltd). Analysis of the data from the controlled environment experiment was performed by ANOVA. Where data were skewed, an arcsine transformation was performed. The contrasts directive within Genstat was used to compare treatment levels. Where many comparisons were possible, a Duncan's multiple range test was performed. Analysis of the data from the controlled environment experiment was performed by an accumulated analysis of variance (Generalised Linear Model with either a binomial distribution error term and logit-link function, or a normal distribution term).

Results and discussion

The presence of *R. solani* AG3-PT inoculum caused significant levels of stem canker symptoms (p<0.001), pruned stolons (p<0.001) and pruned primary (p=0.049) and secondary stems (p<0.001) compared with potato plants that had been growing in soil with no inoculum (Table 35). The level of moisture applied (from planting to tuber bulking) had no effect on the level of stem canker, incidence of pruned stolons and dead primary stems. However, moisture did affect the % incidence of pruned secondary stems. The moderate moisture treatment (30% moisture) produced a higher level of pruned secondary stems than in plants grown under the low moisture treatment (P=0.015).

Table 35. Effect of moisture and presence of *R. solani* AG3 inoculum on level of infected and pruned stolons (% incidence, arcsine transformed), % pruned primary stems and % pruned secondary stems at time of tuber bulking, 7 weeks after planting. Different letters following the means within the same column, indicate differences at p<0.05.

Treatment No.	Inoculum	Moisture	Stem canker index (0-4)		Pruned stolons (arcsine %)		Pruned primary stems (arcsine %)		Pruned secondary stems (arcsine %)	
1	Nil	30%	0.0	a	0.0	a	0.0	a	3.4	a
2	AG3-PT	20%	2.6	b	14.8	b	10.0	ab	55.2	b
3	AG3-PT	30%	2.4	b	22.4	b	11.3	ab	100.0	c
4	AG3-PT	40%	3.2	b	21.9	b	25.7	b	71.4	bc

At the end of the growing period, plants grown in pots where *R. solani* AG3-PT inoculum had been added produced tubers with black scurf and elephant hide (Table 36). Tubers grown in pots where no inoculum had been added had no visible signs of either black scurf or elephant hide. The effect of moisture on tuber blemishing was evident. The low moisture treatment (20%) produced more black scurf than the high (40%) moisture treatment (p=0.020). Also, the low moisture treatment produced more elephant hide than either the moderate or high moisture treatments (p<0.001). There was no difference between the 'low to high' and 'high to low' moisture treatments on either black scurf or elephant hide severity. In general, tuber blemish levels in the combined moisture treatments were comparable to the moderate moisture treatment.

Table 36. Effect of moisture and presence of *R. solani* AG3-PT inoculum on mean severity of black scurf and elephant hide severity (arcsine transformed) and tuber weight. (mean g per pot) at harvest. Different letters following the means within the same column, indicate differences at $P<0.05$.

Treatment No.	Inoculum	Moisture	Black scurf (arcsine)		Elephant hide (arcsine)		Tuber weight (g)	
5	Nil	30%	0	a	0	a	178.1	c
6	AG3-PT	20%	14.4	c	16.63	c	73.4	a
7	AG3-PT	30%	12.1	bc	9.146	b	91.6	ab
8	AG3 -PT	40%	8.2	b	3.519	ab	151.0	bc
9	AG3-PT	20% to 40%	12.2	bc	4.186	ab	66.0	a
10	AG3-PT	40% to 20%	10.9	bc	2.925	ab	65.3	a

In this study, the levels of soil moisture appeared to have little or no effect on stem canker development but did influence black scurf and elephant hide levels. In the high moisture treatment, lower levels of elephant hide and black scurf were observed and tuber yield (by weight) was also higher. This is concordant with finding from some previous studies; Kyritsis and Wale (2002) found that black scurf was lower in higher moisture levels (60% WHC) compared to lower soil moisture levels. Irrigation of dry soils before planting is a recommended practice in areas where soil is irrigated to reduce disease levels (Banville *et al*., 1996). The reduction in infection at higher moisture levels has been ascribed to insufficient aeration for growth of *R. solani* in soil at least in poinstettia and bean crops (Bateman, 1963; van Bruggen and Arneson 1986). However, the effect of moisture may be more complicated, some authors recommend avoiding planting in cold, wet, heavy, poorly drained soil to reduce the risk of root and stem cankers (Jeger *et al.,* 1996). It is likely to be interplay of factors concerning moisture, soil type and other environmental factors.

4.3.6. Field trial to study the interaction between seed or soil inoculum level, soil organic matter levels and disease development

As R. solani *has proved unpredictable in its detection, for example, in fields where the crop had black scurf, it has often been undetectable in the soil when sampled only months post-harvest, questions arise as to what enables hyphae or sclerotia to persist (at detectable levels) in some soils and not in others. One factor which may affect the persistence of inoculum is the presence of soil organic matter which could act as a substrate for growth and survival. With this in mind, a field trial was set up in 2011 to monitor soil inoculum levels during the potato crop and for a period thereafter, in plots with low and high levels of organic matter, which have been amended with either R. solani sclerotia or mycelia.*

The aim of this experiment was to investigate the effect of seed- and soil-borne R. solani *(AG3) inoculum (in the form of either sclerotia or hyphae) on black scurf and on tuber size distribution, and the interaction with the level of soil organic matter under field conditions.*

JHI experiment 2011

Materials and methods

A field was selected which was naturally very low in organic matter; 0.2 g organic matter per kg of soil. A split plot design was created, with high and low organic matter areas of the field as main plots. A high level of soil organic matter was created with the incorporation of cattle manure mixed with barley straw (approximately 35 t/ha) which increased the organic matter to 0.5g per kg, which whilst being referred to a high is, in fact, still relatively low.

Seed

Mini-tubers of the cultivar Markies were kept at 4 °C in the dark until required. A visual assessment of disease was made on 50 tubers, and 10 tubers peeled and assessed individually for *R. solani* contamination using real-time PCR tested negative for both *R. solani* AG3 and AG 2-1.

Soil inoculum

Four treatments were created in each of the main-plots:

i) Treatment A. soil inoculated with sclerotia and planted with mini-tubers
ii) Treatment B. soil inoculated with hyphae and planted with mini-tubers
iii) Treatment C. soil un-inoculated, planted with mini-tubers
iv) Treatment D. soil un-inoculated but planted with mini-tubers inoculated with *R. solani.*

Treatment A. Sclerotia in the soil

To eight batches of 2 litres of vermiculite, 5g of dried sclerotia [*R. solani* isolate Rs08 (provided by Fera)] were added to each and mixed. The contents of one bag were spread along the drill at planting to each plot of treatment A. The plot area was 6.5 m x 0.8 m = 5.2 m^2. The soil volume if taken to be 0.2 m deep, would be 1.04 m^3. One litre of soil weighed approximately 1 kg, therefore, 1.04 m3 of soil would weigh 1040kg.

With 5 g of sclerotia added to this soil, it approximates to 0.005g sclerotia per kg soil (0.000005 w/w). However, the inoculum will almost certainly not have been homogenous throughout this volume of soil, and will be more concentrated around the planted tubers.

Treatment B. Hyphae in the soil
Sand/cornmeal mix was inoculated with *R. solani* isolate Rs08 (provided by Fera). The mix was prepared as 100 parts sand: 15 parts distilled water: 5 parts cornmeal, autoclaved, inoculated and shaken each day for 3 weeks. This was applied directly to the drill in Treatment B.

Treatment C. Control
No inoculum added to soil and untreated mini-tubers

Treatment D. Inoculated seed
Ten plates of *R. solani* (Rs08) were blended in 3 litres of tap water. Mini-tubers were placed in a net bag and dipped into the inoculum suspension, ensuring all were submerged for 2 minutes. Excess inoculum was shaken off, and tubers allowed to-air dry overnight.

There were four replicates for each treatment. Each plot consists of a single row of 13 plants with a guard at either end, surrounded by a guard row. Irrigation and crop management were as for a commercial crop.

The trial was planted on the 12th May 2011; emergence was recorded at regular intervals. A sample harvest, consisting of a single plant per plot was dug up on the 23 August (103 DAP). The number of main and secondary stems per plant were recorded, as were the severity of stem canker on a scale of 0 to 3 (0 = no stem canker, 1= lesions with 1-33% circumference of stems infected, 2= 34-66 % and 3= lesions over 67 % of circumference of stem infected), the total number of stolons and the number of pruned stolons per plant. At the time of final harvest 4 October (145 DAP), all progeny tubers from the remaining 12 plants per plot were assessed for black scurf and results expressed as the mean incidence and severity of black scurf disease per plot. Tubers from each plot were graded to give tuber size distribution and yield.

Soil samples
Two sets of samples were taken prior to the trial being planted to ensure that no detectable inoculum was in the soil prior to the creation of the treatments. The first was taken to establish that the trial area had no detectable inoculum, the second to ensure that no inoculum was introduced with the addition of organic matter into the split plot design. Both these samples consisted of a bulk of 100 x 10g cores (top 15 cm of the soil profile), the first taken across the whole trial area, the second from each of the two main-plots, once the area amended with organic matter had been incorporated.

After the treatments had been established and the trial planted, the area was left for one week, a soil sample was then taken from each of the plots, each consisting of a bulk of 25 cores per plot. Similarly, soil samples were taken from each plot at the time of the mid-season sampling (23rd August) and immediately after the final harvest.

To establish if the incorporation of additional organic matter into the soil affected the persistence of inoculum, soil samples were taken from each of the two main-plot areas, in December 2011 (10 weeks post-harvest) and February 2012 (18 weeks post-harvest).

Results and discussion

Within 5 weeks of planting the control and seed inoculated treatments in both the low- and high-organic plots had reached over 90 % emergence. Emergence was progressively delayed in the low-organic sclerotia, low-organic hyphae, high-organic hyphae and high organic sclerotia plots respectively, with the later taking over 13 weeks from planting to reach over 90% emergence (Figure 35).

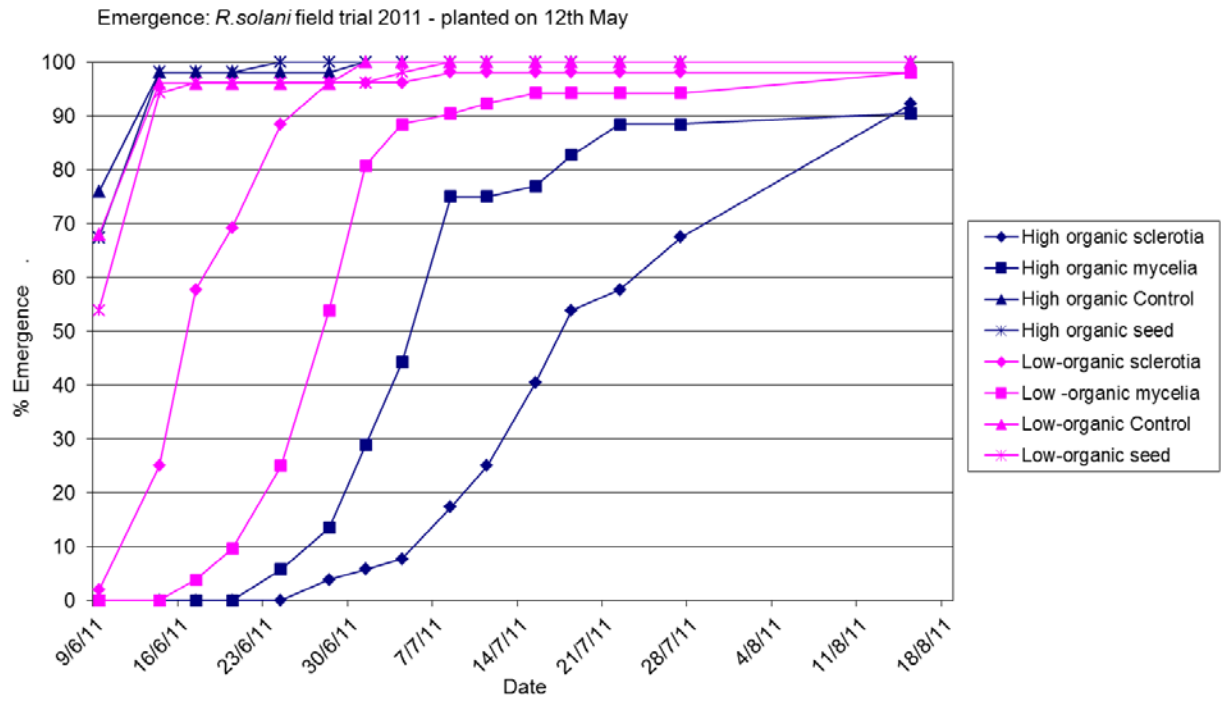


Figure 35. Emergence of plants in the 8 treatments over time. Mean of four replicate plots per treatment.

Mid-season harvest

At the time of the sample harvest, there was no difference in the number of main-stems per plant or the total number of stolons per plant between treatments (Figures 36A and 36D). The soil inoculum (sclerotia and hyphae) in the high-organic plots and the hyphae soil inoculum in the low-organic plots were associated with fewer secondary stems than other treatments ($p<0.05$)- Figure 36B.

The severity of stem canker was slightly, but significantly ($P<0.05$) higher in plots with hyphal inoculum in high-organic soils, and both hyphal and sclerotial inoculum in low-organic soils (Figure 36C). The relatively low levels of stem canker in the high-organic

soil inoculated with sclerotia may be associated with the delay in emergence in this treatment. If growing stems become infected early on, and stem canker develops with time, then stems from the high organic sclerotial plots may have been initiated later, after initial stems were pruned, and will have had less time for stem canker to develop by the time of the sample harvest. Both the high-organic soil inoculum types were associated with greater numbers of pruned stolons ($p<0.05$) than any other treatments (Figure 36E).

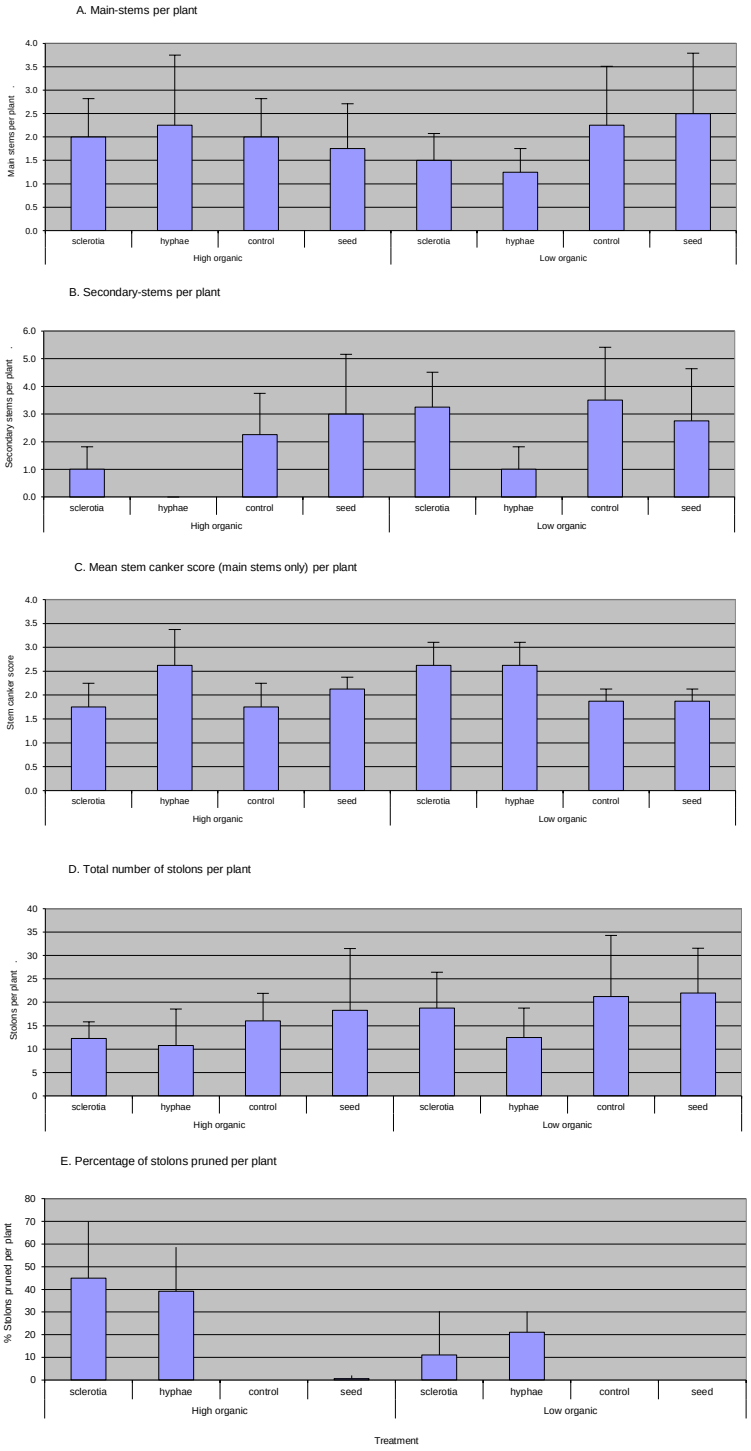


Figure 36. The effect of seed- and soil-borne inoculum treatments on A. main-stems and B. secondary-stems (mean number per plant), C. the mean severity of stem canker on main-stems, D. the total number of stolons per plant and E. the percentage of stolons which had been pruned per plant. Mean of four replicates + standard error.

Final harvest

In the high organic soil, the addition of inoculum either as sclerotia or mycelia caused significantly more black scurf, both incidence and severity (Figure 37) than controls.

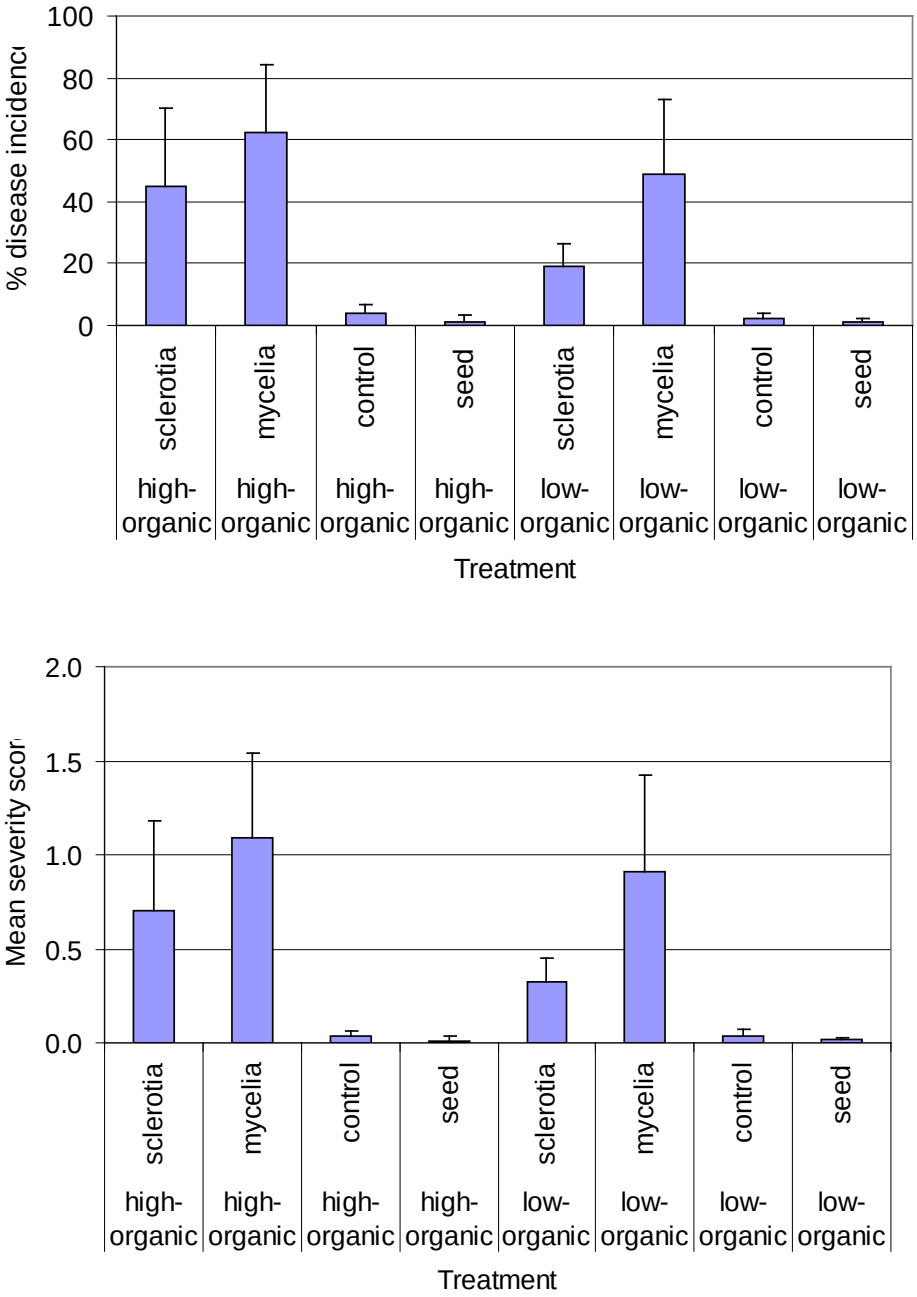


Figure 37. The effect of seed- and soil-borne inoculum treatments on the black scurf incidence and severity score at final harvest (mean of four replicates).

Whilst there was no significant difference in the extent of black scurf caused by mycelia in the low organic treatments compared to high organic, sclerotia added to low organic soil resulted in less disease than either mycelia in low organic soil, or either inoculum source in high organic soils.

There was no significant difference between the seed inoculum treatment and the control, but this may have been due to the method of inoculation, which whilst having proved successful in other experiments, does not appear to have contributed inoculum in to the system. It is possible that the hyphae did not survive on the tubers prior to planting.

The yield of tubers (Figure 38) was significantly lower in treatments with soil inoculum (both sclerotia and hyphae) and the effects were worse in the high-organic soils. The progressive reduction in yield from a maximum of 25kg per plot (equivalent to 52 t/ha) in low-organic sclerotia, low-organic hyphae, high-organic hyphae and high organic sclerotia reflects the length of delay in emergence between these treatments.

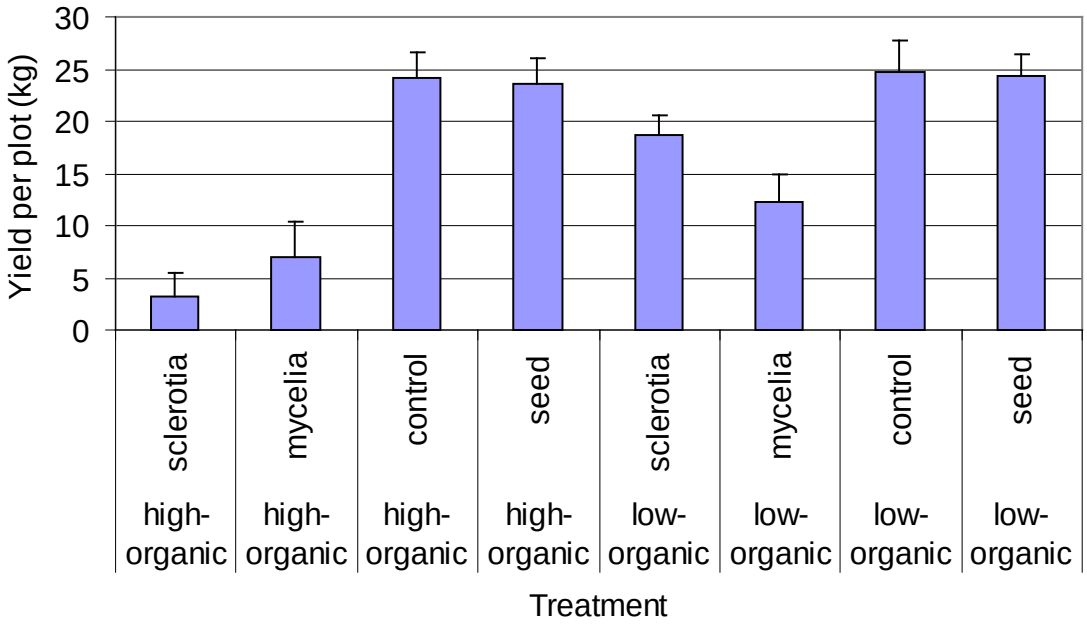


Figure 38. The effect of seed- and soil-borne inoculum treatments and organic matter on the yield of tubers (kg per plot) at final harvest (mean of four replicate plots).

Not only was the total yield of tubers affected by the soil-inoculum treatments, but the tuber size distribution also (Figure 39). The two soil inoculum treatments in the high-organic soils resulted in tubers predominantly in the <45 mm grade. The soil inoculum treatments in low-organic soils resulted in tubers predominantly in the 45-65 grade. Whilst progeny tubers from the seed and control treatments in both low- and high-organic soils were predominantly 65-85 mm.

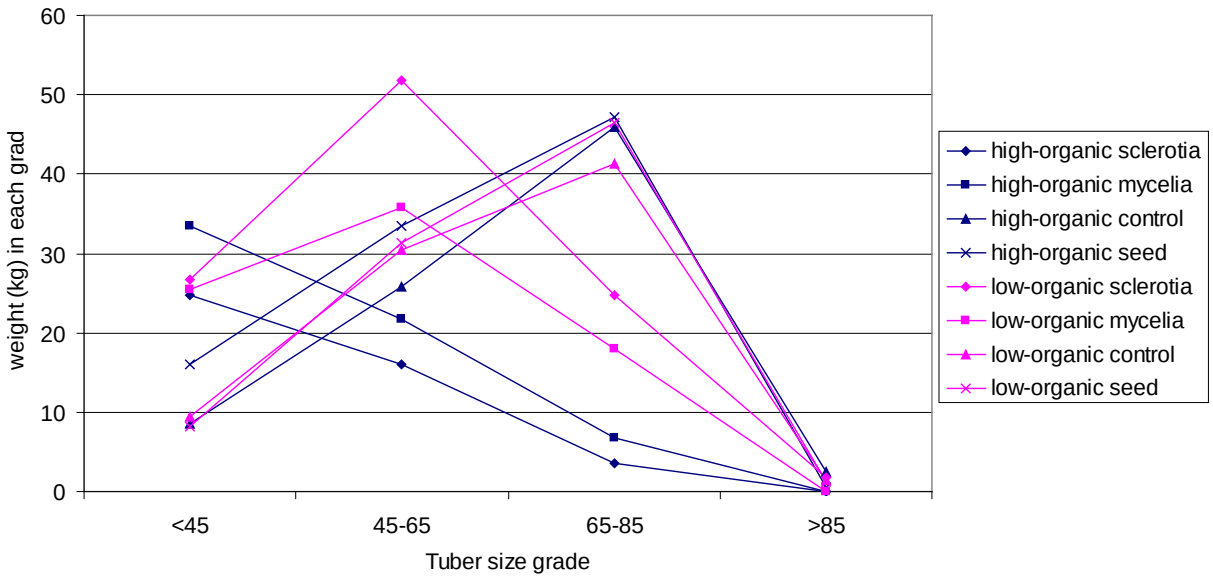


Figure 39. The effect of seed- and soil-borne inoculum treatments on the tuber size distribution at final harvest (mean of four replicate plots).

Soil samples:
Soil samples were collected from each plot on three occasions, the first, one week after the trial was planted and the plots inoculated; the second at the time of the sample harvest; and the third immediately after harvest. There were very few samples at any occasion in which inoculum was detected.

Post planting, inoculum was detected in only two plots, both low-organic amended with hyphae. In the mid season samples, two plots, one of the above and a high-organic soil amended with hyphae had detectable levels of inoculum. Finally, post-harvest, one plot of the high organic soil amended with sclerotia, one of the low organic soil with inoculated seed, and one plot of high organic soil with inoculated seed were found with detectable levels of inoculum.

In the soil samples taken from each of the main-plots in December and February following harvest, only a trace amount of inoculum was detected in two samples. In December this was in the low organic main-plot and in February in the high organic main-plot. Therefore the results indicate that very low levels of inoculum (around the threshold of detection) are present in the soil, but increasing the level of organic matter in the soil did not have a significant, consistent effect on persistence of pathogen inoculum.

4.3.7. Controlled environment experiments to investigate the importance of anastomosis group (AG) in determining the severity of stem and tuber disease

Two controlled environment experiments were carried out .The first experiment compared 12 different R. solani *isolates representative of AG2-1, AG3 (all three subgroups), AG4 (two subgroups) and AG5 plus an isolate of a previously undescribed binucleate* Rhizoctonia *(BNR) species isolated from a UK potato tuber. BNR species are distinct from multinucleate* R. solani*. BNR species have previously been isolated from potato plants (Carling et al., 1986; Lehtonen et al., 2008) although are widely believed to have little or no role in causing disease on potato (Tsror, 2010).The second experiment compared the aggressiveness of more isolates from three different subgroups of AG4*.

Experiment 1:Fera experiment 2010

Materials and methods

Inoculum consisted of four 18 mm fully colonised PDA plugs taken from a culture of the appropriate isolate (Table 37). This inoculum was placed on top of a mini-tuber (cv. Santé) in a 2 litre pot. Tubers were planted at approximately 15 cm depth. Pots (height 22 cm, diameter 25 cm) were used and each contained 3.75 kg of compost. Tubers for this experiment were in the size range of 35-45 cm. Pots were placed in a controlled environment room; each treatment consisted of eight replicate plants in separate pots containing John Innes No. 3 compost as the growing medium. After four weeks, four replicates were harvested and were assessed using the disease keys for stems from Carling & Leiner (1990). The relative surface area of black scurf on the seed tuber was also recorded using the black scurf severity key from Woodhall *et al*., (2008). After a further 14 weeks, the haulms of the plants were removed and after an additional 3 weeks the tubers were harvested and assessed for black scurf severity. Incidence and severity of elephant hide was also recorded.

All statistical analyses were carried out using Genstat release 13 (VSN International Ltd). Analysis of the data from the controlled environment experiment was performed by ANOVA. Where data were skewed, an arcsine transformation was performed. The contrasts directive within Genstat was used to compare treatment levels where many comparisons were possible.

Table 37. Code, source, anastomosis group, original host, origin and year of isolation for the reference isolates of *R. solani* used in this study

Isolate code	Other codes	AG & subgroup	Original host	Origin
cc2023		2-1	Cauliflower	UK
Y3		2-1	Potato	UK
cc2314		2-1	Cauliflower	UK
rs09B		2-1	Potato	UK
rs08		3-PT[1]	Potato	UK
rs09C		3-PT	Potato	UK
GH3		3-TB[2]	Tobacco	USA
Tom19a		3-TOM[3]	Tomato	USA
cc1903	R25, IMI303162	4 HG-II[4]	Bean	UK
cc2317		4 HG-II	Cauliflower	UK
44Rs	ATCC 14007	4 HG-III	Sugar beet	Japan
Rs09A		5	Potato	UK
cc43		BNR[5]	Potato	UK

[1]Potato subgroup of AG3; [2]Tobacco subgroup of AG3; [3]Tomato subgroup of AG3; [4]HG denotes a homogenous group – in total three HGs exist within AG4; [5]Binucleate Rhizoctonia species

Table 38. Effect of isolate from different AG/subgroups on severity of stem canker, stem death, root disease and development of black scurf of mother tuber

Isolate code	AG & subgroup	Stem disease key(0-4)	Stems pruned (%)	Mother tuber black scurf severity key (0-5)	Incidence Black scurf on daughter tubers	Incidence of Elephant hide on daughter tubers
cc2023	2-1	0.0 (0.0)[b]	0	0.0 (0.0)	0	0
Y3	2-1	0.0 (0.0)	0	0.0 (0.0)	0	0
cc2314	2-1	1.1 (6.1)	0	0.0 (0.0)	0	0
rs09B	2-1	0.4 (2.4)	0	0.0 (0.0)	0	0
rs08	3-Potato	2.9 (9.8)	54.2	2.0 (8.1)	0	25
rs09C	3-Potato	0.6 (3.7)	0	1.5 (6.1)	54.2	0
GH3	3-Tobacco	0.0 (0.0)	0	0.0 (0.0)	0	0
Tom19a	3-Tomato	0.0 (0.0)	0	0.0 (0.0)	0	0
cc1903	4 HG-II	0.4 (2.4)	0	0.0 (0.0)	0	0
cc2317	4 HG-II	0.0 (0.0)	0	0.0 (0.0)	0	0
4Rs	4 HG-III	1.4 (6.8)	0	0.0 (0.0)	0	0
Rs09A	5	2.0 (8.1)	0	0.0 (0.0)	0	11.8
cc43	BNR	1.0 (5.7)	0	0.0 (0.0)	0	20.91
Control		0.0 (0.0)	0	0.0 (0.0)	0	0
LSD (P<0.05; df 55)		(1.99)	15.93	(1.55)	15.89	14.42

[b]Arcsine transformed values for non-normal data given in parenthesis.

Results and discussion

Results of the controlled environment trial comparing different isolates representative of several different AGs are given in Table 38. Eight of the isolates tested were able to infect potato stems and were successfully re-isolated from at least one inoculated plant. Two of the four AG2-1 isolates infected potato stems producing slight stem canker lesions, similar to the BNR isolate. Of the AG4 isolates tested, one AG4 HG-II isolate did not appear to infect potatoes whilst the other isolates did. The AG5 isolate caused relatively severe lesions, second only to the AG3-PT isolate, Rs08, which was the most aggressive isolate tested. This was the only isolate to cause stem death. In addition, sclerotia only developed on the mother tubers of plants inoculated with isolates of AG3-PT. No disease was observed in the non-inoculated control plants. Black scurf was only observed in one AG3-PT treatment (isolate Rs09C) but in the other AG3-PT treatment high levels of elephant hide were observed. Elephant hide symptoms were also observed in potatoes inoculated with AG5 and BNR. Although scored as elephant hide, the symptoms caused by the BNR isolate was less visibly severe than the elephant hide caused by AG3-PT and AG5 appearing lighter in colour.
.

This study confirms that isolates of AG3-PT are amongst the most aggressive to potato stems. However, other AGs also caused severe stem disease, including isolates belonging to AG2-1, AG4 and AG5. However, only the AG3-PT isolates caused sclerotia to develop on the mother tuber and the progeny tubers. The development of such sclerotia on progeny tubers could facilitate its survival and dispersal in seed, perhaps at least partially explaining why AG3-PT is the predominant AG in UK potato crops. Interestingly, the aggressive stem canker isolate of AG3-PT caused elephant hide and no black scurf. Elephant hide symptoms were also observed with the BNR isolate and AG5.

Isolates from tomato and tobacco subgroups of AG3 failed to cause disease on potato stems, suggesting that these subgroups may be host specific. Amongst AG2-1 isolates, a wide range of pathogenicity existed. AG2-1 is a genetically diverse group and also one with a wide host range. A large variation in pathogenicity to potato has been observed previously (Woodhall *et al*., 2008) which was why four isolates of AG2-1 were tested in this experiment. Further work to investigate the determinants of pathogenicity and diversity amongst AG2-1 is required.

Experiment 2

This experiment follows on from the previous experiment by comparing disease caused by more isolates from the three different subgroups of AG4 with an AG3 isolate included for comparison. Previous published studies have shown that AG4 can cause disease on potato in a variety of climates including Peru (Anguiz and Martin, 1989) and Canada (Bains and Bisht, 1995). Isolates of AG4 are present in the UK (Budge et al., 2009a) yet have not been found in UK potatoes. An experiment was therefore undertaken to determine the risk AG4 isolates from three different subgroups (homogenous groups or 'HG') can pose to UK potato crops. The experiment was carried out at 21°C in a controlled environment room.

Fera experiment 2010

Materials and methods

Mini-tubers of the cultivar Santé were visually checked for diseases and healthy tubers selected were disinfected by immersion in sodium hypochlorite (1% available chlorine) for 10 minutes and rinsed twice in distilled water. Details of isolates are given in Table 39.

Mini-tubers were planted individually in 2 litre pots (174 mm height, 150 mm diameter) filled with compost (John Innes No.3) to a depth of 150 mm. The mini-tuber was then covered with approximately 20 mm of compost and pots were inoculated with seven, 17 mm plugs taken from 10 day old PDA plates of the appropriate isolate. Eleven different isolates of AG4 and one isolate of AG3-PT (Rs08) were tested. One treatment remained un-inoculated as a control. The remainder of the pot was filled with compost. Each treatment was replicated four times.

After 21 days, plants were harvested, washed and assessed for stem disease according to the key of Carling & Leiner (1990). Root disease was assessed as follows: 0 = no visible damage, 1 = 1 to 10% of the root area infected, 2 = 11 to 25% of the root area infected, 3 = 26 to 50% of the root area infected, 4 = 51% or more of the root area infected. Black scurf development on the seed tuber was also assessed as in Woodhall *et al.,* (2008).

Analysis of the data from the controlled environment experiment was performed by an accumulated analysis of variance (Generalised Linear Model with either a binomial distribution error term and logit-link function, or a normal distribution term).

Table 39. Code, source, AG, host and country of origin for the isolates used in the AG4 experiment study.

Isolate code	Other codes for isolate	AG	Original host	Origin
Rs08		AG3-PT3	Potato	UK
1873	MWR5-2	AG4 HG-I	Rose	Japan
AH-1	4i_AB012	AG4 HG-I	Peanut	Japan
Chr3		AG4 HG-I	Chrysanthemum	Japan
1903	R25, IMI303162	AG4 HG-II	Bean	UK
2307		AG4 HG-II	Cauliflower	UK
2316		AG4 HG-II	Cauliflower	UK
2317		AG4 HG-II	Cauliflower	UK
4K		AG4 HG-II	Not known	Japan
R90	Rh165, 04-03,	AG4 HG-II	Sugar beet	Japan
44Rs	ATCC 14007	AG4 HG-III	Sugar beet	US
Rs185		AG4 HG-III	Peanut	USA

Results and discussion

Differences in the aggressiveness to potato could be observed between the different AG4 subgroups. All isolates of AG4 HG-I and AG4 HG-III infected 100% of the stems present; with an average stem infection score of at least 2 in all cases. Stem death was present in some instances for the AG4 HG-I isolates but not the AG4 HG-III isolates. Root infection was present in plants infected with AG4 HG-I and AG4 HG-III with an average score of 0.75 or 1 in all instances. Between 16.7% to 37.5% of stolons were infected in AG4 HG-I and AG4 HG-III inoculated plants. No infection of stems, stolons and roots was observed for AG4 HG-II inoculated plants except for one isolate (4K) where several small lesions no bigger than 5 mm were observed on stems in a some instances. For all AG4 infected plants, no sclerotia or blemishes were present on the mother potato tuber, unlike the AG3-PT inoculated plant where sclerotia were frequently observed. The AG3-PT isolate caused the most severe stem disease (in terms of stem canker index) (Table 40, $P<0.001$). In addition, AG3-PT caused death in 18% of stems. Although this is numerically higher that the incidence of stem death caused by isolates in the other AGs tested, this difference was not significant (Table 40, $P=0.153$). In all instances of disease symptoms occurring above, Koch's postulates were completed, by successfully re-isolating onto PDA the strain used to inoculate the plant. No disease symptoms were observed in plants in the un-inoculated control treatment.

Table 40. Percentage stem infection, stem death, secondary stem death, stolon infection and disease keys (0 to 4 scale based on surface area affected) for stem disease, root lesions and black scurf on mother tubers for mini-tubers inoculated with isolates of *Rhizctonia solani* AG4 or AG3-PT in a controlled environment experiment. LSDs and significance levels were calculated using a generalised linear modelling analysis.

Code	AG & Subgroup	% Stems Killed	% Stems infected	% Stolons Infected	Stem canker index (0-4)	Root infection severity	Black Scurf on Mother Tuber
Rs08	AG3-PT	18.3	100.0	0.0	2.9	1.0	1.8
AH-1	AG4 HG-I	0.0	100.0	16. 7	2.3	1.0	0
Chr3	AG4 HG-I	5.0	100.0	25.0	2.2	0.8	0
1873	AG4 HG-I	12.5	100.0	37.5	2.5	1.0	0
4K	AG4 HG-II	0.0	33.3	0.0	0.4	0.0	0
1903	AG4 HG-II	0.0	0.0	0.0	0.0	0.0	0
R90	AG4 HG-II	0.0	0.0	0.0	0.0	0.0	0
2317	AG4 HG-II	0.0	0.0	0.0	0.0	0.0	0
2316	AG4 HG-II	0.0	0.0	0.0	0.0	0.0	0
2307	AG4 HG-II	0.0	0.0	0.0	0.0	0.0	0
44RS	AG4 HG-III	0.0	100.0	31.3	2.0	1.0	0
Rs-185	AG4 HG-III	0.0	100.0	37.5	2.4	1.0	0
control	Negative	0.0	0.0	0	0.0	0.0	0
$LSD_{(P=0.05)}$		13.63	18.70	40.52	0.46	0.198	0.38
P (51 df)		0.153	<0.01	0.286	<0.01	<0.01	<0.01

Some differences in pathogenicity occurred with the three AG4 subgroups tested. Isolates of AG4 HG-I and AG4 HG-III both caused disease whilst on the whole AG4 HG-II did not. Isolates from these subgroups have previously been isolated from solanaceous weeds in Brazil and shown to have pathogenicty to potato (Silva-Barreto *et al.,* 2010). Although AG4 HG-II is widespread in the UK (Budge *et al.,* 2009a) it has never been reported in UK potatoes. Literature searches suggest that AG4 HG-I and AG4 HG-III are both not present in the UK. The results from this study suggest that the AG4 HG-II subgroup of AG4 is less aggressive to potatoes compared to the other sungroups. This subgroup has been reported in potatoes in Idaho (Woodhall *et al.*, 2012) and Finland (Lehtonen *et al.,* 2009), although was detected at low frequencies compared to AG3-PT. It is unclear why, despite being present in the UK in other crops, it is not more prevalent in potatoes. This could be because UK cropping practices do not select for the AG in soil or the UK climate is not conducive to disease development. This experiment shows that subgroups of AG4 can differ in terms of pathogenicity to potato. AG4 HG-II, is less pathogenic to potato than the other two AG4 subgroups. Due to its apparent absence in UK potatoes crops and limited pathogenicity, it may be that assays for this subgroup do not need to be included, at least initially, in screening for different AGs in soil and tuber material.

4.3.8. Monitoring individual *R. solani* anastomosis groups (AG) in tubers and soil

The aim of this monitoring programme was to determine the prevalence of AGs in British seed and soils and to quantify AG3-PT DNA in soil pre-planting to determine if disease risk can be predicted. In addition, the aim of the survey was to confirm if AG3-PT was still the predominant AG in UK crops with the use of direct molecular testing.

Monitoring individual *Rhizoctonia solani* AGs in seed and ware tubers Fera experiment 2009-2011:

Materials and methods

Individual potato tuber samples (from a combination of crops being grown for both ware and seed) were tested for AG2-1, AG3-PT, AG5 and AG8 using real-time PCR. 25 kg samples of tubers were collected from the main potato-growing regions at harvest as part of another Potato Council project. Eighty-six samples were tested (50 from 2008 harvest and 36 from 2009). From each sample, five subsamples were taken, each consisting of five tubers. DNA was extracted from potato tubers by removing a strip of peel from each tuber (from rose to stolon end). In addition, small cores (approx. 3-5 mm in depth and width) were taken from both the rose and stolon ends. Material from each sub-sample of five tubers was placed into an extraction bag (Bioreba AG, Switzerland) containing 7.5 ml PB7 (2 ml tetrasodium pyrophosphate in 100 ml phosphate buffer pH7). The contents within each extraction bag were pulverised using a large sample grinder (Bioreba AG) and the contents transferred into a 5 ml sample tube. Sample tubes were spun at 1000 rpm for 10 minutes at 4°C (Sigma 4K15 centrifuge) and the supernatant transferred to a clean, labelled 5 ml tube and spun again at 6200 rpm for 15 minutes at 4°C to pellet any sediment. DNA was extracted from each pellet using the Wizard Food kit (Promega) in conjunction with a Kingfisher ML magnetic particle processor (Thermo Electron Corporation) as above. DNA underwent a fivefold dilution prior to PCR and 10 µl DNA was used in each 25 µl reaction.

Results

AG3-PT was the most prevalent AG being present in a little over half the total number of tuber samples (Table 41). AG2-1 was the next most commonly occurring AG detected in 45% of all samples. AG5 was only detected in one sample in 2008 whilst AG8 was not detected at all.

Table 41. Incidence of *Rhizoctonia solani* AGs in 60 tuber samples harvested in 2008 and 2009, 26 tuber samples were found to not contain any AG.

Group	Number of tuber sub samples positive					
	Total positive*	1	2	3	4	5
AG2-1	39 (15)	9	4	5	5	16
AG3-PT	44 (20)	15	4	11	7	7
AG5	1 (0)	1	0	0	0	0
AG8	0					

*Figures in parenthesis are number of samples where that particular AG was the only AG detected. i.e. not mixed samples.

Detecting Rhizoctonia in Soil
Fera experiment 2009-2011
Materials and methods

Over three years (2009-2011) soil sampling was carried out from 86 potato fields pre-planting and 64 fields post planting of potatoes. Samples were predominantly from England, with less from Scotland (6 sites pre-planting, 10 post-planting). The following English counties were represented: Cambridgeshire, Cornwall, East Yorkshire, Essex, Lincolnshire, Norfolk, North Yorkshire, Nottinghamshire, Shropshire and Staffordshire.

Each sample consisted of at least 25 sample points taken per hectare. Each individual sample per point weighed approximately 40 g. Soils were air dried and DNA was extracted from 250 g of soil using standard Fera methods. After extraction, soil DNA samples were tested for *Rhizoctonia solani* AG2-1, AG5 AG8 using the primers Budge *et al*., (2009b) and for AG3-PT using the Fera assay for AG3-PT.

Where possible, fields were sampled pre and post planting although this was not always possible. A selection of fields was sampled one year after potatoes were grown to monitor decline in soils. Tuber samples (at least 30 individual tubers) were taken at harvest where possible. In 2009, some fields were sampled 6 weeks post planting.

Results and Discussion

The relative incidence of individual AGs of *R. solani* in soil pre-planting and at harvest is shown in Tables 42 to 44. At planting, only five sites were positive for AG3 for all years. At harvest, more sites were found (23) with AG3-PT. Four of the sites positive pre-planting were positive at the end of the season, it was not possible to obtain a sample for the other pre-planting AG3-PT positive site. At three of these sites black scurf was present in progeny tubers. AG2-1 was frequently detected in soils pre-planting but declined in number post planting. AG5 was found at small number of sites pre-planting and at harvest. AG8 was detected in 26 sites at planting and 16 sites at harvest.

From 12 AG3-PT positive sites (at harvest) which were sampled at both harvest and pre-planting in one year, AG3-PT was only detected in three soils at planting the following season (Table 45). Symptoms on tubers from harvest were present at all sites except one. This site was one of three sites where soil was not classed as free draining (soil classification taken from the LANDIS website) so the soil may be less conducive for disease compared to the other sites.

In one site where AG5 was detected, levels of AG5 increased in the soil one year after planting. No AG5 was detected at planting. However AG5 was detected soon after harvest (when winter wheat was planted). Levels of AG5 increased in the soil the following year. One year post planting, AG5 isolates were taken from volunteer potato stems and tubers. AG5 was also taken from the stem base of a wheat plant displaying brown lesions towards the stem base.

Table 42. AGs detected in potato soils in 2009

	No. soils at planting	No. soils at 6 weeks post planting	No. soils at harvest
AG2-1	10	5	8
AG3-PT	0	0	6
AG5	0	0	0
AG8	7	nt	3
Total number of samples	26	9	28

nt = not tested

Table 43. AGs detected in potato soils in 2010

	No. soils at planting	No. soils at harvest
AG2-1	11	4
AG3PT	2	3
AG5	0	1
AG8	8	4
Total number of samples	30	7

Table 44. AGs detected in potato soils in 2011

	No. soils at planting	No. soils at harvest
AG2-1	19	17
AG3PT	3	14
AG5	2	2
AG8	11	9
Total number of samples	27	29

Table 45. Levels of AG3-PT in soil samples monitored both at planting and at harvest for all years 2009-2011

Sample[1]	AG in soil	Soil type[2]	DNA (pg DNA/ g soil)			AG present on tuber at harvest
			Pre-planting	At harvest	One year later	
XA	5	Freely draining slightly acid sandy soils	0	455.6	2084.4	AG5[3]
XB	3-PT	Slowly permeable seasonally wet slightly acid but base-rich loamy and clayey soils	0	40.1	0	AG3-PT
XE	2-1	Freely draining slightly acid loamy soils	2030.4	266.4	225.6	nd
XE	3-PT	Freely draining slightly acid loamy soils	0	120.4	83.2[4]	AG3-PT
XZ	3-PT	Freely draining slightly acid loamy soils	0	1412.7	-	AG3-PT
YQ	3-PT	Slightly acid loamy and clayey soils with impeded drainage	19.08	3.7	-	AG3-PT
ZA	3-PT	Slowly permeable seasonally wet slightly acid but base-rich loamy and clayey soils	37.1	3.0	-	nd
ZH	3-PT	Naturally wet very acid sandy and loamy soils	0	0.4	-	AG3-PT
ZI	3-PT	Freely draining lime-rich loamy soils	0.4	903.7	-	AG3-PT
ZJ	3-PT	Freely draining lime-rich loamy soils	0	1457.5	-	AG3-PT
ZK	3-PT	Freely draining lime-rich loamy soils	2.2	187.8	-	AG3-PT
ZO	3-PT	Slowly permeable seasonally wet slightly acid but base-rich loamy and clayey soils	0	3.5	-	AG3-PT
ZX	3-PT	Freely draining slightly acid sandy soils	0	25.1	-	AG3-PT
ZY	3-PT	Freely draining slightly acid sandy soils	0	9.0[2]	-	AG3-PT

[1] Samples beginning with X denotes 2009; Y denotes 2010 and Z denotes 2011; [2] Soil type given as described in the LANDIS website.

[3]AG5 was isolated from wheat and a volunteer potato plant;[4]AG-3PT isolate from potato volunteers as well as harvested tubers; Nd: not detected

In this study, DNA from seed tubers and soils were directly tested for *R. solani* AG3-PT, AG2-1, AG5 and AG8. The proportions of different AGs in the different samples were largely concordant with a previously published study, which had used isolation techniques (Woodhall *et al.,* 2007) although the incidence of AG2-1 using direct PCR testing was much higher than by isolation. In the current study, an isolate of BNR was also found. Using direct testing AG3-PT appeared to be the predominant AG in tubers. The lack of isolation of AG2-1 from seed tubers maybe due to isolates of AG2-1 being relatively poor producers of sclerotia (Woodhall et al., 2008) and they display a broad range of pathogenicity to potatoes with some isolates causing little or no disease (see above). AG2-1 was also widely present in soil both pre-planting and post planting. However, AG3-PT was rarely detected in soil pre-planting (five times from 83 samples) but was detected in 35% of samples at harvest. Given that AG3-PT was detected in the majority of seed borne samples this may suggest that seed borne inoculum is of particular importance in UK potato crops.

AG8 was not detected in any seed tuber sample but was detected in soils at planting and at harvest. As AG8 appears to almost exclusively infect roots (Hide & Firmager, 1990; Woodhall et al., 2008) this result was as expected.

Isolating *R. solani* from tubers originating from the seed and soils monitored

Fera experiment 2009-2011

Materials and methods

Direct isolation was used on a subset of samples to validate the real-time PCR data. From 30 samples from the tuber survey and 40 samples originating from the soil survey (which were taken from fields at harvest where possible), 25 tubers from each sample was visually examined. Isolations were attempted from a range of blemishes and asymptomatic tubers. Pure colonies were obtained and isolates were identified using the appropriate real-time PCR assay or sequencing of the rDNA ITS region with primers ITS1 and ITS4 (White *et al.*, 1990).

Results and Discussion

R. solani was isolated from 36 of the 70 tubers taken from both tuber and soil surveys (Table 46). The majority of isolates belonged to AG3-PT and were isolated from black scurf and elephant hide symptoms. AG2-1 was isolated from elephant hide symptoms. AG5 was isolated from sclerotia on potatoes. One isolate from a tuber sample was consistently recovered from areas of less severe elephant hide symptoms, which often covered as much as 50% of the tuber surface. This could not be assigned to a specific AG by this PCR for AG2-1, 3-PT, 5 or 8. To determine the identity of this isolate, the hyphae were stained with trypan blue in lactoglycerol to visualise nuclei. Under 400x magnification, each cell had two nuclei indicating the strain was a binucleate *Rhizoctonia* species (BNR). The mean width of 20 mature hyphae was 5.1 µm (range 3.81 to 7.63 µm,s.d.= 0.89 µm) and consistent with BNR species. Sequencing of the rDNA ITS region was undertaken for the isolate with primers ITS1 and ITS4 (White *et al.,*1990). The resulting sequence (GenBank Accession No. FR828480) was compared to other sequences in GenBank. No near identical matches were found suggesting the

isolate could be a unique strain of a particular BNR or belong to a previously undescribed AG.

Table 46. AG of isolates taken from 30 seed and ware tuber samples and 40 tubers originating from soil monitoring

AG	Black scurf	Elephant hide	Total
2-1		2	2
3	28	4	32
5	1		1
BNR		1	1
negative			34

An overview of all monitoring data for all years including that of a previous study is given in Table 47. This confirms that AG3-PT is the predominant AG in potato material overall, although AG2-1 is frequently detected in soils.

Table 47. Summary of all data monitoring AGs in UK potato crops and all field soil samples

	Isolation			Real-time PCR all years		
	Stems, stolons & roots*	Tubers*	Tubers	Tubers	Soil at planting	Soil at harvest
AG2-1	4	5	2	40	40	29
AG3-PT	61	64	32	45	5	23
AG5	0	1	1	1	2	3
AG8	0	0	0	0	26	16
BNR	0	0	1	nt	nt	nt
Total number of samples	176		70	86	83	64

*Results from Woodhall *et al*., 2007, included for comparison

Monitoring decline of Rhizoctonia solani AG3-PT in potato field soil

Soil from 18 plots across two fields (sampled as part of work described above) was analysed by real-time PCR to determine the level of AG3-PT decline after harvest.

Fera experiment 2009-2011

Materials and methods

Two AG3-PT positive fields were selected for an experiment to determine the ability of *R. solani* to survive in the soil post-harvest (see Table 45 for information about the

soils). Prior to harvest, field ZJ was divided into 13 x 30m^2 plots and Field ZI was divided into 5 plots of a similar size. At the time of harvest at least 25 tubers in each plot were assessed visually for black scurf and disease incidence was recorded. Shortly after harvest, soil sampling was undertaken of at least 15 cores over a 'w' sampling pattern within each plot. 18 weeks post-harvest soil was sampled again. For both sampling times, DNA was extracted from a 250 g soil sample.

Results and discussion

Black scurf or elephant hide was observed in all but two of the 30 m^2 plots sampled from two AG3-PT positive fields (Table 48). In all of the plots where black scurf was observed, AG3-PT was detected in soil by real-time PCR. For all plots, levels of AG3 DNA was significantly ($P<0.05$) less 18 weeks post harvest. This indicates that AG3-PT may not survive well in fields in the absence of the host crop.

Table 48. Disease presence, DNA level at time of harvest and DNA level 18 weeks post harvest for 18 plots across two fields

Field	Plot number	Disease present on tubers	DNA at harvest (pg DNA/ g soil)	DNA at +18 weeks (pg DNA/ g soil)
ZJ	1	Yes	3157.5	3.2
ZJ	2	Yes	6853.7	2.3
ZJ	3	Yes	658.9	0
ZJ	4	No	0	0
ZJ	5	Yes	406.1	0
ZJ	6	No	0	0
ZJ	7	Yes	904.9	0
ZJ	8	Yes	1136.2	266.4
ZJ	9	Yes*	0	0
ZJ	10	Yes	185.1	0
ZJ	11	Yes	761.1	13.5
ZJ	12	Yes	903.7	0
ZJ	13	Yes	8347.9	5626.5
ZI	1	Yes	2249.3	0
ZI	2	Yes	1827.0	7.7
ZI	3	Yes	323.8	225.8
ZI	4	Yes	181.7	1.5
ZI	5	Yes	187.1	78.6
Mean			1560.2	345.9

*disease was present as elephant hide in this sample and *Rhizoctonia solani* AG3-PT was isolated from the tuber. In all other plots black scurf was present.

4.3.9. Determining how *R. solani* AG3-PT survives in field soil

AG3-PT positive soils (by real-time PCR) were analysed by isolation and real-time PCR of the organic/non organic matter components in order to determine how the pathogen is surviving in soil.

Fera experiment 2009-2011

Materials & Methods

Nine naturally infested positive samples and one negative sample were tested for how AG3-PT survives in the soil. In addition three artificially inoculated samples were also tested: compost from a controlled environment experiment at Fera from 2010; and sandy loam originating from the James Hutton Institute field trial with two levels of organic matter. Each sample consisted of 750 g of soil. DNA was extracted from 250 g using the Fera soil method. The remaining 500 g was sieved through a 2.36 mm sieve and organic matter visible by eye was removed by forceps. The type of organic matter was characterised by visual identification or using a microscope. The total organic matter was then weighed before approx 0.2 ml was placed into each of 5 x 2ml tubes and processed using the Wizard® Magnetic DNA Purification System for Food (Promega) in conjunction with a Kingfisher Ml (Thermo corporation). In addition, 25 fragments (typically ranging between 2 -5 mm in size) of organic matter were plated onto tap water agar amended with penicillin and streptomycin.

Soil pellets of approximately 5 mm in diameter were formed from the sieved soil and these were directly plated onto amended tap water agar (5 per plate for five plates). DNA was also extracted from five 1 g subsamples of the sieved soil using the Fera soil DNA extraction method. DNA samples were analysed using real-time PCR for AG3-PT (Fera primers) and DNA was quantified. Inoculated TWA plates were visually checked for *Rhizoctonia* colonies 2-4 days after inoculation and number of colonies was recorded. Incidence of any *Rhizoctonia* colony ocurring on a plate was scored and a subset of isolates were subcultured for confirmation of AG.

Results and discussion

Three of the soil samples tested were from experiments at JHI or Fera and so were not naturally infested with AG3-PT. One soil was known to be AG3-PT free (no disease was observed in the crop in this field) and was therefore used as a control soil (Table 49). No AG3-PT was detected in any OM or non-OM component of this control soil sample. AG3-PT was detected and isolated more frequently from the organic matter of AG3-PT positive soils than the predominantly non-organic matter of the soil. Interestingly isolates of AG3-PT and AG5 were both recovered from decaying cereal stems at one site. At a two further sites AG3-PT was isolated from decaying cereal plant material. This may suggest that cereal plant material could be an important material for *Rhizoctonia* to survive on in fields between potato crops. In the case of AG5, wheat and barley are likely to be important alternative hosts for the pathogen.

Table 49. Soil type, origin of organic matter, total AG3 DNA and incidence of AG3 detection by real-time PCR and isolation from organic matter (OM) and non-organic matter components of soil.

Soil ref	Soil type[1]	OM type	Total soil AG3 DNA (pg DNA/g soil)	OM per kg	Real-time PCR (incidence out of 5)		Isolation (incidence out of 5)	
					OM	Non OM	OM	Non OM
XZ	Freely draining slightly acid loamy soil	Potato fragments	1412.7	6	5	1	5	0
ZZ	Freely draining slightly acid sandy soils	Cereal/potato[2]	9.1	1	5	2	5	0
ZAZ	Freely draining slightly acid loamy soil	Cereal, potato, weed	6.7	0.5	2	0	5	0
ZH1	Naturally wet very acid sandy and loamy soils	Cereal & potato fragments	415.2	0.9	0	0	2	0
ZH2	Naturally wet very acid sandy and loamy soils	Cereal & potato fragments	4286.0	0.1	0	0	3	1
ZH3	Naturally wet very acid sandy and loamy soils	Cereal & potato fragments	92.0	1	0	0	3	0
ZH4 (Control as	Naturally wet very acid	Cereal & potato fragments	0	0.7	0	0	0	0

no AG3 present)	sandy and loamy soils							
JH Low	Sandy loam	Mostly Cereal fragments	4.8	0.2	2	2	2	1
JH High	Sandy loam	Mostly Cereal fragments	0	0.5	0	0	0	0
0.005% soil spike	John Innes Compost	Potato fragments	431.4	20	4	2	5	0
ZK	Freely draining lime-rich loamy soils	Cereal & potato fragments	187.8	6	0	0	4	0
ZI	Freely draining lime-rich loamy soils	Mostly cereal fragments	903.7	4	4	2	5	0
ZJ	Freely draining lime-rich loamy soils	Cereal & potato fragments	1457.5	14	3	3	2	0
Mean					2.1	1	3.4	0.2

[1]Soil type taken from the LANDIS website
[2]Both AG5 and AG3-PT isolates from decaying barley stem

4.4. *Pathogen distribution and soil sampling*

Fera and SARDI experiments and analysis

Introduction

There is little published information on sampling strategies for soil-borne pathogens of potato (i.e. *Spongospora subterranea, Rhizoctonia solani* and *Colletotrichum coccodes*). Potts (2005) reviewed sampling strategies for soil borne fungal pathogens as part of an earlier Potato Council-funded diagnostics project 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 some research into sampling for potato cyst nematodes (Turner, 1993; Schomaker & Been, 1999). The conclusion is that an 'X' or 'W' pattern of 40 sample points over a 4 Ha sample area adequately represented the overall field mean (cyst counts per unit soil). Of more general interest, Ettema & Wardle (2002) reviewed work in the area of geostatistics relating to soil-borne microbes. Their main conclusion was that the spatial variability and patterns of soil organisms appears to be random. However, this variability often has a predictable spatial structure. Studies have shown that spatial heterogeneity of populations of soil organisms can be found at scales ranging from millimetres to hundreds of meters.

Research has been undertaken at SARDI, Australia and Fera, UK to determine spatial variability of *R. solani* AG3 based on intensively sampled grids. These data were analysed across scales ranging from 4 ha to 50 Ha.

Material and methods

Sample collection and preparation

A total of 8 data sets from intensively sampled pivots (i.e. circular fields under pivot irrigation) for *R. solani* were available from SARDI, Australian. Cores were collected to a depth of c. 15 cm in a uniform grid pattern of either 50 m or 100 m, apart. In GB, two 10 x 10 point grids each sampled from an area of approximately 4 ha (collected by SAC and JHI) were tested for *R. solani* AG3. In addition, a further two 10 x 10 point grids were sampled from areas of approximately 40 ha by Fera. Soils were air dried and DNA was extracted from 60 g (SAC, JHI, SARDI) or 250 g (Fera) of soil using methods in use at each laboratory. Soil DNA samples were tested by the laboratory that collected the samples for *R. solani* AG3-PT levels using pathogen-specific primers and probe.

Analysis of data

Analysis was carried out using the following geostatistical methods: analysis and modelling of semi-variance, plotting contour maps generated by kriging, analysis of Ripley's K-function. All were analysed using Genstat 14th Edition (VSN International Ltd).

Table 50. List of intensively samples fields. Of the 12 fields sampled, 4 provided suitable data to enable geostatistical analysis (C27, O34M, C1M and Portsoy).

Field name	Country Location	Sample time	Approx. area sampled (ha)	Mean pathogen level (pg DNA/g soil)
P4	W Australia	Pre plant	50	1.2
O7	W Australia	Pre plant	50	1.8
C27	W Australia	Pre plant	50	121.3
O34M	W Australia	Pre plant	50	138.3
O21L	W Australia	Pre plant	50	20.9
P6M	W Australia	Pre plant	50	2.8
C1M	W Australia	Pre plant	50	59.4
C4L	W Australia	Pre plant	50 (30 data points)	105.5
Portsoy	Scotland	After crop	4	6627.3
Keam	Scotland	After crop	4	<1.0
ZA	England	Pre plant	40	<1.0
F22	England	Pre plant	40	<1.0

These data were analysed to determine the degree of pathogen aggregation (uniform, aggregated, random) in soil for each of the *R. solani*. An estimate of aggregation (*K*) and mean pathogen level will be obtained by fitting a negative binomial model to each data set. This will be done on different field scales and using a number of sampling patterns (c. five). The analysis will be used to test whether a single sampling strategy is suitable for providing a reasonable estimate of the 'true' mean for the target pathogen. It is possible that a pathogen with a highly aggregated distribution will require a modified sampling strategy. For example, a threshold value of *K*, as well as a large difference between measured and true means, will indicate that the sampling unit is inappropriate and that a different sampling area needs to be considered.

Results

Contour maps of Kriged estimates of *Rhizoctonia solani* AG3 DNA levels in intensively sampled soil samples are shown in Figure 40. The levels of *R. solani* AG3 are highly variable within sites. In addition, the patterns of *R. solani* distribution vary considerably from site to site. The distribution patterns display chaotic features. This means that it is not possible to draw general conclusions about the distribution of R*. solani* in soil from geostatistical contour maps.

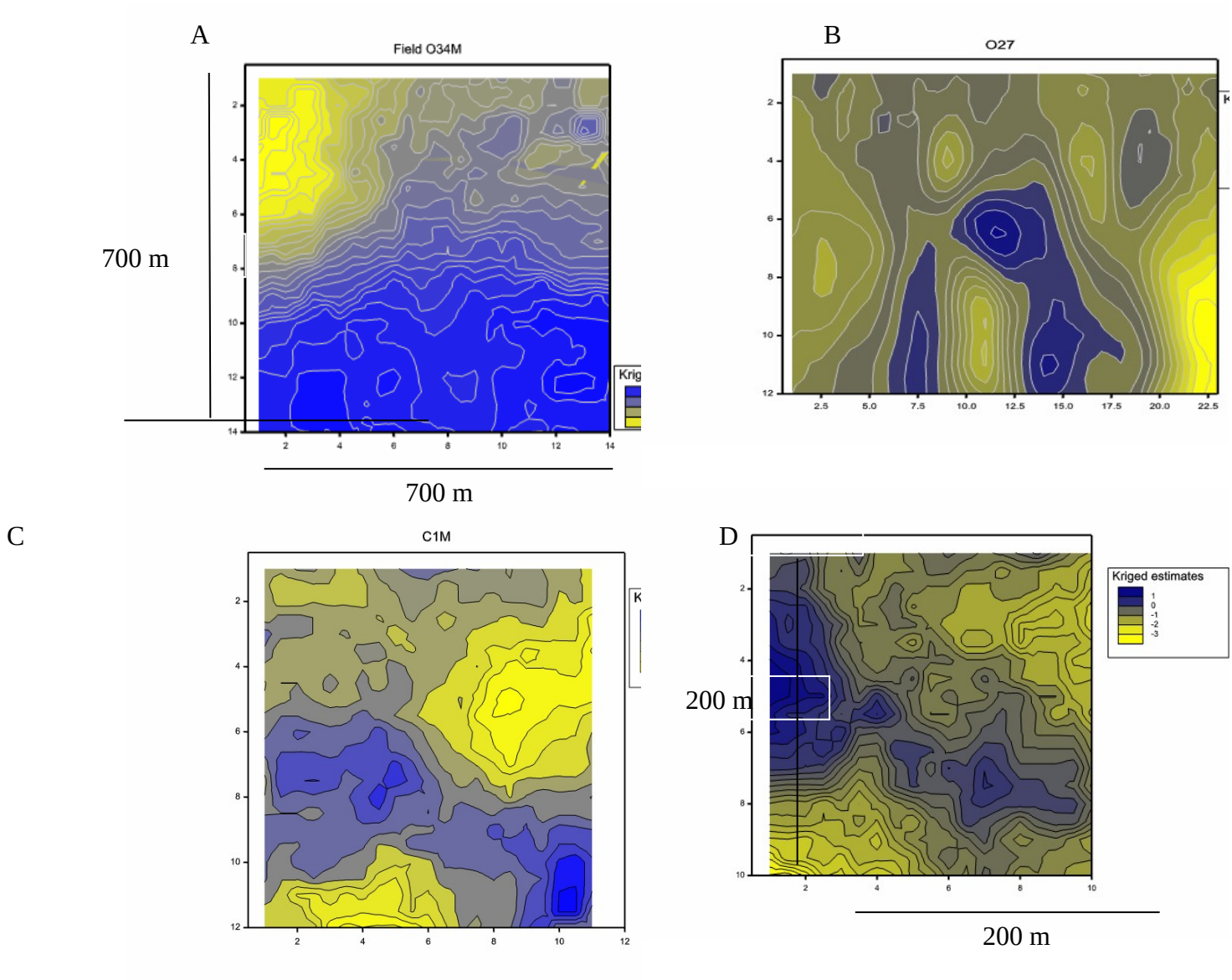


Figure 40. Contour maps showing distribution of Kriged estimates (log pg DNA *Rhizoctonia solani* AG3) in four intensively sampled fields. A, B, C fields O34, O27, C1M, 50 Ha fields in Australia (data supplied by R. Harding); D, a 4 Ha area of field in Aberdeenshire.

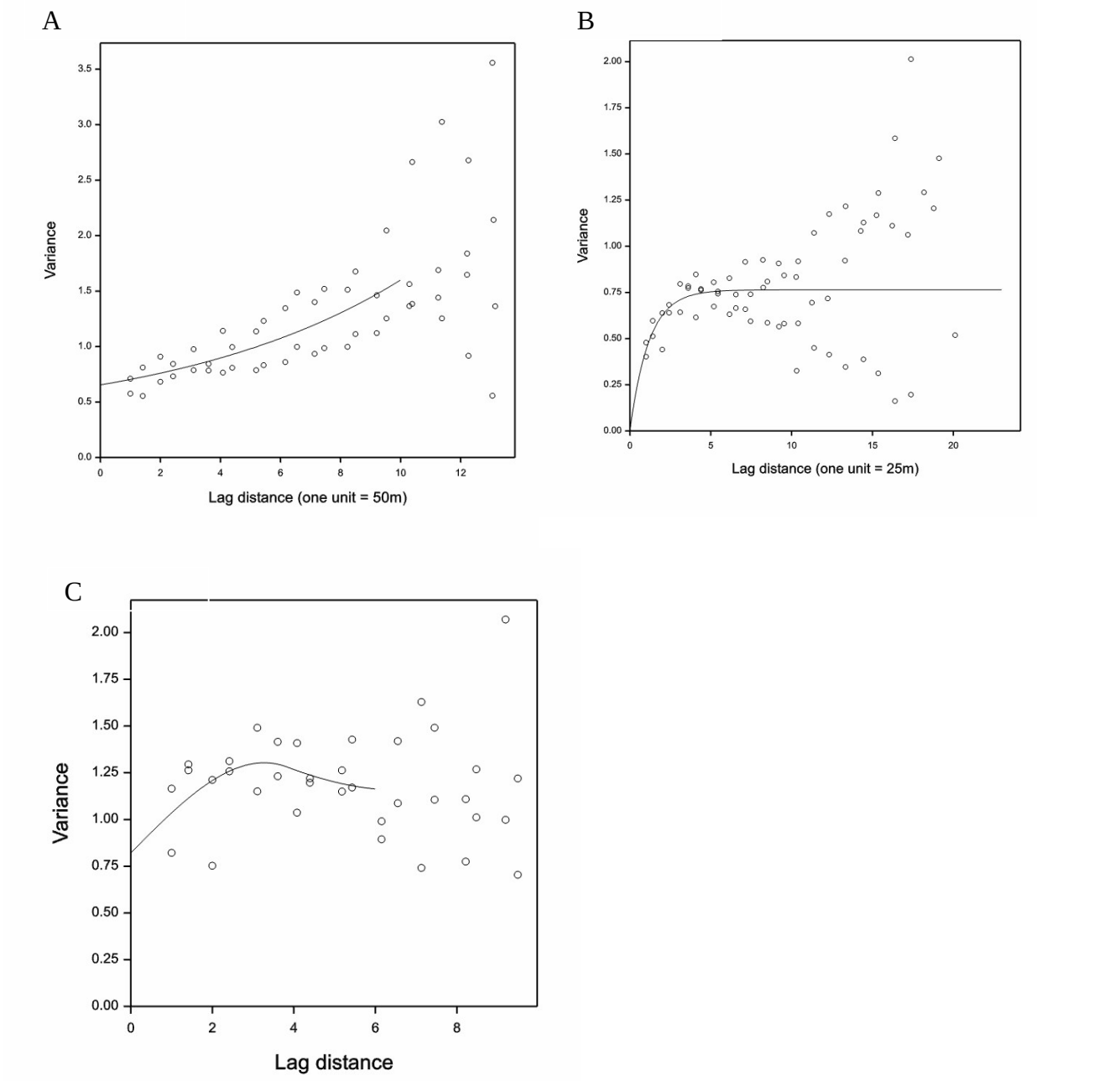


Figure 41. Semivariograms showing variance plotted against distance for *R. solani* AG3 in: A, field O34 in Australia (data supplied by R. Harding). B. field O27 in Australia (data supplied by R. Harding); C, a field in Aberdeenshire, UK. Data were analysed in segments at 45° angles.

In order to determine how the variance changes with distance from the sample points, semi-variograms of *R. solani* AG3 data were analysed by comparing the data at transects that were 45° angles from each other (Figure 41). This shows that as distance between

samples increases, the degree of unpredictability increases. Beyond 120 to 500m apart, the data become highly unreliable. In field O34, the pattern of inoculum was characterised by a mainly uniform patch of *R. solani* in the approximately 70% of field. Subsequently, the variance continued to increase to approximately 500m lag distance (after which the variance became unpredictable). Thus, inoculum within this distance is stochastically dependent. In the case of site O27 and the Aberdeenshire field, the asymptotes of the exponential and cubic models, respectively, were reached within 80 to 100m, indicating that inoculum patch sizes were of that order.

When data from parallel slices, are plotted as semi-variograms (Figure 42), this shows that in the O34 pivot, the data show large-scale heterogeneity where patches are few and large, resembling gradients. In O27 and the Aberdeenshre sites, the data displayed nested heterogeneity. That is, multiple scales of patchiness were evident possibly because factors influencing pattern operate at different scales (ie microbial antagonists at the micro scale, plant structures at the medium scale, and geological features at the macro scale). This is further backed up by the results from the analysis of the K-function from the Aberdeenshire data **(**Figure 43), which showed that clustering occurred across many scales (120, 150 and 170 m).

Discussion

At present, it is standard practice to base decisions on whether fields have high or low levels of inocula on single, mean, values of target DNA that are obtained from between 40 and 100, bulked, core samples. These are typically collected using a 'W' pattern within a 4 Ha sample area. This mean value is used to determine the risk of disease in a crop within the area sampled. This mean is likely to be a good representation of disease risk where the pathogen is uniformly distributed (for example, *Colletotrichum coccodes*). However, in situations where the pathogens are not uniformly distributed, the mean DNA values per unit area might not be a good representation of true disease risk. From the work presented in this report, the distribution of *R. solani* AG3 is either highly variable (O26 & Portsoy) or stratified (ie field pivot O34). In cases where the fields are highly variable for pathogen inoculum, it is vital that samples are collected throughout the field area. In addition, data become highly unreliable when collected further than 80 to 120 m apart. This strongly suggests that clusters of sample points should be collected at most every 50 m and ideally as close together as possible.

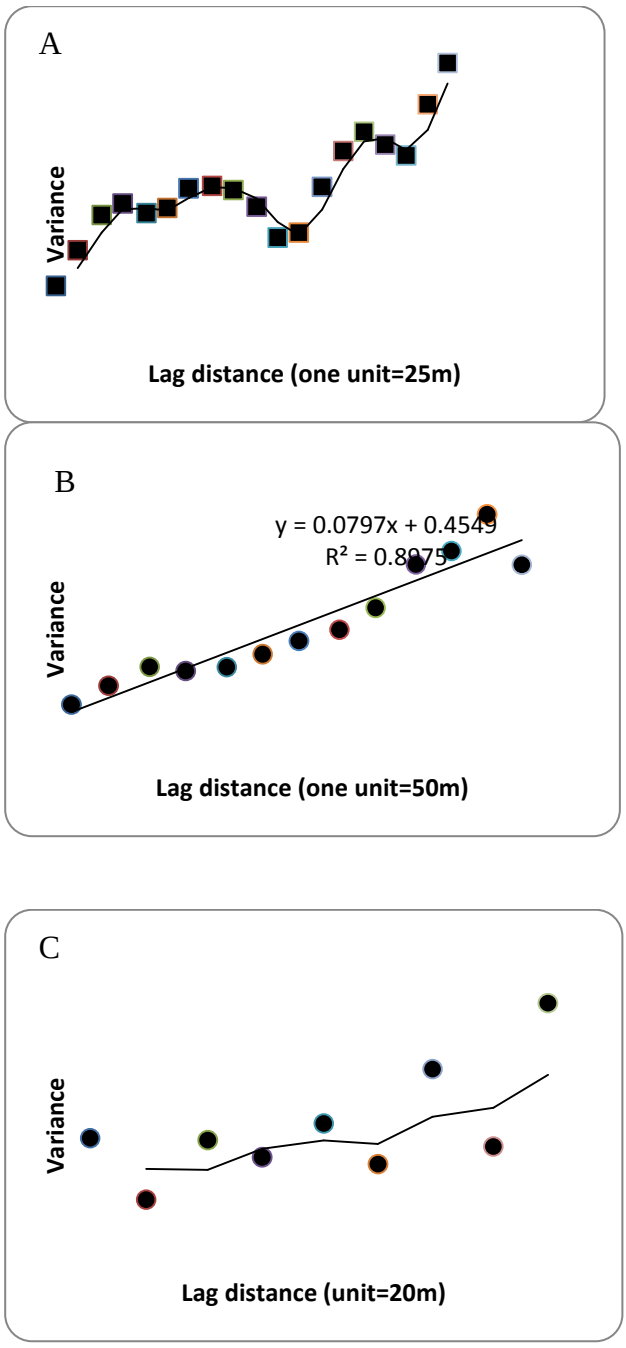


Figure 42. Pattern of semi-variance plotted against lag distance for Rhizoctonia solani AG3 in three fields, A) field O34 and B) field O27 both from Australia (data supplied by R. Harding); C, a field in Aberdeenshire, UK.

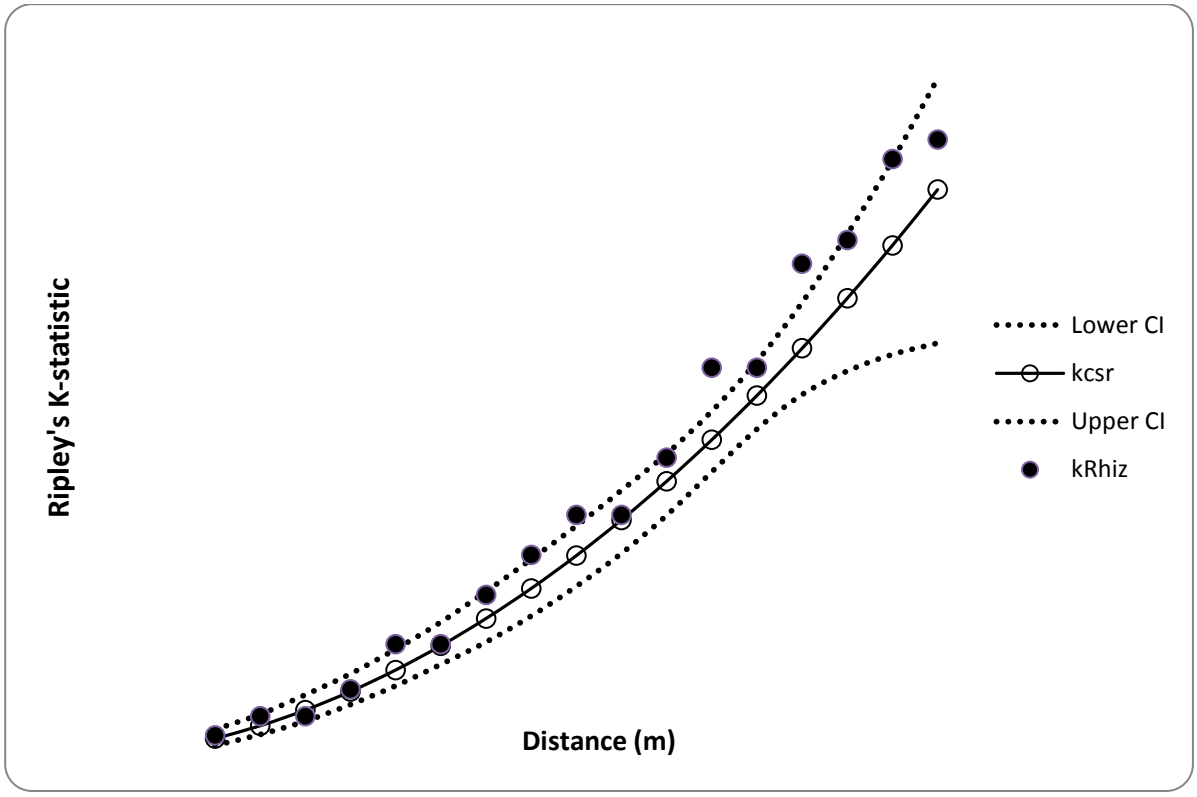


Figure 43. Plot showing Ripley's K-statistic calculated for the Aberdeenshire site *R. solani* AG3 binomial data (kRhiz) as a function of how far apart (in meters) two sample points are. KRhiz points are significantly clustered if greater than the upper confidence interval.

Conclusions

Some conclusions can be drawn from the results presented here:

1. As distance increases beyond 120 to 500m, the data becomes increasingly unreliable. This suggests that sample units in excess of 500 x 500 m should be divided into small units.

2. Sites displayed either large-scale heterogeneity or nested heterogeneity where patchiness was either few and large, or were observed across multiple scales.

3. In terms of informing future sampling, the results here suggest that 4 ha units are suitable for sampling clustering patterns observed for *R. solani* AG3 soil inoculum.

4.5. *Diagnostics: inter-lab comparison*

Introduction

As part of the Potato Council funded Potato Diagnostics Project (R253), molecular tools for detecting and quantifying a range of potato pathogens (including *Colletotrichum coccodes*, *Rhizoctonia solani* and *Spongospora subterranea*) have been developed and refined. As part of the current project (R422) an international consortium was set up to jointly investigate the detection of soil-borne pathogens (Table 51). To ensure that participating laboratories are producing accurate and critically comparable results, standardised test material was produced and distributed by Fera for testing at each laboratory. The objective was to produce a comparable DNA value for similar samples or, failing that, to calibrate test results so that there is a common understanding of what a specific tests result means across laboratories.

Each laboratory (nationally and inter-nationally) currently uses different protocols for DNA extraction from soil and, for some tests, different primers and probes for each of the tests. Whilst DNA extraction protocols cannot be standardised, for the purposes of an inter-lab comparison the same assays have been used by all laboratories. In addition, Fera produced and distributed pathogen standards to aid (and standardise) the conversion of Ct values to DNA levels that can be compared across labs.

Table 51. List of participating labs

Laboratory	Country
James Hutton Institute	Scotland, UK
Scottish Agricultural College	Scotland, UK
Food and Environment Research Agency	England, UK
Plant and Food Research	New Zealand
PIRSA-SARDI	Australia

Materials and methods

Soil samples were ‘spiked’ with known quantities of pathogen and sent to participating laboratories. Participating laboratories used their own extraction methods. In order to determine where differences in test results occurred, the same DNA assay (primers and probes) were applied to DNA extracted by each laboratory. Standard DNA was also distributed by Fera to the participating laboratories, to provide uniformity in the creation of standard curves. Samples were sent by courier to the four international ring test partners (Table 61) plus one set retained for blind testing at Fera. Samples were dispatched on 14 April 2010 (*C. coccodes* set 1), 8 July 2010 (*C. coccodes* set 2), 20 July 2010 (*R. solani* AG3) and 8 March 2011 (*S. subterranea*). Samples were sent in triplicate to each participating lab. See Appendix 3 for protocol and instructions to labs.

Preparation of standard material

DNA standards

For *Rhizoctonia solani* and *Colletotrichum coccodes*, isolates Rs08 and cc1554 respectively, were removed from long term storage and transferred onto potato dextrose agar (PDA) plates and incubated at room temperature. After 10 days hyphal growth was removed from the plates using a scalpel taking care to avoid agar contamination. For the *Spongospora* DNA standard, spore ball material on tuber peel was received from JHI for use. Harvested hyphae/spore balls were pulverised using a pestle and mortar. DNA from *R. solani*, *C. coccodes* and *S. subterranea* was extracted using the method of Saitoh et al. (2006). DNA was suspended in 1.5 ml of molecular grade water to provide the following concentrations for the DNA standards: *R. solani*, 131.6 ng DNA/uL; *C. coccodes*, 68.7 ng DNA/uL; *S. subterranea*, 13.5 ng DNA/uL. For each participant laboratory, a 200 µl aliquot of the DNA suspension was placed into a 1.5 ml tube. This was fully dried using a DNA Speed Vac (DNA 120, Savant) prior to dispatching to each ring test participant.

Soil samples

Soil samples were prepared as follows. Soil (sandy loam) was obtained from a permanent pasture situated in North Yorkshire where no other crops had been grown for at least 60 years. Soil was sieved at 4 mm to remove stones, large debris and organic matter before oven drying at 40ºC for at least 48 hours.

Preparation of inoculum

Colletotrichum

Isolate cc1554 was removed from long term storage and grown on PDA for at least six weeks. Conidia were removed using a scalpel and dried for two days at room temperature. Inoculum was added to soil to produce the following dilution range: 0.1 mg/g (high) and 0.01 mg/g (medium). Low naturally infested soil was used for the two low categories (Fera field soil from potato plots). This was because diluting at lower volumes than 0.01 mg/g, produced inconsistent detection of DNA by real-time PCR. Two sets of *C. coccodes* inter-laboratory comparison samples were prepared three months apart, using the same method.

Rhizoctonia

Isolate Rs08 was removed from long term storage and grown on PDA for six weeks. Sclerotia were remove using a scalpel and dried for two days at room temperature. Inoculum was added to soil to produce the following dilution range: 2.2 mg/g (high), 0.2 mg/g (medium) and 0.1 mg/g (low).

Spongospora

Spore balls were obtained from Agria tubers with visible and extensive powdery scab pustules supplied by JHI. Approximately, 0.2 g of material (spore balls plus plant tissue) was ground using a pestle and mortar and SDW added to form a suspension. The number of cystosori in each suspension was adjusted, after determining the number of cystosori per µl using a haemocytometer, such that the following levels of spore balls/g soil was added to each individual 60g sample: 936 cystosori/g (high), 80 cystosori/g (medium) and 8 cystosori/g (low).

DNA testing
Participating labs tested soils for DNA levels using their own methods. Two replicate extracts were produced for each test sample and each sample was tested in triplicate by qPCR. Each participating lab retuned the results (pg DNA/g soil sample). 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.

Analysis
On receipt of all results, a 'consensus mean' was calculated for each inoculum level. This is the arithmetic mean across labs at each inoculum level. From these data, an estimate of the degree to which the individual laboratory means deviated from the consensus mean was calculated (the z-score). 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.
These are Standard Deviations from the consensus mean (Equation 1; Anon, 2002). The z-score reflects 'best practice' or fitness for purpose. Values for z-scores within |2| are generally deemed 'acceptable' in standard inter-laboratory tests. Note, however, that 11 participants are usually considered the minimum required to provide reliable z-scores.

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

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

Results
Colletotrichum coccodes
Pre-dispatch sample testing showed that DNA did not deteriorate during one week storage at room temperature (data not shown). The coefficients of variation (cv%) were within the target limit of acceptability (<10%) for High and Medium samples but variation between replicate sub-samples was higher than the notional limit of acceptability in the low sample (Table 52). It is likely that low inoculum levels will inevitably have higher cv% than higher inoculum levels no matter how the samples are prepared.

Table 52. Pre-dispatch sample coefficients of variation for *C. coccodes* samples.

Sample	CV%
High	3.7
Medium	8.3
Low	24.4
Nil	0.0

The consensus means shown in Figure 44 and Table 53 show that the range of DNA levels in the samples were appropriate for the Low, Medium and High sample labels. All returned values from participating labs were well within the 2xSD tolerance (including the

one non-compliant result). In addition, one lab also detected low levels of pathogen in the Nil sample.

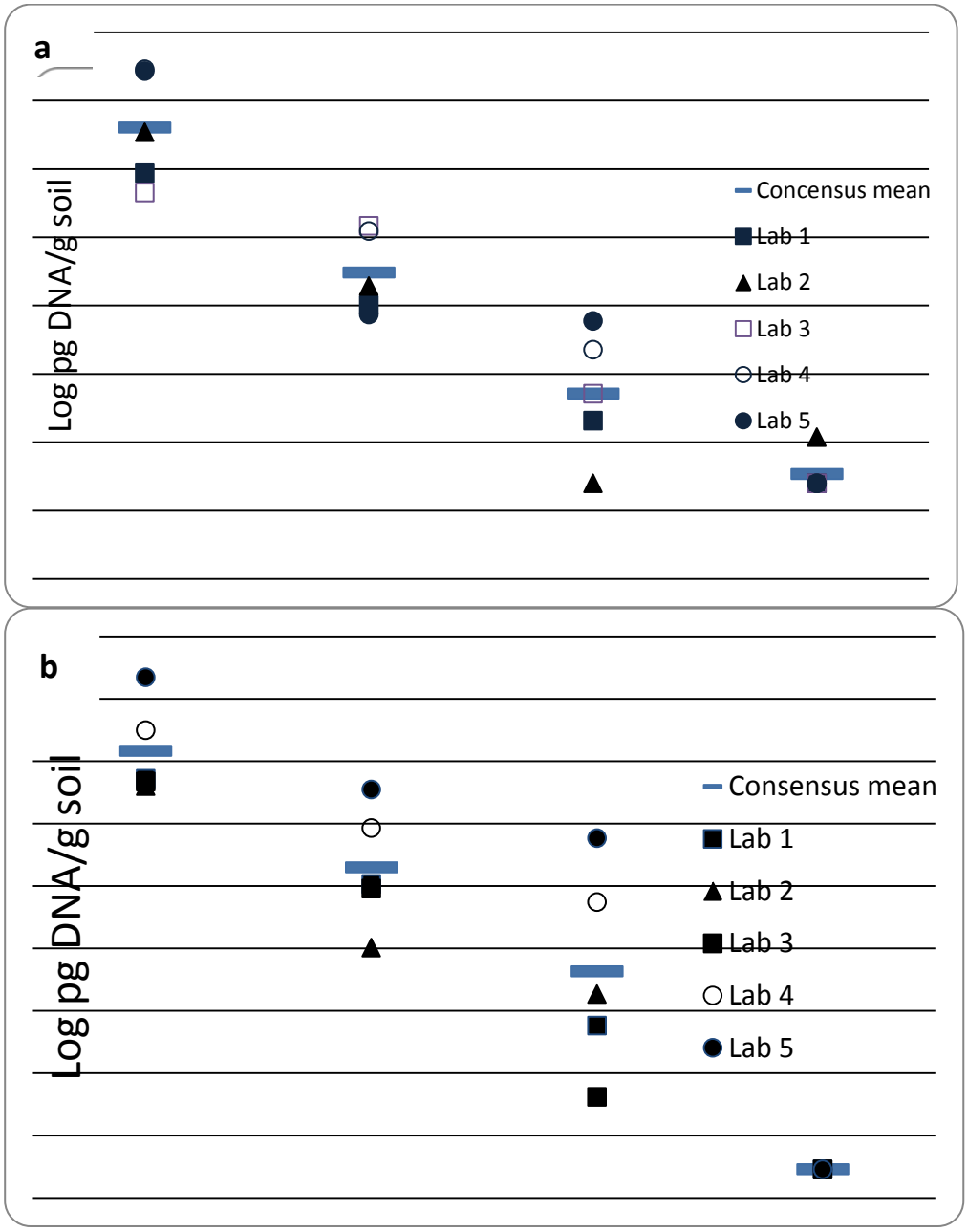


Figure 44. Shows the spread of data from individual labs around the consensus mean for *Colletotrichum coccodes* (a, set 1; and b, set 2).

Table 53a. Log mean pg DNA/g soil sample for each participating lab and associated z-scores (*C. coccodes* set 1).

Sample	Lab 1		Lab 2		Lab 3		Lab 4		Lab 5		Consensus mean
	Mean	z-score	Mean	z-score	Mean	z-score	Mean	z-score	Mean	z-score	
High	2.97	-0.809	3.27	-0.080	2.83	-1.148	3.73	1.036	3.72	1.002	3.30
Medium	2.01	-0.591	2.14	-0.245	2.58	0.850	2.55	0.753	1.93	-0.767	2.24
Low	1.16	-0.446	0.70[nc]	-1.480	1.36	-0.001	1.68	0.725	1.89	1.203	1.36
Nil	0.70[†]	-0.258	1.04	1.034	0.70	-0.258	0.70	-0.258	0.70	-0.258	0.77

[nc] = non-compliant; † 'undetected' values were treated as at the limit of detection (5 pg/g) for the purpose of the analysis.

Table 53b. Log mean pg DNA/g soil sample for each participating lab and associated z-scores (*C. coccodes* set 2).

Sample	Lab 1		Lab 2		Lab 3		Lab 4		Lab 5		Consensus mean
	Mean	z-score	Mean	z-score	Mean	z-score	Mean	z-score	Mean	z-score	
High	3.36	-0.63	3.30	-0.80	3.34	-0.70	3.75	0.47	4.17	1.67	3.58
Medium	2.51	-0.29	2.01	-1.39	2.48	-0.36	2.96	0.69	3.27	1.35	2.65
Low	1.38	-0.55	1.63	-0.26	0.81	-1.21	2.37	0.59	2.88	1.18	1.82
Nil	0.23	0.00	0.23	0.00	0.23	0.00	0.23	0.00	0.23	0.00	0.23

***Rhizoctonia solani* AG3**

Pre-dispatch sample testing showed that DNA did not deteriorate during one week storage at room temperature (data not shown). The coefficients of variation (cv%) were within the target limit of acceptability (<10%) for High. However, the variation between replicate sub-samples for Medium and Low samples was higher than the notional limit of acceptability (Table 54).

Table 54. Pre-dispatch sample coefficients of variation for R. solani AG3 samples.

Sample	CV%
High	2.0
Medium	17.9
Low	50.5
Nil	0.0

The consensus means (shown in Figure 45) indicates that the range of DNA levels in the samples were appropriate for the Low, Medium and High sample labels. There was good agreement between participating labs except for samples labelled Medium and Low, where Fera's extractions failed and so returned results that were out of step with those provided by the other labs.

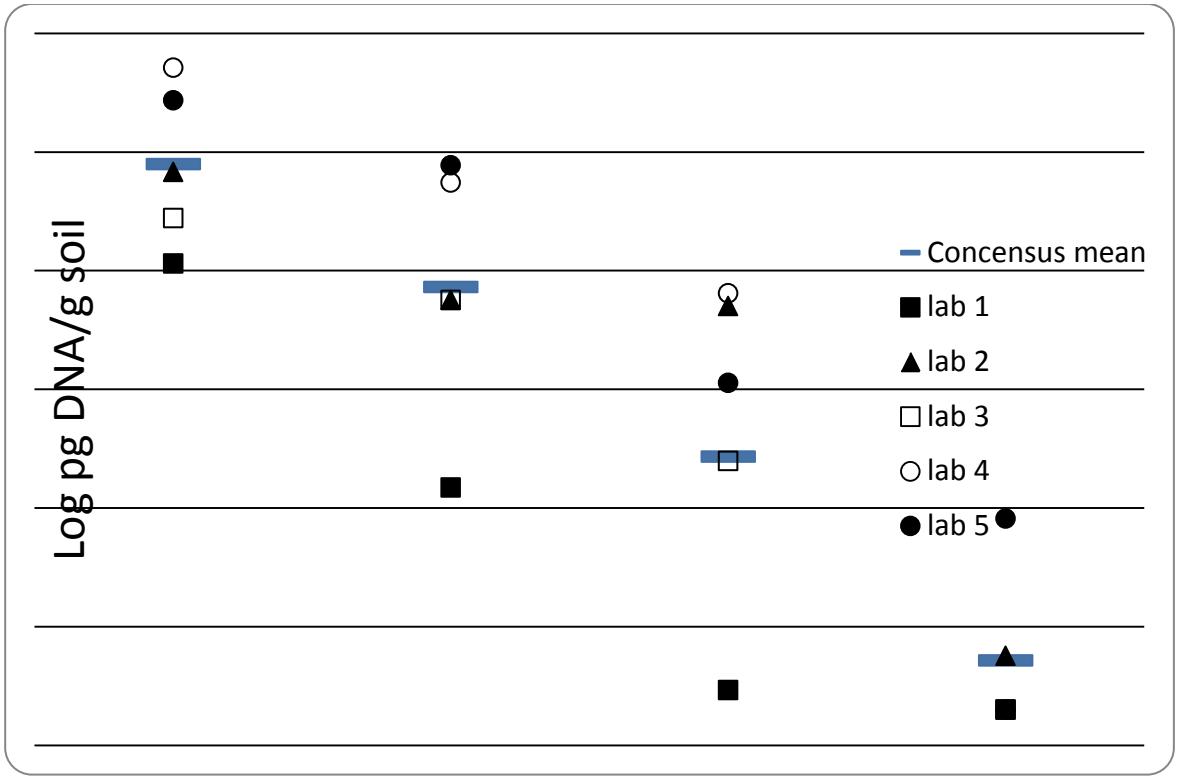


Figure 42. The spread of data from individual labs around the consensus mean for *Rhizoctonia solani* AG3.

Spongospora subterranea

Pre-dispatch sample testing showed that DNA did not deteriorate during one week storage at room temperature (data not shown). The coefficients of variation (cv%) were within the target limit of acceptability (<10%) for High and Medium samples. However, the variation between replicate sub-samples for Low samples was higher than the notional limit of acceptability (Table 55).

Table 55. Pre-dispatch sample coefficients of variation for *S. subterranea* samples.

Sample	CV%
High	3.5
Medium	9.8
Low	27.7
Nil	0.0

The consensus means (shown in Figure 43) indicates that the range of DNA levels in the samples were appropriate for the Low, Medium and High sample labels. There was good agreement between participating labs.

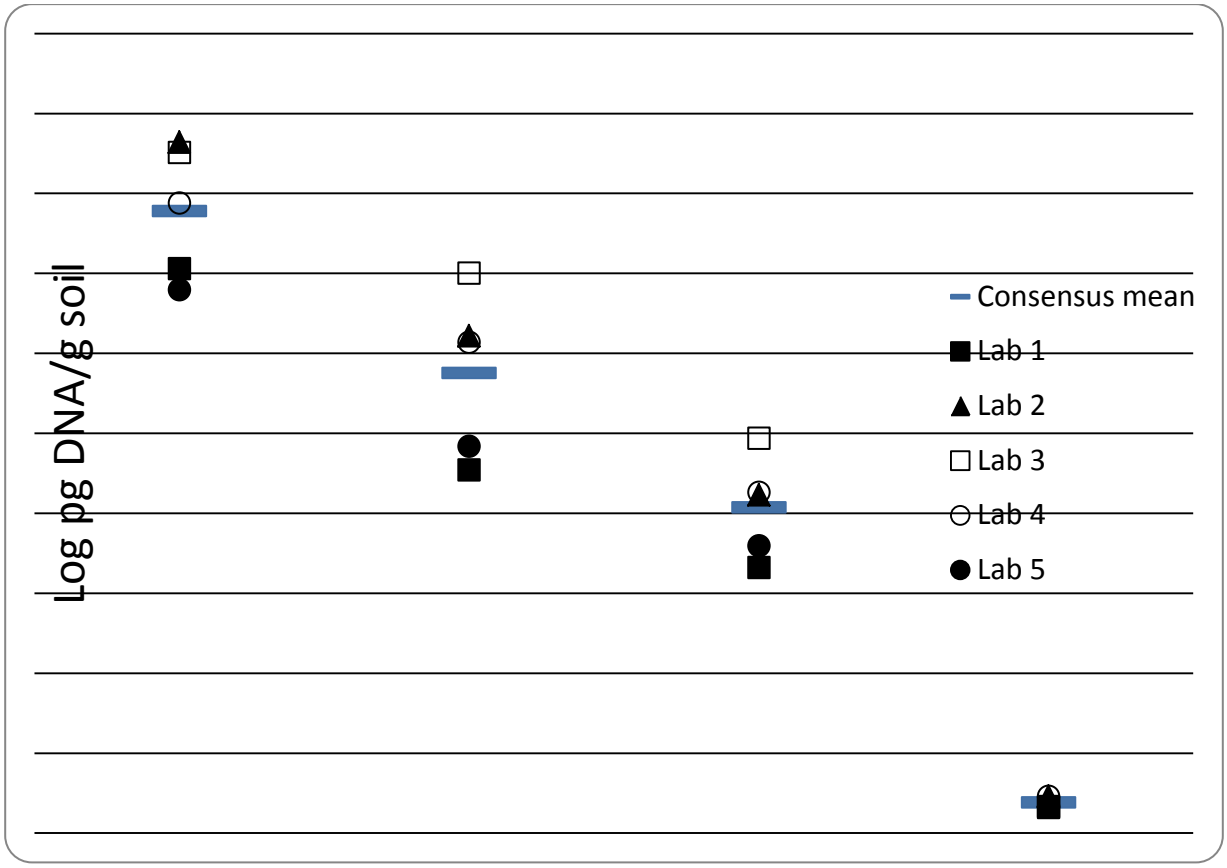


Figure 46. The spread of data from individual labs around the consensus mean for *Spongospora subterranea*.

Discussion

Given that each lab performed their own (and in some cases different) extraction methods and used a range of real-time PCR thermal cycling equipment, it is surprising how similar the results were between participants. The level of agreement varied considerably depending on the target pathogen. The targets *C. coccodes* and *S. subterranea* gave the best agreement whereby all labs were within approximately 1 log of each other (except when low levels of *C. coccodes* were compared). *Rhizoctonia solani* samples gave the poorest consensus (some participants were 2 to 3 logs apart). This was probably due to Lab 1 detecting the pathogen at lower levels than the other labs. Notwithstanding absolute differences in pathogen DNA levels between labs, participants were able to discriminate between High, Medium, Low and Nil inoculum samples in most cases. Only one participant, Lab 5, detected pathogen in the Nil sample. It is not clear whether the sample was contaminated or whether the assay cross reacted with non-target DNA.

Conclusions

1. In all four sets of inter-laboratory comparison data, the inoculum levels (low, medium and high) provided a good range for testing the ability of laboratories to detect and quantify the target pathogen.
2. Coefficients of variation for ‘medium’ and ‘high’ level samples were generally within the accepted limit (<10%). However, ‘low’ samples always had higher than 10% coefficients of variation and illustrates the difficulty in producing samples with low variation when pathogen inoculum levels are low.
3. In general there was reasonable agreement between all participating laboratories.
4. This study shows that results generated from research and commercial testing carried out at participating laboratories in both GB, Australia and New Zealand are comparable with each other.

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Appendix 1.

Powdery scab gall assessment key

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**

Appendix 2

Powdery scab: methods for processing root and tuber samples; nucleic acid extractions; and real-time PCR

Preparation of root samples
Ensure 4^{o}C centrifuge is switched on
Defrost frozen root samples at room temp.
Take a 10g fresh weight sub-sample (splitting root section vertically) and refreeze any remaining root material, if 10 g is not available take the entire root sample.
Record weight of root sample.
Chop up the sample and place in a 50 ml centrifuge tube, re-freeze and then freeze dry.
Grind the root sample in LN_2 using mortar and pestle. (3 lots of LN_2 used to achieve a free flowing powder)
Cool the original 50ml tube in LN_2 and add the ground sample.
Add 30 ml of PB7 extraction buffer to the tube and mix thoroughly.
Centrifuge the 50ml tube at 1000rpm @ 4^{o}C for 10 min to remove large particulate material.
Avoiding the floating debris, pipette 15ml of the supernatant into a new 50ml tube and spin at 1000rpm @ 4^{o}C for 5 min.
Dispense 2x 5ml aliquots of the supernatant into separate 15ml centrifuge tubes.

Preparation of tuber samples
Ensure 4^{o}C centrifuge is switched on
If frozen, defrost sample, then roughly chop and mix each tuber peel sample.
Take a 10g fresh weight sub-sample and refreeze any remaining peel, if 10 g is not available take the entire peel sample.
Record weight of tuber peel sample.
Place the 10g peel sample into a Bioreba grinding bag (Long Special Universal bags: 15 x 28.5cm Cat No 470100b) and add 30ml PB7 buffer
Pulverise the sample to an oatmeal consistency using the Homex grinder.
Pipette the supernatant into a labelled 50ml universal tube (place on ice) and centrifuge for 10 min at 1000rpm at 4C to remove large particulate material
Dispense 2 x 5ml aliquots of the supernatant into separate 15ml centrifuge tubes.

NA extraction from both tuber and root samples:
NA extraction using Promega Reagents: Lysis Buffer B, Food (Cat no. Z3191)
Precipitation Solution, Food (Cat no. Z3201)
NB Calculate the required amount of each reagent and aliquot each into a fresh sterile tube. This may not be returned to the original reagent pot.
Centrifuge the 15ml tubes for 15 mins @ 5000rpm @ 4^{o}C
Set centrifuge to room temp
Remove and discard 4ml supernatant and re- suspend the pellet in the remaining 1ml
Add 250µl Buffer B to each tube
Add 750µl precipitation solution to each tube, vortex and incubate at room temp. for 5min

Centrifuge @ 5000rpm for 15mins **at room temp**.
While tubes are centrifuging label 2ml tubes.

Pipette off 750µl of supernatant from each sample tube, avoiding pellet, and add to labelled 2ml tubes.
Add 750µl -20^{o}C Isopropanol
Add 75µl of Sodium Acetate (3M)
Invert gently and incubate for 1 hour @ room temp.
Centrifuge tubes @13200rpm for 4mins
Remove supernatant
Add 150µl 70% ethanol, vortex
Centrifuge @13200rpm for 2mins
Remove Ethanol and allow to air dry no longer than 10mins
Re-suspend pellet in 100µl TE buffer
Store Neat NA at –20 ºC until required

Extraction Buffer PB7 (Phosphate Buffer pH 7.0 + antioxidant)

$Na_2HPO_4.12H_2O$	2.7g	Sodium phosphate dibasic dodecahydrate
NaH_2PO_4	0.4g	Sodium phosphate monobasic
SDW	1000ml	

Autoclave then add 2ml per 100ml of 0.2M tetrasodium pyrophosphate antioxidant

Real-time PCR assays

All real-time PCR reactions were performed in 96-well reaction plates using TaqMan Universal PCR MasterMix (Applied Biosystems Cat:4324020, no amperase).
Plates were cycled at generic RT PCR system conditions (95ºC for 10 min, and 40 cycles of 60ºC for 1 min plus 95ºC for 15 sec).
Non template controls, with 2 µl HPLC, instead of DNA were included in every assay.
All samples were tested in duplicate and results averaged.

PMTV assay

R. A. Mumford, K. Walsh, I. Barker, and N. Boonham. (2000). Detection of *Potato mop top virus* and *Tobacco rattle virus* Using a Multiplex Real-Time Fluorescent Reverse-TranJHIption Polymerase Chain Reaction Assay. Phytopathology. 90, No. 5, 448-453.

Primers & Probe

PMTV 1948F (forward): GTG-ATC-AGA-TCC-GCG-TCC-TT
PMTV 2017R (reverse): CCA-CTG-CAA-AAG-AAC-CGA-TTT-C
PMTV Probe (FAM labelled): ACC-AGA-ACT-ACG-GTG-CCG-CGT-CG

Hydrate the lyophilised product with RNase free water and use RNase free water in the Master Mix

	x1 reaction (µl)	
RNase free water	6.875	
Taqman mix (x1)	12.5	
PMTV 1948F (0.3µM)	1.5	
PMTV 2017R (0.3µM)	1.5	
PMTV 1970 (0.1µM)	0.5	
Reverse TranJHIptase (25 units)	0.125	(Promega cat no. M1701)
NA (neat)	2.0	

25µl

- A +ve PMTV sample was included in each run
- PMTV results were expressed as Ct values.

***Spongospora subterranea* assay**

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

Primers and Probe

SsTQF1 (forward):	5-CCG GCA GAC CCA AAA CC-3
SsTQR1 (reverse):	5-CGG GCG TCA CCCTTC A-3
SsTQP1 Probe (FAM labelled):	5-CAG ACA ATC GCA CCC AGG TTC TCA TG-3

	x1reaction (µl)
HPLC Water	7.0
Taqman mix (x1)	12.5
For Primer (0.3µM)	1.5
Rev Primer (0.3µM	1.5
Probe (0.1µM)	0.5
NA (1/20 dilution)	2.0
	25µl

A range of standards containing DNA (extracted from a known number of spore balls and diluted) were included in the real-time PCR assays.
The standards were used to create a standard curve of the critical threshold (Ct) value against the logarithm of the amount of spore balls.
Using the standard curves, the amount of DNA in each unknown sample was expressed as spore ball equivalents for *S. subterranea* on the basis of their Ct values.
All samples were tested in duplicate and results averaged.
Results were adjusted for the amount of material from which the DNA was extracted from and expressed as pg DNA / gfwt.

DNA calculation, ng DNA per g fwt tuber peel or root material

If 10g fwt in 30 ml buffer:
1.67 g fwt in each 5 ml aliquot of suspension removed. From this initial aliquot, a further aliquot is removed during the processing of the sample (750 µl from a 2000 µl suspension), equivalent to 0.63 g fwt which continues through extraction and is resuspended in 100 µl TE buffer; 2 µl of this is added to the PCR assay then, the amount of NA can be calculated to per g fwt basis.

x 20 dilution
x adjustment for g fwt root material
x 50 (2 µl out of total of 100)
/ 1000,000 to get ng rather than fg / gfwt root material

For calculating *S. subterranea* as DNA rather than sporeball equivalents a conversion factor is applied; 1 sporeball equals 90 pg DNA.

Appendix 3

Inter lab comparison testing

Introduction

Authors: Jeff Peters/ Kathy Ophelkeller 8/11/2010

Each participating laboratory currently uses different protocols for DNA extraction from soil and, for some target pathogens, different primers and probes. Confidentiality agreements that have been signed by a representative from each participating lab has enabled the sharing of protocols. However, it is still necessary to compare results from each lab involved in the collaboration as there will be lab-to-lab variation in execution of protocols as well as differences in DNA quantification standards, reagents and equipment in each laboratory which will influence the results.

The objective of this inter-laboratory testing is to allow the comparison of data from the participating research labs.

As part of the global potato disease diagnostics collaborative project 'Optimisation of potato DNA diagnostic tools for strategic disease management', each participating lab will test soil samples prepared by Fera and SARDI that have been spiked with known levels of pathogen (*Colletotrichum coccodes*, *Rhizoctonia solani* AG 3 and *Spongospora subterranea*).

Soil samples will be 'spiked' with known quantities of pathogen and the sample plus freeze-dried standards sent to participating laboratories for analysis using current protocols developed through PCL-funded R411. In order to determine where differences in test results occur, the same DNA assay (primers and probe) will be applied to DNA extracted by each laboratory using their own protocols. Data for the DNA quantification from each lab will be analysed by Fera and the results from the inter-laboratory comparison exchanged with all participants.

Scope

This SOP covers the inter laboratory reproducibility test. Participating institutes are: SARDI, SAC, SCRI and Fera. Horticulture New Zealand cannot receive soil from overseas so will only be able to receive DNA extracts and send samples out.

MATERIALS

See SOPs below (Fera and SARDI):

PLH/422b – sample preparation and homogeneity testing for Interlaboratory Reproducibility Testing.

Cullen DW, Lees AK, Toth IK, Duncan JM (2002). Detection of *Colletotrichum coccodes* from soil and potato tubers by conventional and quantitative real-time PCR. *Plant Pathology*. 51: 281-292.

Lees AK, Cullen DW, Sullivan L, Nicolson MJ (2002), Development of conventional and quantitative real-time PCR assays for the detection and identification of Rhizoctonia solani AG-3 in potato and soil, *Plant Pathology*. **51:** 293–302.

van de Graaf P, Lees AK, Cullen DW, Duncan JM (2003). Detection and quantification of *Spongospora subterranea* in soil, water and plant tissue samples using real-time PCR. *European Journal of Plant Pathology* 109: 589–597.

Equipment

-20 □C Freezer
+4 □C Fridge
Microcentrifuge
Microcentrifuge tubes (2ml, 1.5ml)
Set of pipettes (1000 □l, 200□, 20□, and 10□) with ster
Vortexer
96 well optical microtitre plates and clear film sealable film (i.e. Starlab StarSeal Polyolefin Film)
Real Time PCR Thermal Cycler (i.e. Applied Biosystems 7900HT)
Other equipment will be specific to individual labs

Reagents

Extraction reagents will be specific to each laboratory
Sterile molecular grade water
Master mix (Fera uses: TaqMan 1000 Rxn Gold/Buffer A pack (AB: 4304441):
Containing Buffer A (+ROX), Magnesium chloride ($MgCl_2$), dNTPs and *AmpliTaq* Gold.
Positive controls (available from Fera and SARDI)

Primers:

C.coccodes F: 5'- TCTATAACCCTTTGTGAACATACCTAACTG -3'
C.coccodes R: 5'- CACTCAGAAGAAACGTCGTTAAAATAGAG -3'

R. solani AG3 F (RsTqF1): 5'- AAGAGTTTGGTTGTAGCTGGTCTATTT-3'
R. solani AG3 R (RsTqR1): 5'-AATTCCCCAACTGTCTCACAAGTT-3'

S. subterranea F (SsTQF1) (5'-CCG GCA GAC CCA AAA CC-3')
S. subterranea R (SsTQR1) (5'-CGG GCG TCA CCCTTC A-3')

Probes:

C.coccodes: FAM 5'- CGCAGGCGGCACCCCCT-3' TAMRA
R. solani AG3 (RQP1): FAM 5'- TTTAGGCATGTGCACACCTCCCTCTTTC-3'
S. Spongospora (SsTQP1): (5'-CAG ACA ATC GCA CCC AGG TTC TCA TG-3')

This probe is labelled at the 5' end with the florescent reporter dye 6-carboxyfluorescein (FAM) and at the 3' end with the quencher dye, 6-carboxy-tetramethyl-rhodamine (TAMRA). Other die sets may be used as appropriate for individual laboratories.

PROTOCOLS

1. Soil sample preparation

Sample will be prepared by Fera and SARDI (see SOP PLH/422b). Each will comprise of natural soil (that has tested negative for each target pathogen DNA) that has been thoroughly air-dried then spiked with *C. coccodes* and *R. solani* AG3PT at the following

levels: zero inoculum, low inoculum (<100 pg DNA/g soil), moderate inoculum (c. 500 pg DNA/g soil), and high inoculum (>1,000 pg DNA/g soil); and *S. subterranea* at zero inoculum, low inoculum <10 sporeballs/g soil), medium inoculum (10 to 50 sporeballs/g soil), and high inoculum (>50 sporeballs/d soil).

2. Sample distribution

Single 250g aliquotes of soil from each pathogen inoculum level will be sent by Fera and, in the future, SARDI, to each participating lab. Each sample will be identified by a code that will not reveal the amount of inoculum. In addition, DNA extracts of pathogen with a known quantity of DNA will be distributed in parallel with soil samples.

3. DNA Extractions

Participating labs will use their own extraction methods. Three extracts should be produced for each soil sample.

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.

4. DNA Testing

Participating labs will test soils and extracts for DNA levels using their own methods but using the primer/probe assays provided in Materials, above. The following master mix is suggested:

Master mix preparation:

a) Defrost the reagents required (except the gold) to make enough target pathogen master mix for at least 3 replicates of each sample extract, at least 2 replicates of a positive control and at least 2 replicates of master mix with sterile molecular grade water as a negative control (Table 1).

Table 1: Reagents and volumes needed for TaqMan assay

Reagent	Concentration	Volume (□l)
Sterile molecular grade water	-	10.375
AB Buffer A (+ROX)	10x	2.5
$MgCl_2$	25mM	5.5
dNTP	6.25mM	2
Primer (forward)	7.5 □M	1
Primer (reverse)	7.5 □M	1
Probe	5 □M	0.5
AmpliTaq Gold	5U/ □l	0.125
Total	-	23

b) Using pipettes and sterile filter pipette tips, make up each master mix in an autoclaved centrifuge tube (size dependant on how many samples are being tested and therefore the final volume of master mix being made).

c) Take the *AmpliTaq* Gold out of the freezer just as it is needed to make the master mix and put it straight back when finished along with the other reagents.

d) Vortex the master mix for a second so all components are mixed.
e) Pipette the required number of 23 □l/well aliqu reaction plate.

DNA spiking: ‘

f) Dilute the extracted DNA solution as appropriate. It is suggested that a 1:20 dilution is prepared (i.e. pipette 19 □l of the grade water and 1 □l of the extracted DNA solution).
g) Prepare a ten-fold dilution series using the DNA standard supplied by Fera diluted in sterile molecular grade water. It is suggested that the following dilution series is prepared: 10^4, 10^3, 10^2, 10, 1.0 pg/uL. This will be used to create a standard curve for converting Sample C_T values (see k & i, below) to DNA concentrations.
h) Load a 96 well optical reaction plate by adding 2 □l of the dilu standards into the appropriate wells, containing 23 µl master mix.
i) Each sample should be tested in triplicate.

Real-time PCR cycling

j) Load the sample extracts and standard DNA dilution series into the thermal cycler (e.g. ABI 7900). Use laboratory-specified cycling parameters (see Table 2 for example programme).
k) At the end of the run, set the baseline reporter threshold manually so that the threshold is above the background fluorescence (negative controls) and just in the linear phase of the positive controls. Obtain C_T (or cycle threshold values at which the fluorescence exceeds the reporter threshold) for all samples and standards.
l) Convert Sample C_T values to pg DNA/uL using the standard curve.
m) The Results should be presented as: sample identifier, C_T, pg target DNA/g soil (or pg DNA/uL for extracts). Labs should aim to return results to Fera and SARDI within a reasonable timescale (3 weeks from receipt of samples is suggested).

Table 2. An example of thermal cycler settings for reaction volumes of 25 uL.

	Thermal profile				Cycle repeats
	Stage 1	Stage 2	Stage 3		
Temperature	50°C	95°C	95°C	60°C	40
Duration (min/seconds)	2 min	10 min	15 s	1 min	

5. Data entry

All relevant information regarding the PCR testing plus results should be entered in a pro-forma spreadsheet. General information includes: date the test was performed, primers/probe used, and volume of sample per reaction. The Ct values and amount of DNA (ug/uL) should be provided in the ‘DNA standards’ table. From these data, each participating lab should construct a standard curve. Enter the values for the slope and the Y (i.e Ct) intercept in the cells provided. Use these to convert sample Cts to pg DNA/g soil and enter the required values in the cells in the sample table.

6. Data analysis

A consensus mean will be calculated for each inoculum level (for each set of samples) by Fera and SARDI (as appropriate). Results from each lab will be compared to the consensus mean to provide an estimation of strength and direction of bias. A report for each set of samples will be prepared by the co-ordinating laboratory and sent to participating labs as soon as possible after the last set of results have been received.

Appendix 4

Results from R. solani field trials to evaluate control options

Results not provided in the main body of the report are included below for each year/trial site.

Woodlands 2009

Assessments of growth

	Ground cover %		Vigour 0-9	
Treatment	30 July 09	26 Aug 09	30 July 09	26 Aug 09
Monceren	100	100	6.3	6
Monceren + inoculum	99	100	5.5	6.5
Monceren + Amistar	99	100	6.0	6.5
No Monceren	98	100	5.3	6.8
LSD Treatment	0.81		1.07	
LSD Date of assessment	0.51***		0.68**	
LSD Treatment x Date	1.15		1.51	

Yield and tuber numbers (1st harvest – 29 Sept 2010)

	Yield (t/ha)					Tuber numbers (000's/ha)				
Treatment	<45 mm	45-65 mm	65-85 mm	>85 mm	Total	<45 mm	45-65 mm	65-85 mm	>85 mm	Total
Monceren	11.1	40.1	9.2	0	60.4	253	340	30	0	623
Monceren + inoculum	23.4	41.7	14.2	0	79.3	199	361	52	0	613
Monceren + Amistar	8.3	38.5	14.9	0	61.8	186	288	50	0	524
No Monceren	10.2	40.8	11.3	0	62.3	286	314	38	0	639
LSD Treatment	21.64	13.45	9.08	-	25.86	98.6	97.9	33.2	-	119.8

Woodlands 2010

Assessments of growth (20 July 2010)

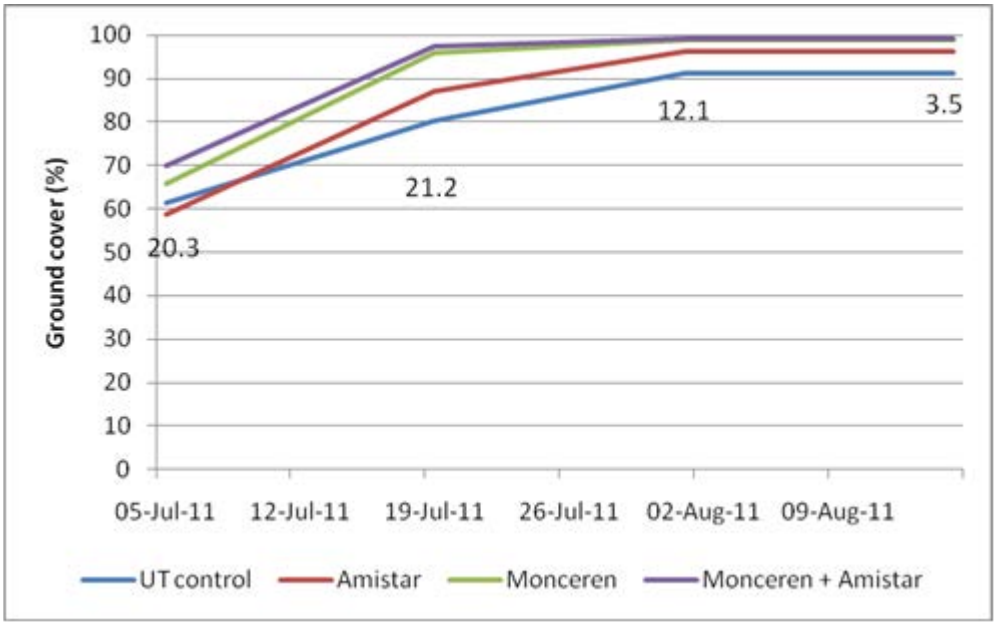
Treatment	Ground cover %	Vigour 0-9
UT control	34	7.3
Amistar	37	7.8
Monceren	30	6.3
Monceren + Amistar	33	7.0
LSD Treatment		

Yield and tuber numbers (1st harvest – 29 Sept 2010)

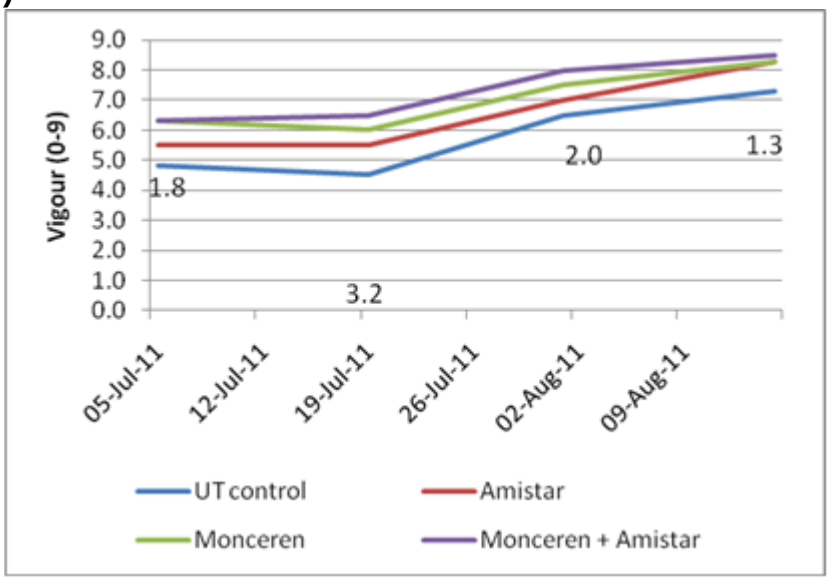
	Yield (t/ha)					Tuber numbers (000's/ha)				
Treatment	<45 mm	45-65 mm	65-85 mm	>85 mm	Total	<45 mm	45-65 mm	65-85 mm	>85 mm	Total
UT control	3.1	16.5	15.5	1.0	36.2	96	127	43.4	2	267
Amistar	1.4	13.5	18.8	1.0	34.7	33	98	54.7	2	188
Monceren	1.8	12.0	9.6	2.3	25.6	39	89	28.6	4	160
Monceren + Amistar	1.4	14.2	12.2	0.4	28.2	39	98	36.5	1	174
LSD Treatment	1.02 *	6.61	5.02 *	2.93	8.29 *	31.0	47.7	14.9 *	4.6	70.7 *

Woodlands 2011

Assessments of growth – Ground cover (Numbers indicate LSD at each assessment)



Assessments of growth – Vigour (Numbers indicate LSD at each assessment)

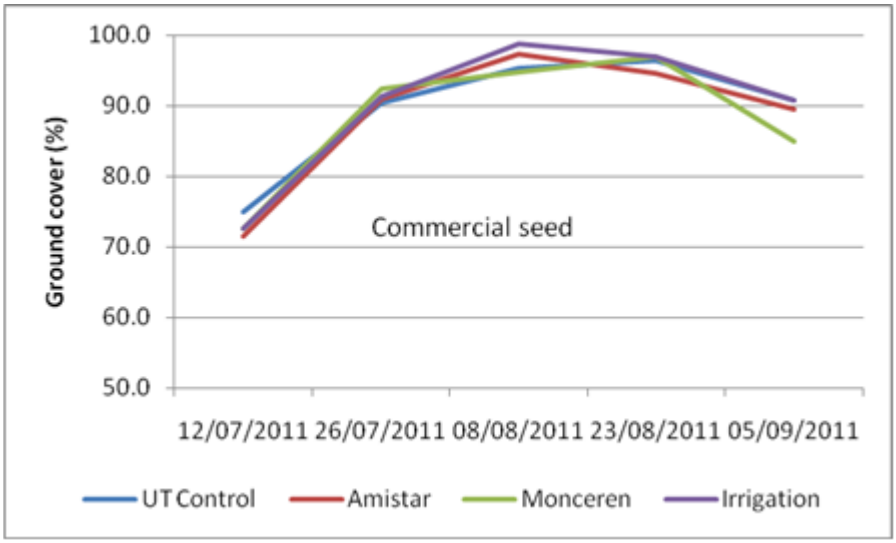


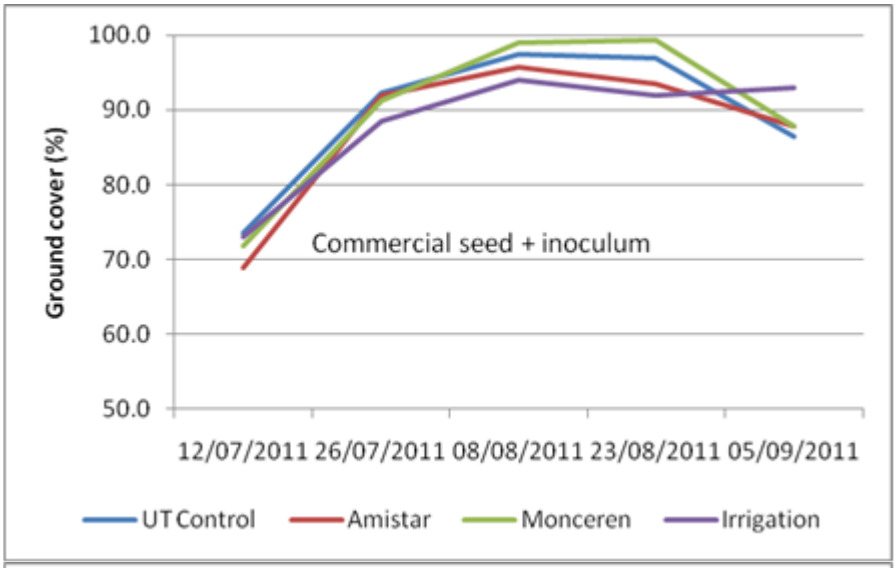
Yield and tuber numbers (mean of two harvest dates)

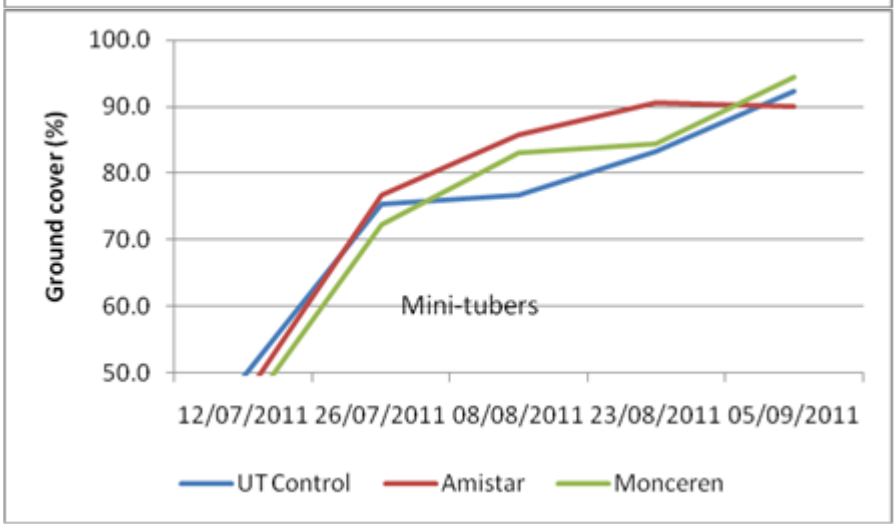
	Yield (t/ha)					Tuber numbers (000's/ha)				
Treatment	<45 mm	45-65 mm	65-85 mm	>85 mm	Total	<45 mm	45-65 mm	65-85 mm	>85 mm	Total
UT control	3.60	17.41	5.65	0.00	26.66	75.28	119.3	21.31	0.00	215.9
Amistar	4.03	16.66	4.68	0.00	25.37	89.49	126.4	17.05	0.00	232.9
Monceren	3.50	10.49	7.81	0.81	22.61	76.70	80.97	29.83	1.42	188.9
Monceren + Amistar	4.27	14.52	3.12	0.81	22.73	92.33	110.8	12.78	1.42	217.3
LSD Treatment										

Fingask 2010

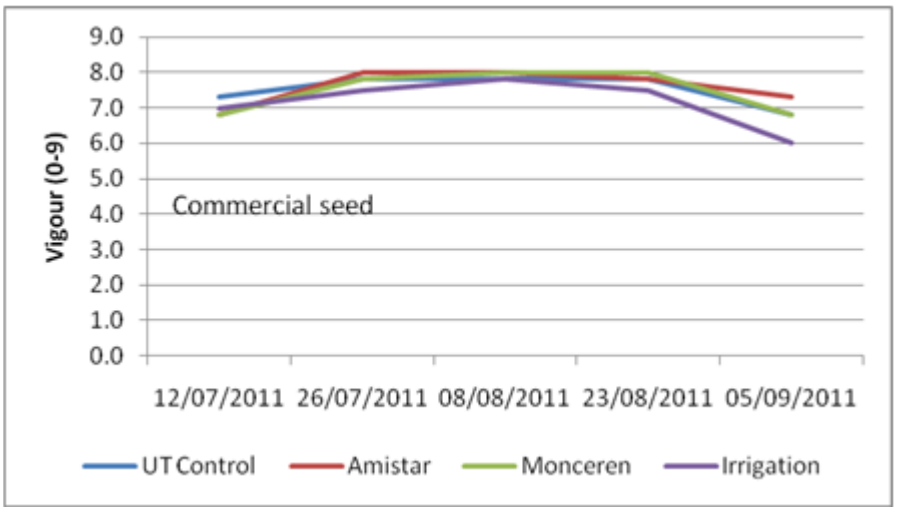
Assessments of growth – Ground cover (Numbers indicate LSD at each assessment).

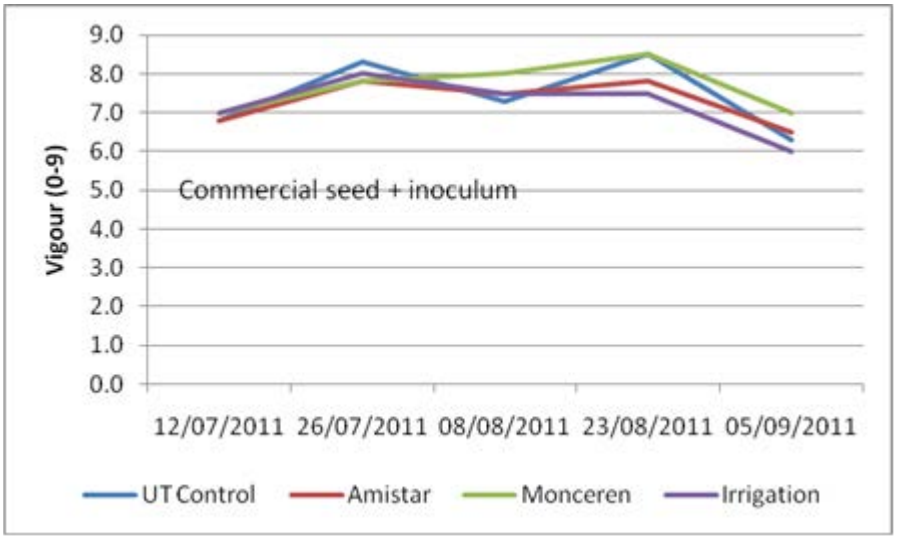


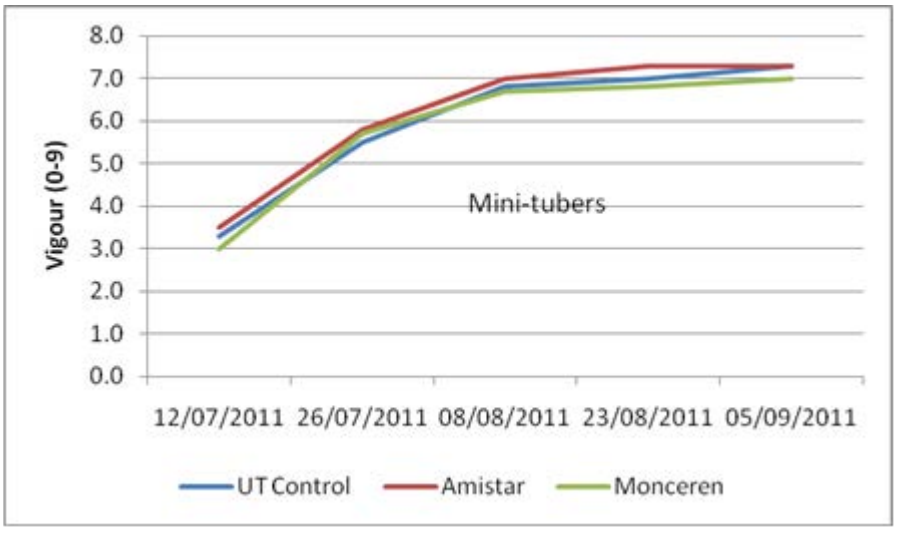




Assessments of growth – Vigour (Numbers indicate LSD at each assessment)

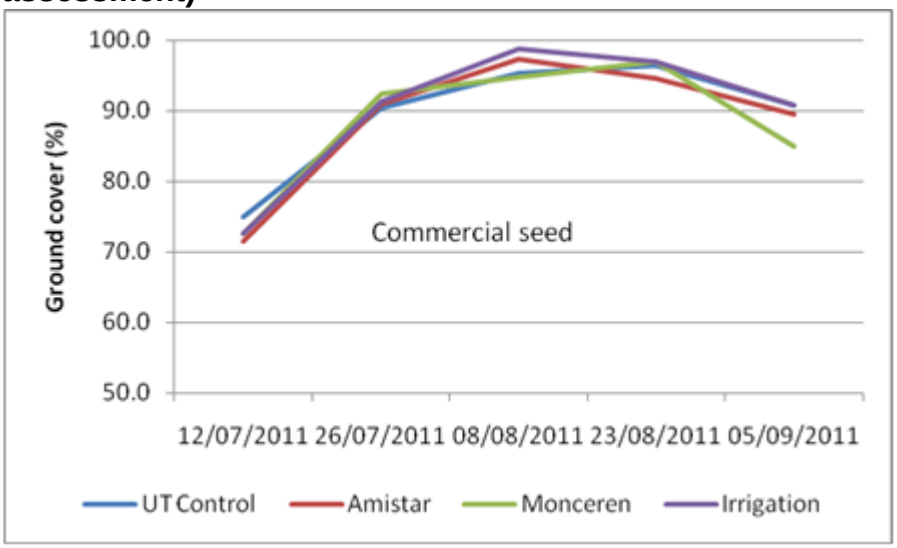


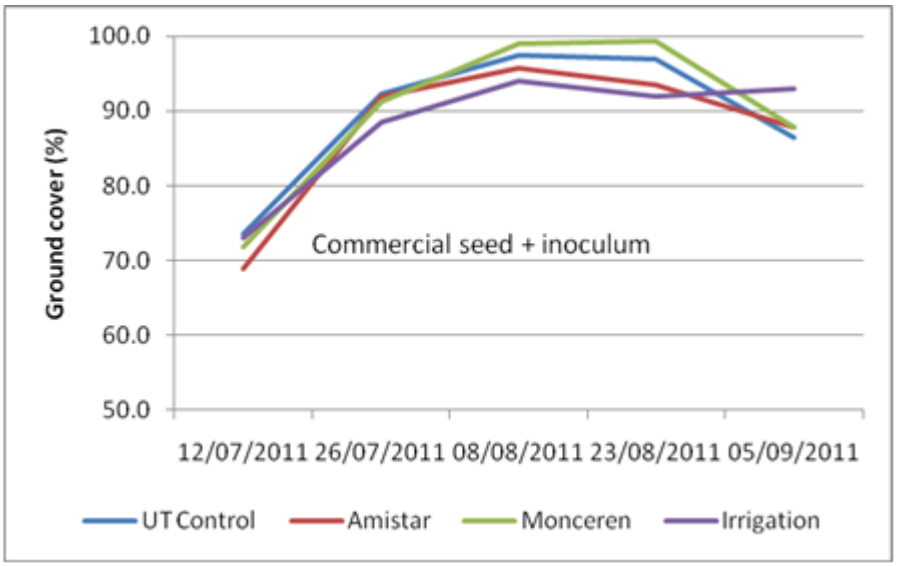


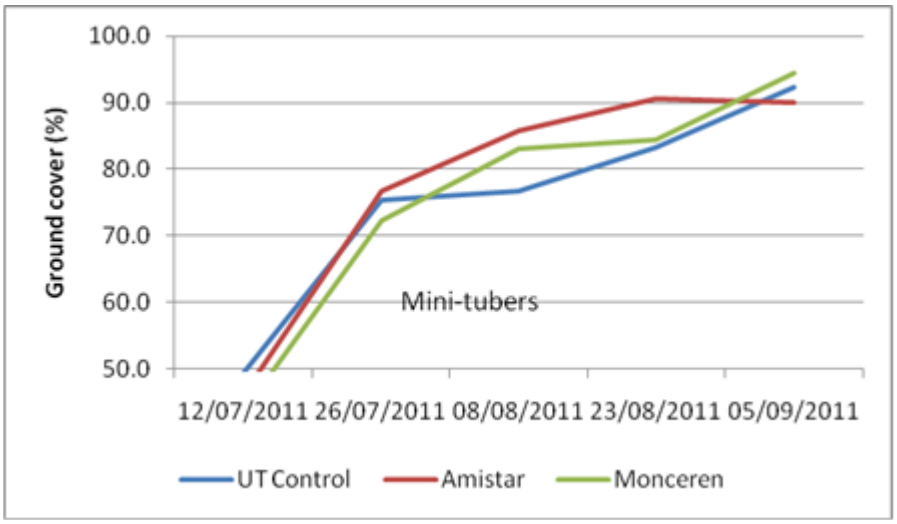


Fingask 2011

Assessments of growth – Ground cover (Numbers indicate LSD at each assessment)







Assessments of growth – Vigour (Numbers indicate LSD at each assessment)