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Prediction and molecular detection of soil-borne pathogens of potato

Dr. Nigel Crump
VIC Department of Primary Industries

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Prediction and molecular detection of soilborne pathogens of potato

Final Report
Horticulture Australia Project PT01019

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Purpose of project

The objectives of this project were to develop innovative diagnostic assays for the detection and quantification of two important potato diseases, Rhizoctonia canker and black scurf and powdery scab, and to lay the groundwork for expansion of the technology to include other pathogens of potatoes and other horticultural crops.

Acknowledgments

Financial support for this project was provided by Horticulture Australia, the Australian Potato industry through contribution to the research and development levy, the Department of Primary Industries Victoria and C-Quentec through a voluntary contribution.

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Media Summary

This project has developed DNA-based soil diagnostic tests for the soil inhabiting organisms or 'pathogens' that cause the common diseases common scab, powdery scab, Rhizoctonia canker & black scurf, Verticillium wilt, black dot and silver scurf of potato plants and tubers. The advantages of DNA tests are that they are quick, reliable, quantitative and specific to a particular pathogen or pathogen strain. Many conventional tests are impractical, being labour intensive and cumbersome, taking several weeks or months to complete and often requiring several additional tests to confirm the identity of a specific pathogen strain.

These tests mean that the research into developing disease management strategies will now be much more efficient and targeted. Scientists now have the tools to track populations of the pathogens in soil and how these populations may change with crop rotations, methods of seedbed preparation, nutrient levels and various organic and chemical treatments applied to the soil. As we learn more about the behaviour of these pathogens, we will be better able to predict disease risk and identify more effective disease management options. These tests will be refined and will become integral to the research being conducted within the National Processing Potato Research and Development Program over the next five years.

Ultimately, DNA-based tests will become part of a commercial service for potato growers, together with decision support tools, to help manage potato crops for maximum profit. These tests can be partially automated, therefore allowing large numbers of soil samples to be processed. Single soil tests will provide results on the disease risk associated with all the pathogens present in a particular field.

Technical Summary

This project has developed DNA-based diagnostic tests, both conventional and real-time, for detecting and quantifying inoculum of several soil-borne potato pathogens in soil samples. In this study, new conventional and real-time PCRs were developed for *Streptomyces scabies*, published conventional and real-time PCRs were adapted for *Rhizoctonia solani*, *Spongospora subterranea* and *Colletotrichum coccodes* and published conventional PCRs adapted for *Verticillium dahliae* and *Helminthosporium solani*.

The process of developing practical PCR tests for soil was demonstrated in this study using the common scab pathogen, *S. scabies* as a model. This process included:

- Detection and quantification of colonies of *Streptomyces* spp. from serial dilution of soils on selective 'STR' agar media.
- Identification of pathogenic strains of *Streptomyces* spp. using thin-layer chromatography to detect the production of the phytotoxin thaxtomin A.
- Development of PCR tests specific to the *nec1* and *txt* genes on the 'Pathogenicity Island' of the *Streptomyces* genome to target only the pathogenic species. The PCR tests were validated against estimates of populations of pathogenic *Streptomyces* spp. using the soil plating method. The population of pathogenic *Streptomyces* in soil samples taken from fields that were severely affected with common scab accounted for a relatively low percentage of the total population of *Streptomyces* species isolated from those fields. Without specificity for pathogenicity, PCR tests in this instance would significantly overestimate pathogen populations.

In addition, several commercial kits for the extraction of DNA from soil were compared. The MoBio™ extraction kit was considerably less expensive and was found to be more robust than the others in terms of DNA extraction and PCR amplification and was, therefore, used as the routine soil DNA extraction system.

Morphological studies confirmed that *S. scabies* was the predominant species of *Streptomyces* isolated from tubers and soil in Australia. The PCR test can be used to quickly distinguish pathogenic from non-pathogenic isolates of *S. scabies* based on the presence of the *nec1* or *txt* genes.

A real-time PCR for pathogenic *Streptomyces* species was tested in a 3-year field study. Micro-plots located within commercial potato crops across Victoria were planted with a disease susceptible variety. The PCR values from soil samples taken at planting were reasonably correlated with the incidence and severity of common scab on tubers harvested from those plots in only one of the three seasons. This result indicates that considerably more validation must be done to develop a robust soil test.

Real-time PCR tests were used to quantify populations of the 'potato attacking strain' of *R. solani,* anastomosis group AG3, (Rhizoctonia canker & black scurf), and for the black dot (*C. coccodes*) and powdery scab pathogens (*S. subterranea*) in field soil samples. An adapted conventional PCR for *V. dahliae* was used to quickly confirm the presence of this pathogen in plant and soil samples from commercial fields severely affected with a 'wilt' disease. In addition, a conventional PCR that was specific to thiabendazole resistant strains of *H. solani,* the cause of silver scurf of potatoes, was used to rapidly screen Australian isolates of the pathogen for fungicide resistance. Conventional tests had taken several weeks to complete.

There are several reports in the literature outlining the development of PCR tests for potato pathogens. These tests were usually validated in the laboratory or glasshouse to produce standard curves relating quantity of DNA against quantity or 'inoculum' of a pathogen. Most of these reported tests were not validated against disease in the field. This study has taken DNA tests to the next level and provided pathologists with workable tools for field research. However, further work is required before these tests will provide a true estimate of disease potential.

Technical Report

Prediction and molecular detection of soil-borne pathogens of potatoes

Chapter 1 Introduction

Soilborne pathogens have a significant economic impact on potato production in Australia and around the world as there is growing pressure for growers to produce high yielding and blemish free crops with reduced reliance on chemical pesticides. These issues emphasise the need for accurate and timely application of pesticides which are economically essential to potato production and which will produce the highest marketable yield. Therefore, there is a need for rapid diagnostics tests to provide the earliest possible warning that a pathogen is present which is capable of causing economic yield loss. Thereby enabling the prescribed application of plant protection technologies at the right time to minimise the losses caused by disease.

The soil is a very complex environment that creates numerous barriers to the identification, isolation and quantification of soilborne pathogens. Consequently, soilborne research has been constrained by limited capability to identify, detect and quantifying pathogens from soil. However, there is a range of classical approaches available. Selective media can be used which exclude the unwanted soil organisms and allow growth of the target pathogen. In most cases, pathogen recovery is method dependent. The target organism is outgrown by better competitors, morphological characters may be common for several species and the bias of the researcher may influence the outcome. For obligate pathogens such as *S. subterranea*, selective media is not an option and therefore plant bioassays have been developed which are time consuming and can be influenced by growing conditions and the variety/cultivar of the host used.

One of the most important aspects of disease prediction and the assessment of control strategies in soilborne diseases is the quantification of inoculum density of a pathogen. In many cases there are large variations using traditional methods, as was demonstrated in an inter-laboratory comparison to quantify micro sclerotia of *V. dahliae* (Termorshuizen *et al.* 1998). This suggests that inoculum density data for *V. dahliae* found *in the literature is only a vague estimate of reality* (Goud and Termorshuizen 2003). In many cases, traditional methods of isolating pathogens from soil can only be used for detection purposes and are not reliable to use for the quantification of inoculum.

Conventional PCR technologies have created many new diagnostic opportunities and are now routinely used in the taxonomic identification of plant pathogens. However, to guide disease management decisions diagnosis requires not only accurate identification, but also accurate quantification so that appropriate disease management strategies can be matched with disease risk. The application of real-time PCR provides the capability to quantify the amount of inoculum present in a sample. A recent review on the use of real-time PCR in plant pathology (Schena *et al.* 2004) summarised the development of PCR detection of fungi that are economically important in agriculture.

PCR is a sensitive technology that provides several advantages over the traditional methods of soilborne pathogen diagnosis.

1. the target pathogen(s) do not need to be cultured,
2. there is potential to identify and detect an individual strain of a pathogen within a mixed sample,
3. when using real-time PCR the amount of the target pathogen can be quantified
4. automation and robotics can be used to enable high throughput sample processing and routine testing.
5. multiple pathogens can be detected in the one sample (using multiplex PCR)
6. in comparison to traditional detection methods, considerably less time is required to complete the diagnosis.

Table 1-1 Examples of the application of real-time PCR for the study and detection of economically important fungi in agriculture (as cited in Schena *et al.* 2004)

Pathogen/antagonist	Crop	Chemistry	Reference
Aphanomices euteiches	Alfalfa	TaqMan	Vandemark et al. (2002)
Aspergillus flavus	Maize, pepper and paprika	TaqMan	Mayer et al. (2003)
Aspergillus fumigatus, Geotricum candidum, Candida albicans, Stachybotrys chartarum	Air, water and dust	TaqMan	Haugland et al. (2002)
Aureobasidium pullulans	Table grape and sweet cherries	Scorpion-PCR	Schena et al. (2000), Finetti Sialer et al. (2000a), Schena et al. (2002a)
Blumeria graminis f. sp *tritici*	Wheat	SYBR Green I	Fraaije et al. (2002)
Cladosporium sp., *Ramularia* sp., and *Microsphaera alphitoides*	Peduncolate oak	SYBR Green I	Heuser and Zimmer, (2002)
Colletotrichum coccodes	Potato and soil	TaqMan	Cullen et al. (2002)
Diaporthe phaseolorum and *Phomopsis longicolla*	Soybeen	TaqMan	Zhang et al. (1999)
Fusarium solani f.sp *phaseoli* and *Glomus intraradices*	Bean and soil	SYBR Green I	Filion et al. (2003a), Filion et al. (2003b)
Fusarium species	Wheat	SYBR Green I	Schnerr et al. (2001)
Glomus mosseae, Phytophthora infestans, and *P. citricola*	Various hosts	TaqMan	Böhm et al. (1999)
Helmintosporium solani	Potato and soil	TaqMan	Cullen et al. (2001)
Heterobasidion annosum	Norway spruce	TaqMan	Hietala et al. (2003)
Magnaporthe grisea	Rice	SYBR Green I	Qi and Yang (2002)
Phaeocryptopus gaeumannii	Douglas-fir	SYBR Green I; TaqMan	Winton et al. (2002), Winton et al. (2003)
Phakopsora pachyrhizi and *P. meibomiae*	Soybean	TaqMan	Frederick et al. (2002)
Phytophthora infestans	Potato	SYBR Green I	Avroa et al. (2003)
Phytophthora medicaginis	Alfalfa	TaqMan	Vandemark and Barker (2003)
Phytophthora nicotianae	Citrus roots and soils	Scorpion-PCR	Ippolito et al. (2000), Ippolito et al. (2004)
Pyrenophora spp. and *P. graminea*	Barley seeds	SYBR Green I	Bates et al. (2001), Taylor et al. (2001b)
Pyrenophora teres	Barley	Scorpion-PCR	Bates and Taylor (2001)
Rizoctonia solani	Potato and soil	TaqMan	Lees et al. (2002)
Rosellinia necatrix	Various hosts and soils	Scorpion-PCR	Schena et al. (2002b), Schena and Ippolito (2003)
Septoria tritici, Stangospora nodorum, Puccinia striiformis and *P. recondita*	Wheat	SYBR Green I	Fraaije et al. (2001)
Spongospora subterranea	Potato, soil and water	TaqMan	Van de Graaf et al. (2003)
Stachybotrys elegans		SYBR Green I	Morissette et al. (2003)
Suillus bovines and *Paxillus involutus*	Soil	Two fluorogenic probes	Landeweert et al. (2003)
Tilletia indica and *T. walkeri*	Wheat	TaqMan	Frederick et al. (2000)
Various pathogens	*Arabidopsis thaliana*	SYBR Green I	Brouwer et al. (2003)
Verticillium dahliae	Olive and soil	Scorpion-PCR	Nigro et al. (2002)

PCR based diagnostics may provide earlier pre-symptomatic detection than that achieved by immunoassays, allowing enhanced exploitation of preventative disease management strategies such a cultivar selection and curative fungicides. Furthermore, few immunoassays are effective in the detection of pathogens in soil. Recent advances in robotics have allowed the automation of PCR diagnostics allowing for rapid throughput and cheaper delivery of diagnostics to end-users.

The ability to routinely quantify the level of pathogen in the soil will allow for more accurate monitoring of pathogen populations resulting in a better understanding of pathogen ecology. Ultimately this will lead to the development of improved disease control strategies.

Chapter 2 Situation analysis of diagnostics for soilborne diseases of potato

Summary. Diagnostic soil tests currently available for most of the soilborne pathogens of potato including *Streptomyces* spp. (common scab) *S. subterranea* (powdery scab) and *R. solani* (Rhizoctonia canker and black scurf) are inadequate and that rapid, sensitive DNA-based tests need to be developed.

Streptomyces spp. can be isolated and quantified from soil using semi selective media. However, not all strains of *Streptomyces* spp. found in soil are capable of causing disease on potato. Therefore, it is necessary to isolate pure cultures of Streptomyces from soil and identify pathogenic strains based on the production of the phytotoxin thaxtomin or by using DNA tests that are specific to pathogenic strains. This is a lengthy process and is extremely labour intensive. There is no routine analysis available for the detection and quantification of *Streptomyces* spp. from soil.

In the case of powdery scab, the causal organism (*S. subterranea*) cannot be cultured on artificial media. Detection has traditionally relied on plant bioassays, which are time consuming and labour intensive. More recently, ELISA has been developed for the detection of *S. subterranea* however this technique lacks sensitivity when used with infested soil. Currently, there is no technique available for the quantification of this pathogen.

The isolation and quantification of *R. solani* from soil is a time consuming and labour intensive procedure that is not reliable or compatible with high-throughput applications. Isolation of the organism relies on the use of selective media and bait plants. Interpretation of the results of these tests is complicated by the fact that *R. solani* exists as three inoculum types, namely sclerotia, colonised plant debris and free hyphae, and that different anastomosis groups may coexist in a single affected field. In order to quantify inoculum of *R. solani*, it is necessary to isolate pure cultures of the fungus and identify the anastomosis group of individual cultures using known tester strains. Quantification relies on comparative counts between soil-isolated inoculum and soil artificially inoculated with a dilution series of inoculum.

The traditional methods of isolation and quantification, for *Streptomyces*, *Rhizoctonia* and *Spongospora*, are time-consuming, do not allow for rapid throughput of samples, and therefore, would be costly in terms of a commercially available test. Therefore, the aim of this project was to:

- develop rapid testing procedures, such as DNA-based diagnostic tests for use with soil and,
- develop quantitative molecular assays to enable disease prediction based on inoculum concentration.

Introduction

Soilborne diseases of potato cause considerable economic loss in Australia. These soilborne diseases include powdery scab (*S. subterranea*), common scab (*S. scabies*) and Rhizoctonia canker and black scurf (*R. solani*). The ability to accurately identify the presence of a pathogen in soil and to determine the threshold levels of inoculum, at which the pathogen causes economic loss, provides information that can be used to better manage disease. Currently, there are no routine diagnostic tests available for soilborne pathogens of potato in Australia.

Diagnostics for soilborne pathogens of potato

Streptomyces spp.

Several species of bacteria belonging to *Streptomyces* spp. are known to cause the soilborne disease common scab of potato. In the overseas literature, it is considered that *S. scabies* is the predominant causal agent of common scab, although *S. acidoscabies* is considered the predominant causal agent in soil with a pH below 5. In Australia, there has been no study of the predominate species of *Streptomyces* that causes common scab of potato. Not all species of *Streptomyces* cause disease on potato and not all *Streptomyces* within the same species are able to cause disease on potato. Pathogenic strains of *Streptomyces* produce a phytotoxin named thaxtomin, which is associated with pathogenicity. Thaxtomin A is the most common phytotoxin produced by both *S. scabies* and *S. acidoscabies.* Nine minor thaxtomins toxins are also produced by *Streptomyces.* Genetic studies have shown that pathogenic strains of *Streptomyces* have a region of genes including *nec 1* and *txt* that are associated with pathogenicity. The *nec* gene codes for the production of a protein, which is thought to have a role in pathogenicity whereas the *txt* gene codes for the production of the thaxtomin. This region of genes, known as a pathogenicity island, is thought to be a transposable element meaning that it can be potentially exchanged into non-pathogenic strains. These genes (nec

and txt) are conserved in all pathogenic strains of *Streptomyces*, including *S. scabies*, *S. acidoscabies* and *S. turgidiscabies* (Bukhalid *et al.* 1998).

Disease symptoms of common scab can vary widely. Lesions on the surface of potatoes can be shallow or deep-pitted (Figure 2-1). The various symptoms can be caused by strains belonging to the same species, currently it is not clear what factors directly determine the nature of the scab symptoms on potato.

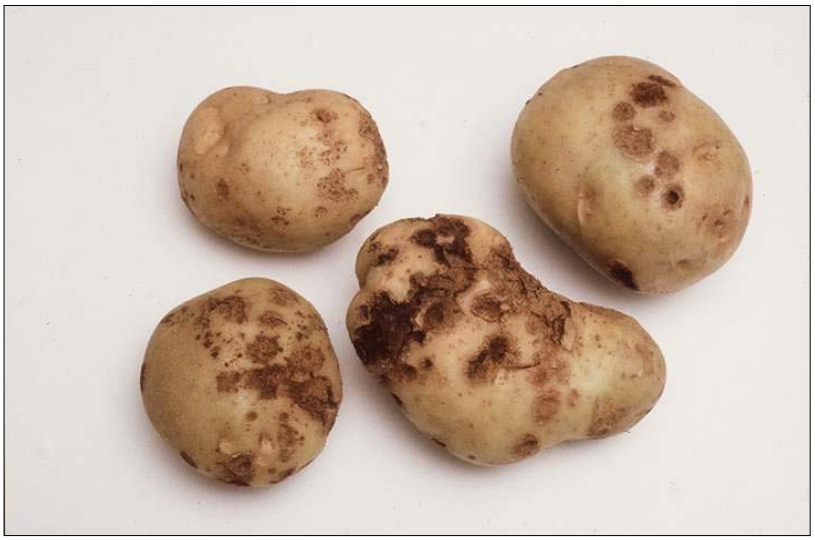


Figure 2-1 Potato tubers with common scab

The detection and quantification of Streptomyces spp. from soil. The development of disease control methods is constrained by the lack of a rapid and quantitative technique for measuring populations of pathogenic *Streptomyces* spp. from soil. Enumeration of total *Streptomyces* from infested soil does not provide an estimation of disease risk as only a small portion comprises of pathogenic strains. Keinath and Loria (1989), using a greenhouse assay, showed that only 3.3% of all *S. scabies* isolates obtained from infested soil were pathogenic to potato. However, the greenhouse method is too time-consuming and labour intensive for routine soil population assessments. The production of melanin on artificial agar has been used as a diagnostic character to identify *S. scabies* (Keinath and Loria 1990), but this does not differentiate pathogenic and non-pathogenic strains. DNA markers that target the 16s rDNA gene have been used to identify species of *Streptomyces* (Takeuchi *et al.* 1996), however they do not differentiate pathogenic and non-pathogenic strains. Other techniques have been used to identify strains of *Streptomyces* such as fatty acid methyl ester profiles (Ndowora *et al.* 1996), RFLP (Doering-Saad *et al.* 1992) and DNA-DNA hybridisation (Healey and Lambert 1991). These methods are labour intensive and time consuming, as they require a pure isolate of *Streptomyces* before conducting the test.

A semi- selective culture medium known as STR was developed to isolate *Streptomyces* spp. from infested soils (Conn *et al.* 1998). This medium allows colonies to express a phenotype that assists in the identification of potentially pathogenic strains. Using this semi- selective media in combination with a rapid thin layered chromatography technique to detect thaxtomin production, the number of pathogenic strains of *Streptomyces* in soil can be determined. However, the method is labour intensive, time consuming (the entire process takes about 1 month), requires some expertise in identification of colonies, and therefore, could not be used for routine diagnosis. Although, the use of this media to determine the population of pathogenic *Streptomyces* in soil does provide a comparative method to evaluate new soil diagnostic tests for *Streptomyces* spp.

Spongospora subterranea

The obligate pathogen *Spongospora subterranea* (Wallr.) Lagerh. f.sp. *subterranea* causes powdery scab of potato. In the absence of a suitable host, the pathogen survives in the soil as cystosori (sporeballs) (Jones and Harrison 1972). Root exudates from host plants stimulate the germination of the resting spores to produce primary zoospores (Kole 1954). These zoospores are able to swim in soil water to infect the epidermal cells of stolons and roots or root hairs, where they form plasmodia. Cleavage of plasmodia result in secondary zoospores that infect surrounding tubers and roots (Kole and Gielink 1963).

Infection of the cortical cells of the roots, stolons and tubers of tuber bearing *Solanum* species and the cortical root cells of other members of the genus results in the development of cystosori.

Symptoms of disease include galls on roots and stolons and scab like eruptions on the surface of tubers (Figure 2-2), all of which, contain masses of cystosori.

Figure 2-2 Potato tubers with powdery scab

The detection and quantification of S. subterranea from soil. The quantification of *S. subterranea* in soil is important to allow studies on the epidemiology and management of powdery scab. Traditionally, bioassays have been used to detect *Spongospora* in soil using glasshouse or nutrient solution bioassays. The methods of Merz (1989) and Fornier (1997) involve the suspension of soil or cystosori in nutrient solution in which tomato seedlings are added as bait plants. After a period of incubation the roots of the tomato seedlings are stained and observed under the microscope and rated using the modified method of Kole (1954). The methods of Merz (1989) and Fornier (1997) differ in the incubation periods used in the bioassays

The glasshouse bioassays of Brereton (1991) allows the detection of cystosori to a very low level and to a limited degree provides some quantification. In these bioassays field soil is placed in pots and directly baited with tomato or potato seedlings. These bioassays take into account the soil factors (such as pH, zinc, and structure) that may affect disease development. Factors such as pre treatment of cystosori (priming), infection period, variety of potato and environmental conditions can influence the outcome of these assays.

For the majority of bioassays, the severity of root hair infection is recorded as opposed to disease on tubers. To date, there has been no conclusive evidence to relate the level of infection of root hair

to disease incidence or severity on potato tubers. The assessment of root hairs requires considerable time and some expertise although some researchers are now measuring level of root infection using Q-PCR (van de Graaf *et al.* 2003). An enzyme linked immunosorbent assay (ELISA) using polyclonal anisterum for quantifying *S. subterranea* is available, but is not sensitive enough to detect low levels of inoculum in soil (Walsh *et al.* 1996). More recently a monoclonal anisterum assay was developed (Merz 2000). However, this assay is specific to cystosori only and does not recognise other forms of the pathogen such as zoosporangia and zoospores (Bouchek-Mechiche *et al.* 2000).

DNA based detection has been developed for *S. subterranea* by several researchers (Bell *et al.* 1999) (Ward *et al.* 2004) (Bulman and Marshall 1998) . Most recently, the conventional PCR and real-time PCR (van de Graaf *et al.* 2003) has shown potential in the detection and quantification of *Spongospora* in soil. To date, this PCR assay has not been evaluated for the detection of *S. subterranea* in Australia.

Rhizoctonia solani

Rhizoctonia canker and black scurf, caused by the fungus *Rhizoctonia solani*, is widespread and common in Australia and can affect emergence, plant growth and tuber yield and quality. *Rhizoctonia* is a soil-inhabiting fungus adapted to survival under a diverse range of conditions, affecting a wide range of crop species world-wide (Anderson 1982; Ogoshi 1987). The fungus can be found in native vegetation as well as in agricultural production areas (Cother 1979; Ogoshi 1987). *R. solani* survives in soil as sclerotia (red/black thick walled structures on potato skins), as thick walled hyphae (fungal threads) in soil and on tubers or in plant debris (Papavizas *et al.* 1975). Under favourable conditions, the fungus grows actively in soil, colonising the roots of potatoes and other plants. The fungus can colonise roots and stems of many plants without causing disease symptoms, thereby maintaining its population. It can survive in organic matter in the absence of hosts.

Anastomosis Groups of R. solani. *R. solani* is taxonomically divided into 13 subspecific groups called Anastomosis Groups (AGs) (Carling *et al.* 2002) based on somatic (vegetative) incompatibility responses between hyphae of genetically distinct isolates (anastomosis reactions) (Carling *et al.* 1988). Each of these groups has different degrees of specialisation to specific families of plants. Some have a high degree of host specificity. AG3, for example, is most commonly isolated from potatoes and AG8 from cereal roots (Banville *et al.* 1996).

Of the 13 different anastomosis groups of *R. solani*, six have been associated with potatoes. They include AG1, AG2 (subgroups 2-1 and 2-2), AG3, AG4, AG5 and AG9 (Banville *et al.* 1996). Traditionally, AG3 has been known as the potato attacking strain, being host-specific to potatoes. However, AG4 and AG5 are also capable of damaging potato plants. Most of the sclerotia isolated from potato tubers are reported to belong to AG3 (Banville *et al.* 1996).

Anastomosis groups of R. solani in potatoes in Australia. Balali and co workers (1995) collected isolates of the fungus from stems, roots, tubers and soil in potato crops grown in Virginia and Lenswood in South Australia. Of 301 isolates tested, 90% were AG3 and 7% and 2% were AG4 and AG5, respectively. AG3 and AG5 caused stem and root cankers and black scurf. AG4 caused stem cankers, severe root cankers and significantly reduced the number and volume of fine roots (feeder roots) but did not produce black scurf (Balali *et al.* 1995).

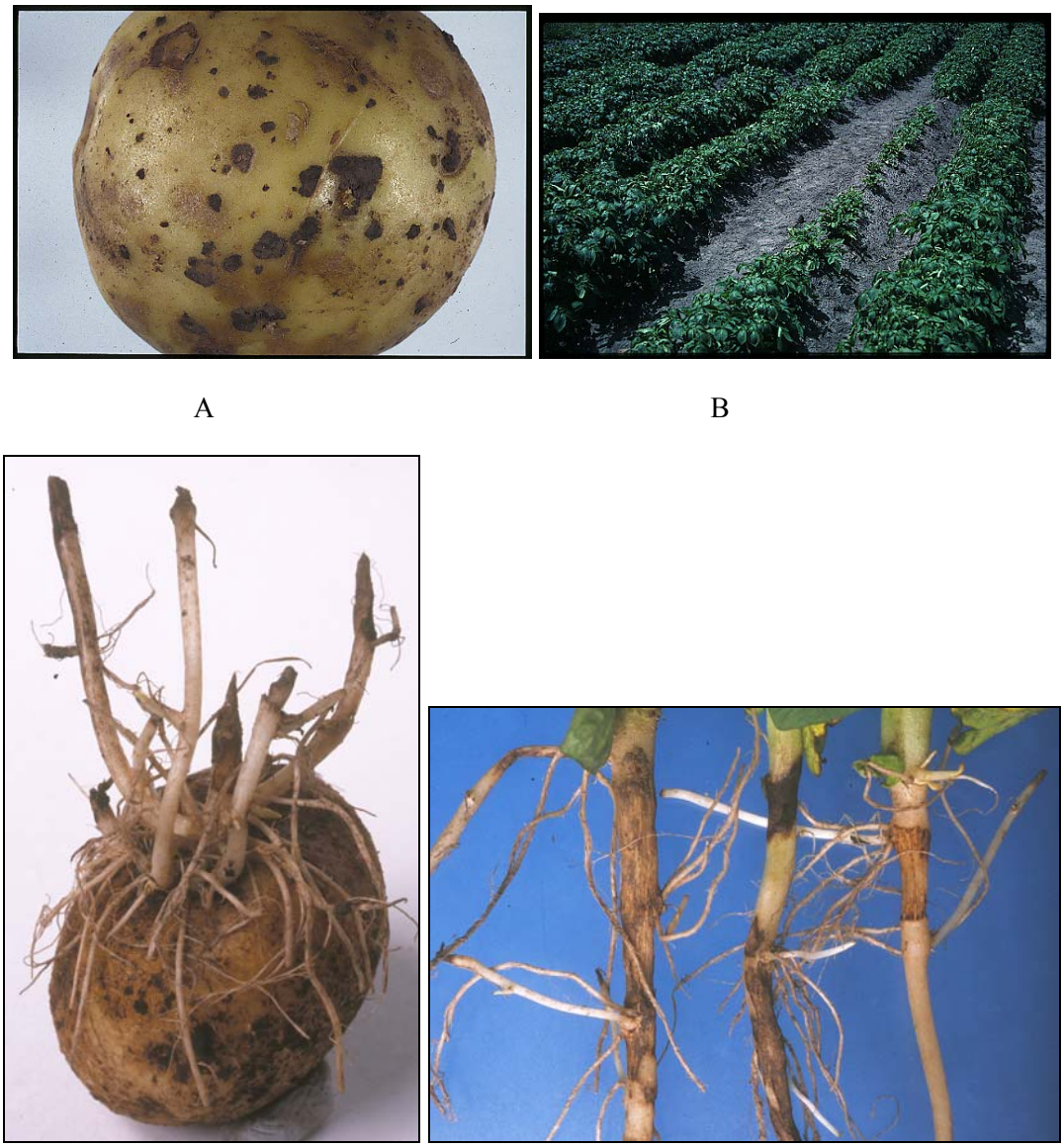
In Australia, AG8 is common in cereal production areas and causes severe root rot of cereals and other field crops (Mazzola *et al.* 1996). Potatoes are now grown in traditional wheat cropping areas and glasshouse studies have shown that AG8 can also cause stem and root cankers and a significant reduction in the number of fine roots of potato plants, but does not produce black scurf on potato tubers (Balali *et al.* 1995).

A study of potato crops in two districts of Victoria (Petkowski *et al.* 2003) found not only AG3, 4 and 5, but also AG2 (subgroups AG2-1 and AG2-2). The AG2 groups are often associated with crucifers (*Brassica*) and legumes, as well as many other crops. The AG3s were the most common,

accounting for more than 60% of the isolates of the fungus, whilst the AG2s accounted for about 25%. The AG2s proved to be very pathogenic to potatoes, as well as *Brassica* species and red clover (*Trifolium repens*). This is the first report of AG2 causing significant damage to potatoes.

R. solani AG2s were also implicated as the cause of a new disease in Victorian potato crops (de Boer *pers. comm.*) where large patches of wilting plants were found in relatively mature crops (post-flowering). This disease has not been described elsewhere and strains of AG2-1 and AG2-2 have been associated with the disease.

It has also been reported that *R. solani* AG3, the 'potato strain' is able to grow and reproduce on the roots of *Brassica* species and clover forming an epiphytic relationship with its hosts (de Boer *et al.* 2003). This demonstrates that, although *R. solani* AG3 is a potato pathogen, it is able to develop non-parasitic relationships with other crop species as described by Carling *et al.* (1986). The AG2s strains were also common on the roots of *Brassica* and clover (*Trifolium* spp.).



A B

C D

Figure 2-3 Disease symptoms caused by *R. solani* . A: Black scurf on a potato tuber, B: A Rhizoctonia patch in the crop caused by stem and stolon pruning, C: sprout pruning by *R. solani*, D: below ground stem lesions caused by *Rhizoctonia solani*

The detection and quantification of R. solani from soil. The available diagnostic techniques for the detection and quantification of *Rhizoctonia* in soil or on plants is best summarised by Neate and Schneider (1996) (Table 2). For each of the techniques, there are potential advantages and disadvantages and these have been summarised in Table 2. In selecting a suitable method, the following points must be taken into consideration

(a) soil isolation methods that involve mechanical disturbance will affect the size and viability of fragments of mycelia that are extracted and therefore the estimation of the amount of Rhizoctonia present in a sample.

(b) the quantities of the fungus present in a soil sample are unlikely to be absolute if the test used is only calibrated using artificial inoculated (spiked) soil.

(c) techniques involving baiting with seedling or seeds may not detect just the *R. solani* potato attacking strains and further testing of isolates is required to determine anastomosis group.

(d) agar techniques only give information about the organisms that are capable of surviving the extraction process and then growing on the medium under the selected conditions.

The extraction of *R. solani* from soil does not provide an indication of the capacity to cause disease. This is because *R. solani* exists as three inoculum types; sclerotia, colonised plant debris and free hyphae, and that different anastomosis groups may coexist in a single affected field.

In order to quantify *R. solani*, and relate it to disease risk, it is necessary to isolate pure cultures of the fungus and identify the anastomosis group of individual cultures using known tester strains. This process requires expertise in the identification of hyphal reactions and takes considerable time to complete. To overcome these constraints, molecular techniques have been developed that are specific to anastomosis group. Whisson and coworkers (1995) developed a diagnostic test for AG8 and more recently, Lees and coworkers (2002) developed a PCR based diagnostic test for AG3. Immunoassays have also been developed, however they are not as sensitive as PCR tests and lack specificity (Thornton *et al.* 1999).

Table 2-1 Limitations to techniques used for the quantification of Rhizoctonia from soil or on plants. From Neate and Schneider (1996)

	Experience in identifying hyphae necessary	Only small quantity of soil assayed	Only detects fungi capable of growth in the test	Relatively tedious technique	Influenced by competition between organisms	Fungus must be close to bait	Does not differentiate dead from live hyphae	Only detects hyphae actively growing in soil	Disturbance caused by technique may affect result	More qualitative than quantitative	d by soil type *	Does not indicate size of CFU	Does not indicate capacity to cause disease
Whole soil / pellet plating	x	x	x	x	x				x	?		x	x
Agar film/membrane	x	x					?		?				x
Soil fraction plating	x		x	x	x				x	x	?	x	x
Baiting with plants	x		x			x		x		?		x	x
Baiting with other material	x		x			x		x		?		x	
Solid surfaces in soil	?		x					x	x	x			x
Direct observation	x	x		x			x			x	?		x
Plating plants on agar media	x		x		x					?		x	x
Molecular							?				?		x
Antibodies							?	?			?		x
Biochemical analysis		?					?			?	?	x	x
Vital stains	x	x	x	x				x	x		?		x
Radio isotopes		?							x		x		x

*May be due to signal quenching in the case of radio isotopes and fluorescent dyes
Note that these are the subjective opinions of the authors and will change as the techniques develop.

Verticillium spp.

Verticillium infects roots and stems of potato plants and inhabits the vascular system of plants causing early senescence of stems or whole plants. The fungus also is part of a complex of pathogens, including the nematode *Pratylenchus penetrans* (root lesion nematode), associated with potato early dying (PED) of potatoes in North America. In Australian potato production, the significance and epidemiology of PED is poorly understood. However, extensive studies have been conducted overseas, in particular the USA where PED is considered to be one of the most important diseases of potato. In North America, the potato yield losses are 10-15% in moderately affected fields and can be up to 50% in severe cases (Powelson and Rowe 1993). In temperate regions of the US, PED is primarily caused by *V. dahliae* while in the cooler areas, such as northern US and southern Canada, *Verticillium albo-atrum* is more dominant. In Australia, the distribution of the two species of *Verticillium* is unknown. The significance of determining the species involved in PED is related to the development of appropriate management strategies.

V. dahliae produces micro-sclerotia that consist of clusters of melanised, thick walled cells that are able to persist in soil and can remain viable for more than 10 years. *V. dahliae* can be introduced to new fields through infested soil carried on machinery and equipment and through infected seed. A survey of certified potato seed in the US showed up to one-third of seed lots tested was infected with *V. dahliae* (Rowe and Powelson 2002). A smaller study in Australia showed that up to 12% of certified seed in Australia was infected with *V. dahliae* (Wicks *et al.* 1998). However, a larger survey is required to validate the levels of *Verticillium* spp. in certified seed. *V. albo-atrum* does not produce micro-sclerotia, but produces dark resting mycelium that does not survive in soil for more than two years.

Co-infection of potatoes with *V. dahliae* and root lesion nematodes in particular *P. penetrans* results in the earlier onset of disease symptoms. In addition, the inoculum levels required to initiate disease is lower than that required if the pathogens were acting individually (Botseas and Rowe 1994). The mechanism(s) of this synergism are unknown.

The detection and quantification of Verticillium spp. from soil. Many methods have been developed to quantify *Verticillium* spp. in soil. These include flotation of micro-sclerotia (Ben-Yephet and Pinkas 1976; Huisman and Ashworth 1974b) wet sieving soil samples (Huisman 1988), and using semi-selective media that usually contains pectin as a carbon source (Huisman and Ashworth 1974a). Using such methods, the amount of *Verticillium* inoculum in soil has been correlated with subsequent disease incidence in crop (Wheeler and Rowe 1995). However, these assays can be influenced by pH, type of carbon source used, length and conditions of incubation. To overcome these influences, PCR based assays have been developed for the detection and quantification at the species level for *V. dahliae* and *V. tricorpus* (Hu *et al.* 1993); (Robb *et al.* 1994) and at the strain level for *V. albo-atrum* (VA1 and VA2 strains) (Robb *et al.* 1993). The use of the PCR based techniques provides a rapid and accurate detection of the correct species/strain without the cross-reaction problems reported for immunoassays (Plasenicia *et al.* 1995). PCR based diagnostic prevents the confusion in the identification of species of *Verticillium*, especially *V. tricorpus* and *V. dahliae*. In addition, the PCR based assays allows the diagnosis of not only soil but also infected plant tissue. These PCR based assays have not been validated using Australian potato soils.

Colletotrichum coccodes

Colletotrichum coccodes causes the tuber blemish disease of potato known as black dot, which is widespread in potato production areas of Australia (de Boer and Wicks 1994). The disease black dot gets its name from the abundant small, dot-like black sclerotia that are commonly found on senescent and dead potato roots, stolons and stems below and above the ground, as well as on tubers (Dillard 1992). The fungus progressively colonises the roots, stems, stolons and tubers of potato plants as the crop develops. Severely affected plants can wilt under stress, although, generally, the fungus does not cause serious damage to potatoes (Harrison 1963). Little is known

about the ecology of *C. coccodes* in Australia. It is reported to survive in soil for up to 8 years as sclerotia in crop debris (Dillard and Cobb 1998). Studies in the USA found the number of propagules of *C. coccodes* in soil to be related to the history of potato cropping (Barkdoll and Davis 1992). The fungus was common in ground with a history of potatoes but undetectable in virgin ground. In Victoria, the disease was relatively common in potatoes grown after 8 years of pasture in a study where disease-free minitubers were planted as seed (de Boer and Curtis 1997). The fungus can colonise roots and stems of many plant species besides potato and these hosts may play an important role in its survival in the period between potato crops (Raid and Pennypacker 1987).

The detection and quantification of C. coccodes from soil. Farley (1972) developed a semi-selective agar for the isolation of *C. coccodes* from soil. Using this method, the author reports detection as low as three propagules of *C. coccodes*/g of oven dried soil. Similarly, Carnegie and associates (2003) used a slightly different semi-selective media to detect *C. coccodes* in soil, however found a glasshouse assay to be more sensitive. The bioassay involves assessing the infection of roots of potato plants grown in infested soil after 9-12 weeks. Using this method, the researchers were able to detect 0.4 micro-sclerotia of *C. coccodes* per gram of soil. There are several limitations in using this method for routine analysis.

- The need to include standard inoculum concentrations in order to transform the results to a numerical basis
- The space and time required to conduct such bait tests
- Variations in environment conditions that may affect infection directly or indirectly by affecting plant growth.

To overcome these constraints, both conventional and quantitative real time PCR tests were developed (Cullen *et al.* 2002). These tests could detect and quantify *C. coccodes* in soil and plant material. To-date, these tests have not been validated using Australian potato soils.

Helminthosporium solani

Silver scurf, caused by the fungal pathogen *Helminthosporium solani*, is one of the most common diseases of potato tubers around the world and the most common disease of seed potatoes in Australia (de Boer and Wicks 1994). The fungus only attacks the potato skin, affecting tuber quality but not yield. Until recently, it was believed that *H. solani* did not survive more that a few months in soil and was mainly perpetuated through the planting of infected seed potatoes. However, recent work in Victoria shows that the fungus is soil-borne and widespread in traditional potato cropping districts. When disease-free mini-tubers (produced in a glasshouse) were planted after 8 years of pasture, 100% of their progeny were affected with silver scurf, indicating that inoculum of *H. solani* was soil-borne. Evidence from other trials in Victoria suggests that the fungus may also occur in virgin soil.

For a disease as common as silver scurf, surprisingly little is known about the ecology of the pathogen and how it survives in soil. *H. solani* is not known to have any other hosts. It does not grow on the roots of potato plants. A study in the USA found that the fungus can multiply on the roots of dead grasses (Merida and Loria 1994) which suggests that it may have some activity as a saprophyte in soil. Volunteer potatoes probably play an important role in the survival of this pathogen in the period between potato crops.

Resistance to the commonly used fungicide thiabendozole (TBZ) has been reported around the world, including Australia (Stewart-Wade *et al.* 2003). Detection of TBZ resistance involves growing a pure culture on a TBZ amended media and measuring the effect on radial growth. Due to the slow growth of *H. solani* on artificial media, this process can take up to 12 weeks. A rapid diagnostic test was developed by McKay and Cooke (1997), that detects a single codon mutation in the β-tublin gene of resistant strains of *H. solani.* Using this test, diagnosis of TBZ resistance can be achieved (within 2 days). To-date, this PCR assay has not been validated in Australia.

Conclusions

- There are a wide variety of methods available for the detection and quantification of soilborne pathogens of potato. However, for the most part, these tests are time consuming and labor intensive and do not provide results that can be related to disease incidence or severity in field. Many diagnostic tests work extremely well, such as the isolation of *Streptomyces* using STR, however they can not be used as routine assays.
- There are some DNA diagnostics tests developed for potato pathogens (*Rhizoctonia* and *Spongospora*) however the majority of these tests have not been validated on pathogens and soils in Australia.

Chapter 3 Development of diagnostics tests for potato pathogens

Introduction

This chapter presents the development of sensitive and rapid PCR diagnostic assays for *Streptomyces* spp., *S. subterranea*, *R. solani and V. dahliae* using conventional and real-time (Taqman) PCR. In addition, a PCR diagnostic assay for the detection of fungicide resistance in *H. solani* is also reported.

Routine extraction of DNA from soil

Various commercial DNA extraction kits were evaluated to determine a suitable soil DNA extraction system for routine use. The commercial kits included were FastPrep 101 and MoBio each with 0.5 g air-dried field soil. The FastPrep kit was validated using;

(1) standard manufacturers protocol with DNA suspended in 50 μL TE buffer,

(2) standard manufacturers protocol with DNA suspended in 100 μL TE buffer,

(3) FastPrep with improved DNA clean up with DNA suspended in 100 μL.

The MoBio kit was used according to the manufacturers protocol with slight modifications (see Appendix I). Briefly, the soil was mechanically agitated using the FastPrep machine (setting 5.5 for 30sec). Extractions suspended in 50 μL and 100 μL were compared.

The results of various DNA extractions are shown in Figure 3-1. More concentrated DNA was obtained using the FastPrep DNA extractions in comparison to the MoBio extraction system. When amplified using PCR the FastPrep 100 μL, FastPrep clean up 100 μL and both MoBio extractions were amplified (data not shown). The MoBio extraction kit is considerably less expensive and is more robust in terms of DNA extraction and PCR amplification. Therefore, the MoBio extraction kit using 100 μL was selected as the routine soil DNA extraction system.

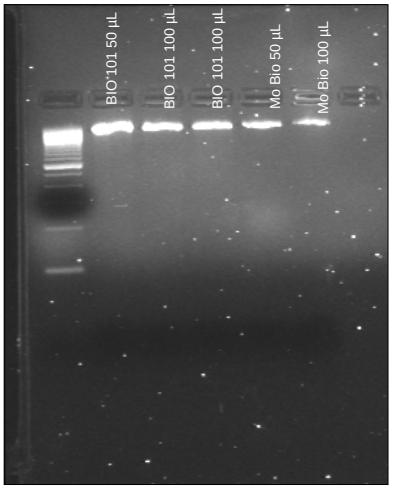


Figure 3-1 A comparison of the efficiency of extracting DNA from soil using different commercial DNA extraction kits with modifications

Development of diagnostics for soilborne pathogens of potato

1. *Streptomyces* spp.

Enumeration of Streptomyces spp. from field soil.

Soil was collected from a field site in the Otway region of Victoria that had a history of common scab. The soil was air-dried and stored at 4° C prior to dilution. A serial dilution of soil was prepared by suspending 10 g soil in 90 mL of 0.1% water agar blended using a stomacher. From this suspension, serial dilutions of 1 mL in 9 mL 0.85% NaCl were made as shown in Figure 5. For each dilution, an aliquot of 100 µL was aseptically transferred and evenly spread onto the 'STR' agar in a 90 mm diameter petri dish. The STR media was prepared according to Conn and Leci (1998) (Appendix II). Three STR plates were inoculated for each dilution. The plates were incubated at room temperature for 10 days before counts were performed.

Colonies with the following criteria were counted on all plates at a given dilution.

- *Streptomyces* colonies with a circular depression in the medium at the edge of the colonies, and,
- *Streptomyces* colonies that were hard and well adhered to the surface of the medium and,
- *Streptomyces* colonies that did not sporulate or produce aerial mycelium with two weeks

These colonies were transferred to a new STR plate and incubated at room temperature for 10 days after which pure colonies were used for further studies.

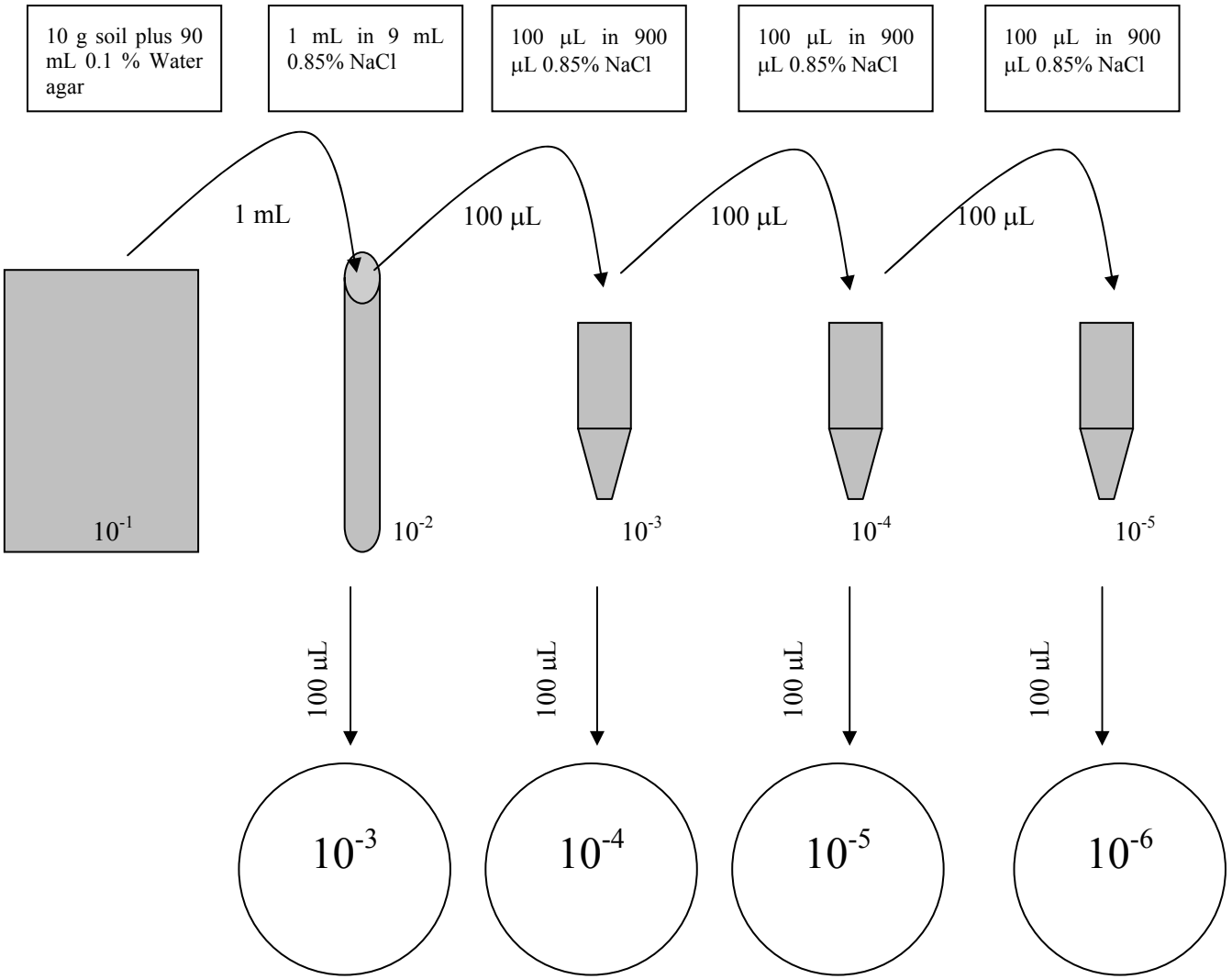


Figure 3-2 Serial dilutions of soil for the isolatation of *Streptomyces* spp. on STR media

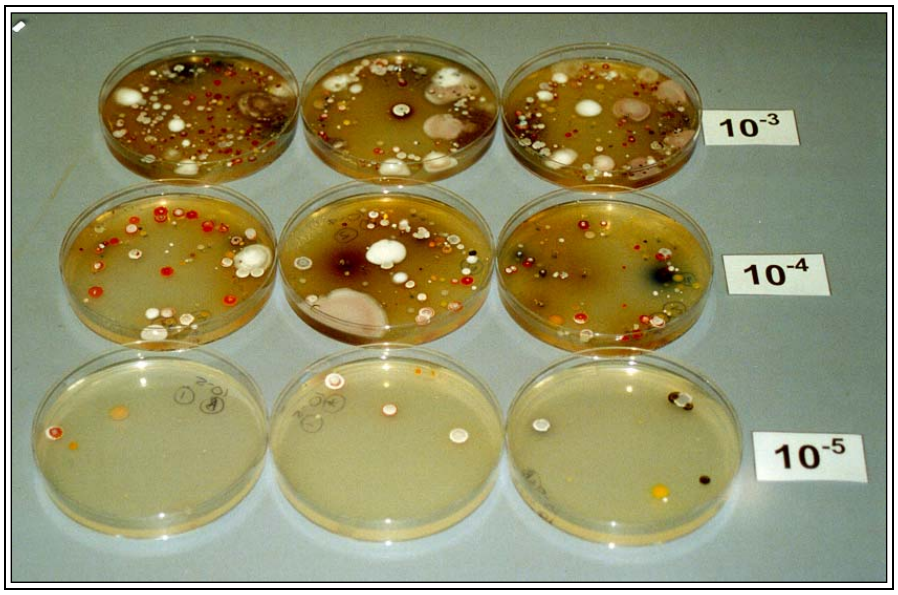


Figure 3-3 Enumeration of *Streptomyces* spp. from field soil using STR agar

To the knowledge of the research team, it is the first time in Australia that *Streptomyces* spp. were isolated from soil collected from potato field using this method. The dilution of 10^{-4} was selected as the best dilution from which to count *Streptomyces* spp. as the colonies were not too crowded. The results for each of the three plates were averaged for each replicate as presented in Table 3-1. From this study, it was estimated that the soil contained 94,000 of *Streptomyces* like colony forming units per gram of soil. However, not all of the *Streptomyces* are able to cause disease on potato. Therefore, the ratio of pathogenic to non-pathogenic strains needs to be determined.

Table 3-1 CFU count of *Streptomyces* spp. isolated from soil taken from a potato field in the Otway region of Victoria

Replicate	Dilution 10^{-4}	# cfus/g soil
1	9.40	94,000
2	9.70	97,000
3	9.20	92,000
Average	9.43	94,333

Identification of pathogenic isolates of Streptomyces spp.

Two methods were used to determine isolates that are pathogenic to potato

a) the production of thaxtomin

b) the presence of the nec gene using PCR

(a) Production of thaxtomin. Thaxtomin was evaluated using the method of Conn and Leci (1998). Isolates of *Streptomyces* spp. collected from the serial dilutions were grown as a lawn culture on oatmeal agar (Appendix II) for a minimum of 5-7 days. After which approximately 3 cm^2 of colonised agar was transferred to a 1.5 mL centrifuge tube. Thaxtomin was extracted with ethyl acetate. For each isolate, 10 µL was loaded onto a silica TLC plate. The TLC was conducted using a solution of 10 % methanol in chloroform. The presence of thaxtomin was confirmed by the appearance of a yellow band (natural light) which absorbs UV_{366} light. Standards of thaxtomin A and B were also included on each TLC plate.

b) Determination of pathogenicity using PCR. Using a sterile pipette tip a small amount of mycelia/spore was taken from a single colony of each isolate and suspended in 100 µL of TE buffer. The suspensions were then stored at -20°C until ready for PCR. PCR was conducted using primers (Nf and Nr Appendix III) that targeted the nec 1 region of the pathogenicity island of *Streptomyces* spp. PCR amplification of all samples was based on a standard set of conditions [initial denaturation at 95ºC for 7 min., then 95ºC for 3 min. followed by 35 cycles of 95°C for 45 sec, 60ºC for 45 sec and 72ºC for 1 min. in a reaction volume of 25 µL. The PCR reaction contained the following components 1 x reaction buffer, 200 µM dNTPs, 3 mM $MgCl_2$, 0.1 µM of each primer, 2 Units of Taq polymerase (Invitrogen) and 40 ηg or 2.5 µL of DNA template. PCR products were separated in 1.0% agarose gels and visualised using ethidium bromide staining.

Of the isolates obtained from the soil sample from the Otway region, 30% were shown to be pathogenic using TLC detection of thaxtomin (Figure 3-5). This result was confirmed by the amplification of the nec region (Figure 3-6). Therefore, Otway soil contained 94,333 *Streptomyces* like cfu/g soil of which, only 28,300 cfu/g were pathogenic to potato. This level of pathogenic *Streptomyces* was similar to that found in Canadian soils with high disease risk (Lazarovits *pers. comm.*)

The method of enumerating *Streptomyces* from soil using STR, and subsequent testing using TLC and PCR, is time consuming, labour intensive, and could not be used for routine testing.

Figure 3-4 Characteristic growth of four isolates of *Streptomyces* spp. on oatmeal agar

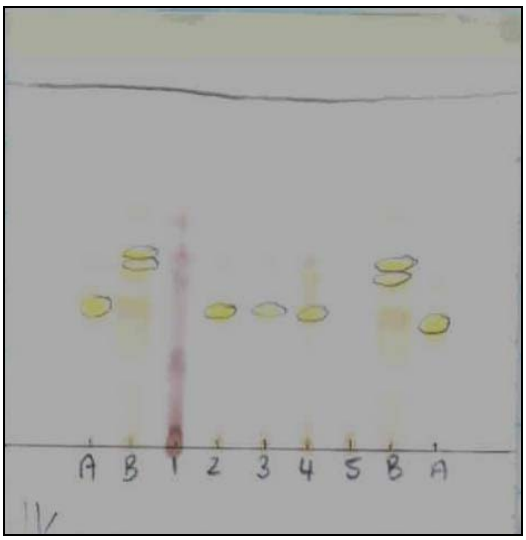


Figure 3-5 Example of Thin Layered Chromatography (TLC) to detect thaxtomin produced by isolates of *Streptomyces* spp.

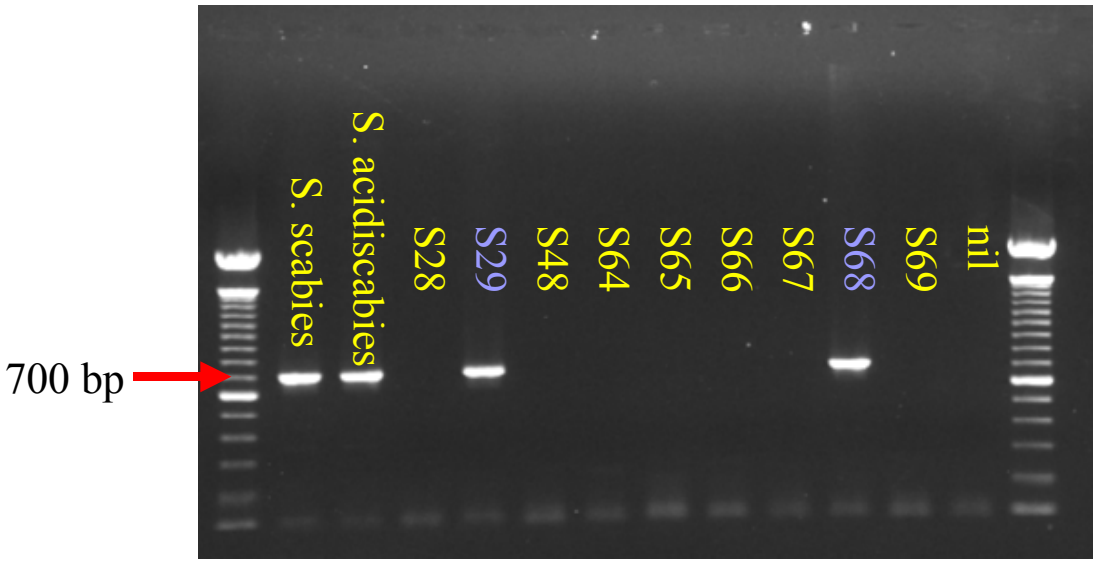


Figure 3-6 PCR amplification of the nec region of isolates of *Streptomyces* spp.

Development of soil DNA diagnostic for pathogenic Streptomyces spp.

A naturally infested soil containing 30,000 cfu/g of pathogenic *Streptomyces* spp. was diluted using sterilised (autoclaved) soil to give soil samples containing 30000, 15000, 7500, 3250, 1625, 813 and 0 cfu of *Streptomyces* per gram of soil. From each of the samples, 0.5 g of soil was used for DNA extraction using MoBio method previously described. DNA extraction was repeated twice on each dilution.

The PCR reaction contained the following components 1 x G buffer (see Appendix IV), 200 µM dNTPs, 0.1 µM of each primer (Nf/Nr), 0.1% DMSO, 160 µg BSA, 2 Units of Taq polymerase (Invitrogen) and 2.5 µL of DNA template. A typed isolate of *S. scabies* was included as a positive control. PCR products were separated in 1.0% agarose gels and visualised using ethidium bromide staining.

All concentrations of the *Streptomyces* spp. in naturally infested soil could be amplified consistently (Figure 3-7). The band intensity was associated with inoculum concentration providing the ability to quantify levels of pathogenic Streptomyces in soil. The concentrations of *Streptomyces* in the assay reflect what is considered high, medium and low disease risk.

To determine if the PCR could be used to predict disease risk of potato field, soil samples were taken from 30 potato fields in Ontario in 2001 and at each site a 10 m row of cv. Shepody was planted as an *in situ* bioassay. At the end of the growing season, potato tubers were assessed for severity of common scab using a score from 0 (nil) to 5 (very high). The predicted risk of common scab was based on the intensity of the 700 bp band from the PCR and was subjectively scored from 0 (nil) to 5 (very high). The predicted disease risk from PCR was found to correlate to actual disease severity on tubers in the field for the majority of fields (Figure 3-8 and Table 3-2).).

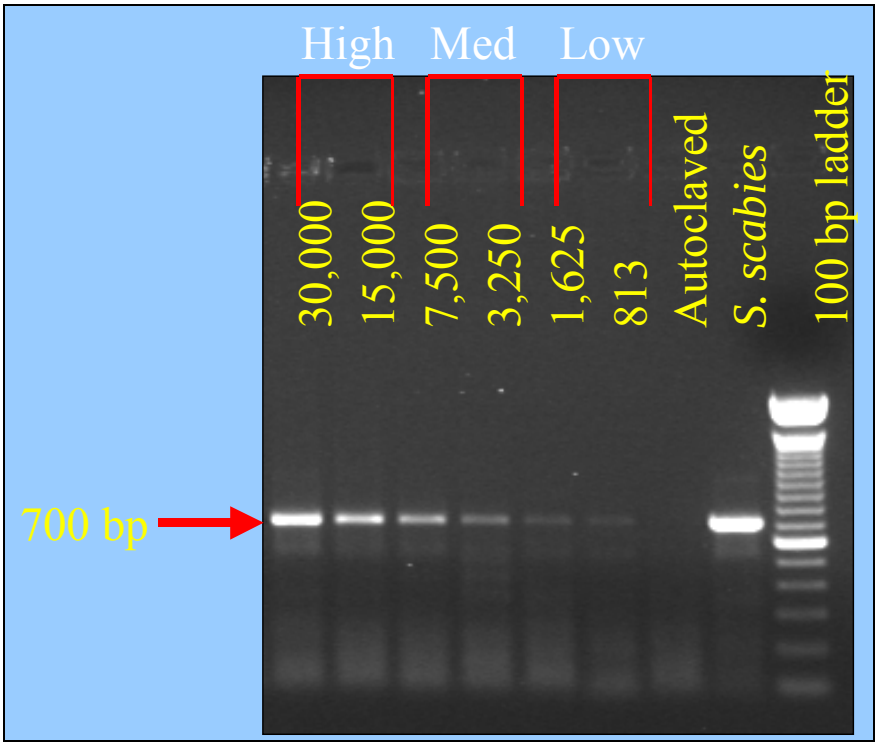


Figure 3-7 Amplification of the nec gene of *Streptomyces* spp. from naturally infested soil with varying amounts of inoculum.

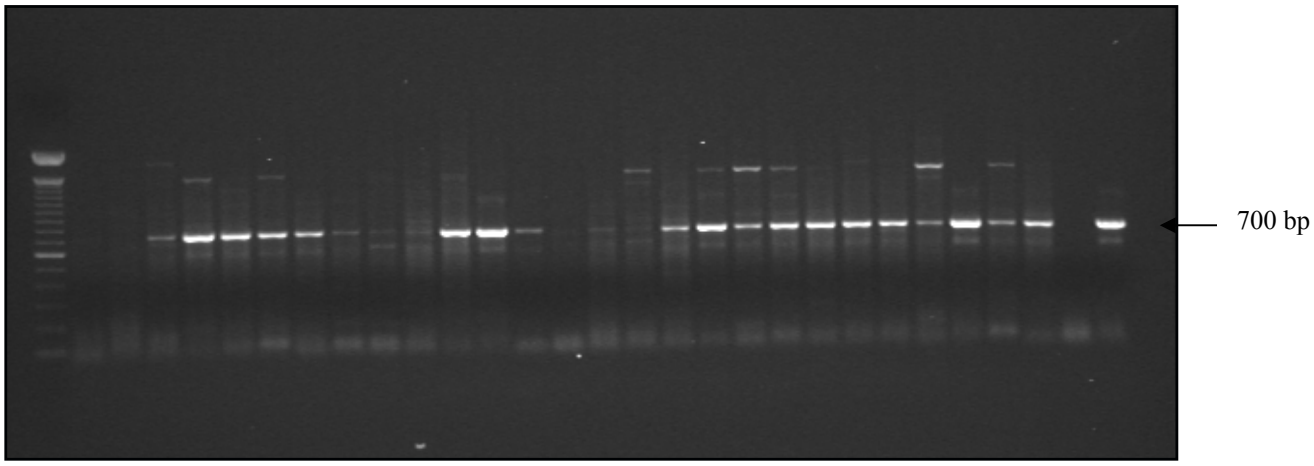


Figure 3-8 Amplification of nec gene of *Streptomyces* spp. from naturally infested soil collected from various regions in Ontario and PEI.

Table 3-2 Comparison of PCR score with disease on tubers

Sample code	PCR score[A]	Tuber scab score (0-5)[B*]	Predictability
B1	0.0	1.5	+++
B3	5.0	4.2	+++
E1	5.0	6.0	+++
E2	5.0	7.0	+++
HV4	2.0	1.8	+++
IR1	5.0	4.5	+++
P3	4.5	5.0	+++
P4	5.0	5.0	+++
R2	4.8	5.8	+++
T1	4.5	4.0	+++
VY1	5.0	5.0	+++
WD2	5.0	4.5	+++
Z2	2.3	2.5	+++
RL2	0.0	1.0	+++
RL1	2.8	3.0	+++
PA1	2.5	3.0	+++
HV3	1.8	1.0	++
JB1	5.0	3.5	++
RM3	4.8	4.0	++
VY2	3.3	2.5	++
BE1	4.8	3.0	++
PA2	4.5	3.0	++
HV2	2.5	1.2	++
JB2	1.3	3.5	+
P1	3.0	5.0	+
R1	4.5	2.3	+
SQ1	4.0	2.6	+
SQ2	2.5	1.5	+
Z1	3.3	5.0	+
B2	0.0	2.3	+

[A] Score = based on band intensity with 0 = no band to 5 very intense.

[B] Disease severity score based on percentage of tuber surface covered with scab lesions

[*] Disease scores: 0 = Nil, 1 = Very low, 2 = Low, 3 = Medium, 4 = High, 5 = Very high

The same PCR assay was evaluated on Australian potato soils however, unexpected results were obtained. The PCR did not amplify on sites known to have high inoculum and in some cases mis-priming occurred (data not shown). To resolve this, it was thought that the *Streptomyces* spp. causing common scab of potato in Australia must be characterised to determine if all strains in Australia have the *nec* gene. Consequently, this would determine if the nec gene is the appropriate target to develop a reliable soil diagnostic test for pathogenic *Streptomyces* spp. in Australia.

Characterisation of Streptomyces spp. from diseased tubers

Note: This work was supplemented with a Monash University seed grant in collaboration with Dr Tonya Wiechel.

To characterise the *Streptomyces* spp. 150 cultures were isolated from diseased tubers collected from various regions in Australia. Overseas studies suggest that the gene for thaxtomin (txt) production maybe present in a higher proportion of isolates than the nec gene.

Primer design for pathogenic Streptomyces spp. The txtA gene of S*. scabies* was accessed on the GenBank database and sequences of isolates were aligned using ANGIS. Putative specific regions were selected and inserted into Primer 3 software to design specific forward and reverse primers TstxtAF1 and TstxtAR1 (Appendix III) for the amplification of the txtA gene and TStxtBF1 and TStxtBR1 (Appendix III) for the amplification of the txtB gene. A primer pair (NosF1 and NosR1 Appendix III) for the nitric oxide synthase gene was also developed. A Taqman fluorescent probe was also designed and was labelled at the 5' end with the reporter dye FAM (6-carboxy-fluorescein), while the 3' end was modified with the quencher dye TAMRA (6-carboxy-tetramethylrhodamine) (Applied Biosystems).

PCR amplification of pathogenic Streptomyces cultures. A spore suspension was used as DNA template in PCR reactions. A small quantity of spores was diluted with 100 µL of TE buffer. Primer pair pA and pH (Edwards *et al.* 1989) was used for the universal amplification of the 16S rDNA of *Streptomyces* spp. as a control to ensure that spore suspensions contained DNA that was suitable for PCR amplification. Primer pair Nf and Nr was used for the amplification of the nec1 gene. Primer pair TstxtAF1 and TstxtAR1 was used for the amplification of the txtA gene. Primer pair TStxtBF1 and TStxtBR1 was used for the amplification of the txtB gene. Primer pair NosF1 and NosR1 was used for the amplification of the nitric oxide synthase gene.

A 25 µL PCR reaction consisted of 1 x reaction buffer, 200 µM dNTPs, 3 mM $MgCl_2$, 0.1 µM of each primer, 2 Units of Taq polymerase (Invitrogen) and 40 ηg or 2.5 µL of DNA template. PCR conditions were initial denaturation at 95ºC for 7 min., then 95ºC for 3 min. followed by 35 cycles of 95°C for 45 sec, 60ºC for 45 sec and 72ºC for 1 min. PCR products were separated in 1.0% agarose gels and visualised using ethidium bromide staining.

To confirm the amplification of the txtA region, PCR products were sequenced. PCR products were cleaned up using QIAGEN 'QIAquick PCR Purification Kit' according to the manufacturers instructions.

Direct PCR product sequencing was carried out on an Applied Biosystems Automated Sequencer using the ABI PRISM Dye terminator Cycle Sequencing Ready Reaction Kit at Monash University. The Big Dye reaction contained 5 µL of PCR product (20 ηg DNA), 1 x Big Dye Buffer, 1 x Big Dye reaction mix and 15 ηM of one primer in a 20 µL reaction volume. Big dye products were precipitated with 2 µL of 3M NaAce (pH 7.5) and 50 µL of 95% EtOH and incubated at room temperature for 1 h tubes were centrifuged for 20 min. at 13 000 rpm and rinsed in 100 µL of 70% EtOH and air dried on the bench for 1h or until dry.

The primers developed for the txtA and nos region amplified 230 bp and 150 bp fragments as expected (Figure 3-9). The blast search of sequences of the txtA region showed that the region amplified by the primers TstxtAF1 and TstxtAR1 was a match for txtA sequences in genbank. The sequences of all isolates of *Streptomyces* amplified using these primers were identical (data available on request). Using primer set Ph/Pa (Appendix III) a 1514 bp region was amplified and this was consistent with the work done by Edwards *et al.* 1989. The sequence of the product of the

txtA reaction confirmed that the correct region was being amplified. Only isolates found to have the txtA region were shown to produce thaxtomin (data not shown). The txtA region was found in more isolates of *Streptomyces* spp. than the nec gene (Figure 3-10) indicating that the txtA region would be a better target for a diagnostic test for the identification of pathogenic *Streptomyces* spp.

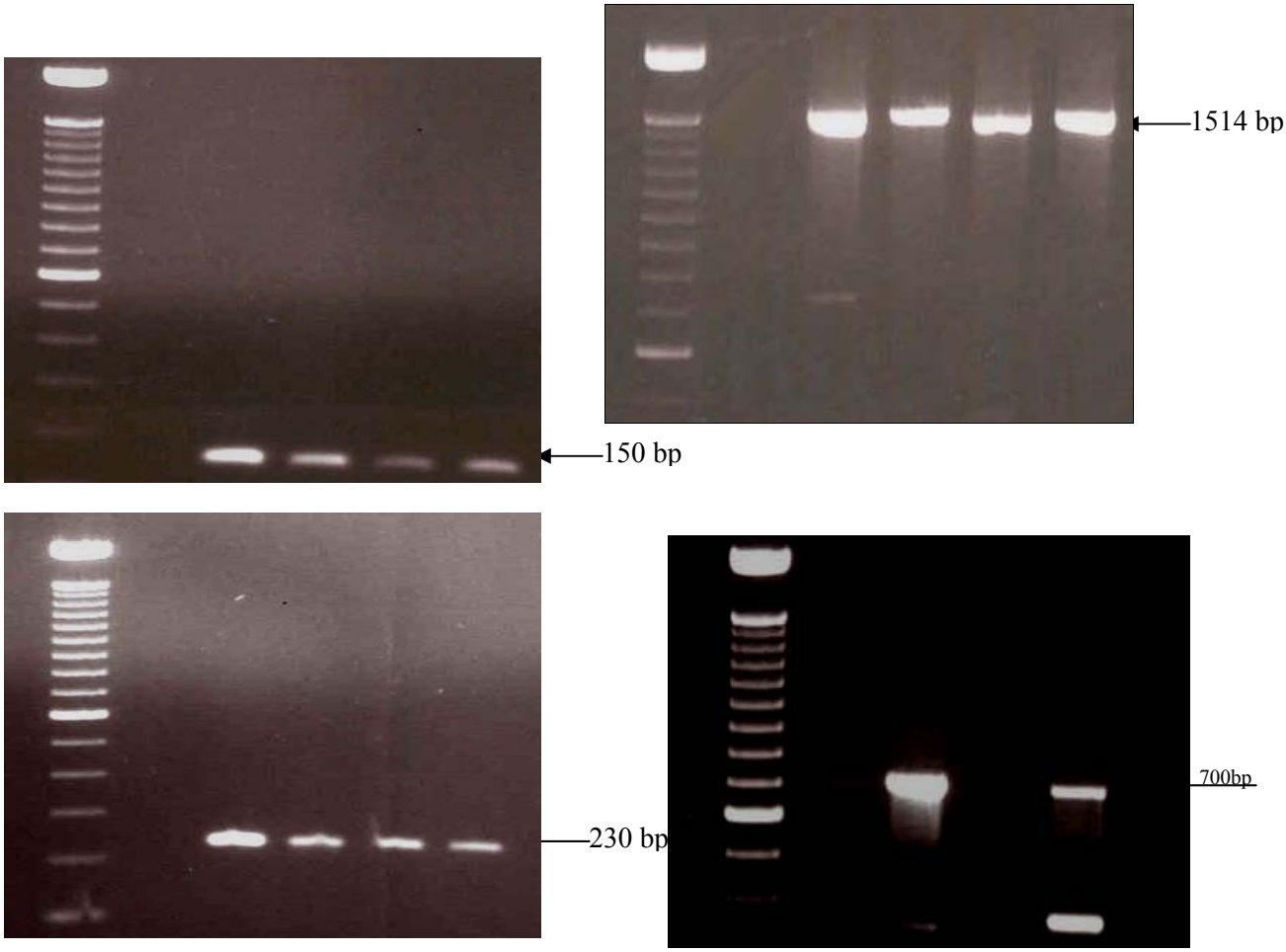


Figure 3-9 Amplification of the 16s rDNA, nec, txtA, and nos regions of *Streptomyces* spp.

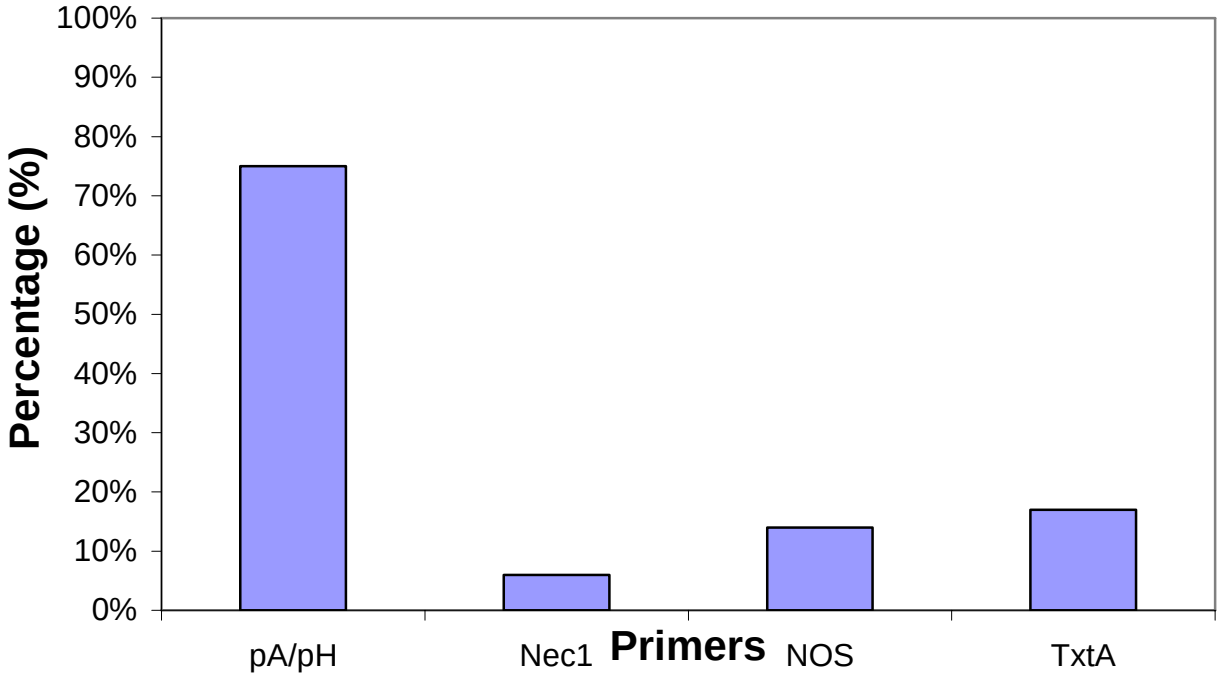


Figure 3-10 The relative amplification of 16s rDNA region using primers pA/Ph, the nec region using primers Nf and Nr and the txt region using primers TstxtaF1 and TstxtaR1.

Conventional PCR for detection of pathogenic Streptomyces in soil

Primer pair TStxtAF1 and TStxtAR1 was used for the amplification of the txtA gene of Streptomyces spp. in spiked soil samples. Sterile distilled water 100 µL per sample containing varying numbers of *S. scabies* spores equivalent to 30000, 15000 and 1000 spores / gram soil were added to samples of dried autoclaved soil (0.5 g each) and the soil was again air-dried before DNA extraction. This was done in triplicate with two isolates of *S. scabies* (Scab path and S146).

The PCR reaction contained the following components 1 x Gitschier buffer (Appendix IV), 200 µM dNTPs, 0.1 µM of each primer, 0.1% DMSO, 160 µg BSA, 2 Units of Taq polymerase (Invitrogen) and 2.5 µL of DNA template. A typed isolate of *S. scabies* was included as a positive control. PCR products were separated in 1.0% agarose gels and visualised using ethidium bromide staining.

All concentrations of the *Streptomyces* in soil were amplified (Figure 3-11). This assay could be used to detect the presence of pathogenic *Streptomyces* spp. in soil. However, as the intensity of the PCR products does not differentiate concentrations of inoculum, this assay does not provide quantification of *Streptomyces* spp.

Quantification of Streptomyces spp. using QPCR of the txt A region.

Real time quantitative (TaqMan) PCR was performed in 0.2 mL tubes in a Rotor Gene 3000 machine (Corbett Research). The 25 µL reaction mix included 2.5 µL template DNA, 1x Universal QPCR Master Mix (Invitrogen), 0.1 µM of each primer and 0.2 µM of the TaqMan probe (**Txt** Appendix III). The thermal cycle protocol was 50ºC for 2 min, 95ºC for 10 min. and 45 cycles of 95ºC for 15 sec and 60ºC for 60 secs. A non-template control of water instead of DNA was always included in each run. A range of standards containing different amounts of pathogen DNA was included in the quantitative PCR assay (described above).

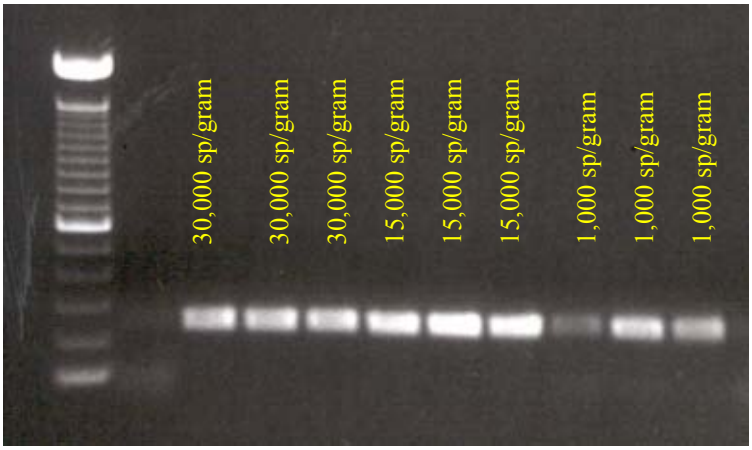


Figure 3-11 Txt A conventional PCR with *Streptomyces* spiked soil.

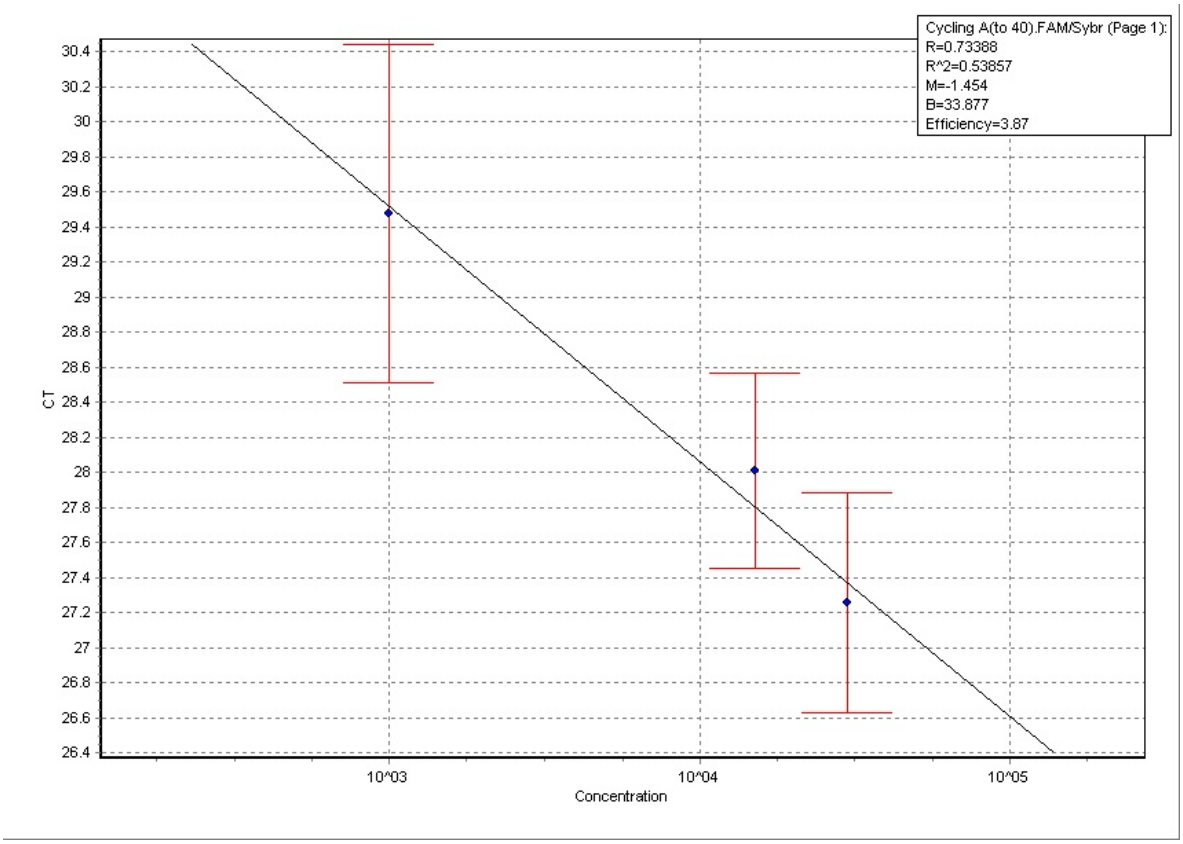


Figure 3-12 Standard curve for the detection and quantification of *Streptomyces* spp. using real-time PCR.

Summary of diagnostic development for Streptomyces spp.

- The txtA gene is more strongly correlated to pathogenic strains of *Streptomyces* spp. than the nec gene making the txtA region a better basis for the development of a DNA diagnostic test. Targeting the region associated with pathogenicity provides a more true reflection of the potential disease risk, as not all *Streptomyces* in soil are pathogenic on potato.
- PCR primers were designed to amplify the txtA region and a TaqMan probe developed to enable use of PCR.
- The conventional PCR developed in this study can be used to detect pathogenic *Streptomyces* spp. in soil.
- Using real-time PCR pathogenic *Streptomyces* in soil could be reliably detected and quantified. A standard curve was established showing the correlation between inoculum concentration and amplification of the txtA region.

2. ***Spongospora subterranea***

Conventional PCR for detection of S. subterranea in soil

This section adapted the methods and primers developed by Bell and coworkers (1999) with some modifications. PCR was performed in 0.2 mL tubes in a theromcycler (Corbett Research). The 25 µL reaction mix included 2.5 µL DNA, 1 x G buffer, 3 mM $MgCl_2$, 0.5 µM of each primer SpS1 and SpS2 (Appendix III) and 0.25 µM of each dNTP, 0.1% DMSO, 160 µg/mL BSA and 2 units Taq polymerase. The thermal cycle protocol was 95ºC for 3 min. and 45 cycles of 95ºC for 20 sec, 55ºC for 25 secs and 72°C for 50 sec followed by a single cycle of 72°C for 10 min. A non-template control of water instead of DNA was always included in each run. For detection standards, autoclaved sand was inoculated with cystosori of *S. subterranea* at 3906 spores/g of sand. A dilution series was prepared by the addition of 1 g of sterile sand to 1 g of inoculated sand to give samples with final concentrations of 7.67, 15, 30, 122, 244, 488, 976, 1953 and 3906 spores / g of sand from which DNA was extracted.

To confirm if the test would work on naturally infested soils, PCR was conducted on DNA extracted from several field soils that had a history of powdery scab.

Results of conventional PCR for detection of S. subterranea in soil

Conventional PCR could be used to detect the presence of *S. subterranea* in soil. DNA of *S. subterranea* could be detected from the soil containing 7.67 cystosori per gram of soil (Figure 3-13). Although this method is suitable for detection, it does not provide a reliable method for the quantification of inoculum in soil.

Amplification of *S. subterranea* was successful over a range of soil types (Figure 3-14) suggesting the DNA extraction and PCR conditions are robust.

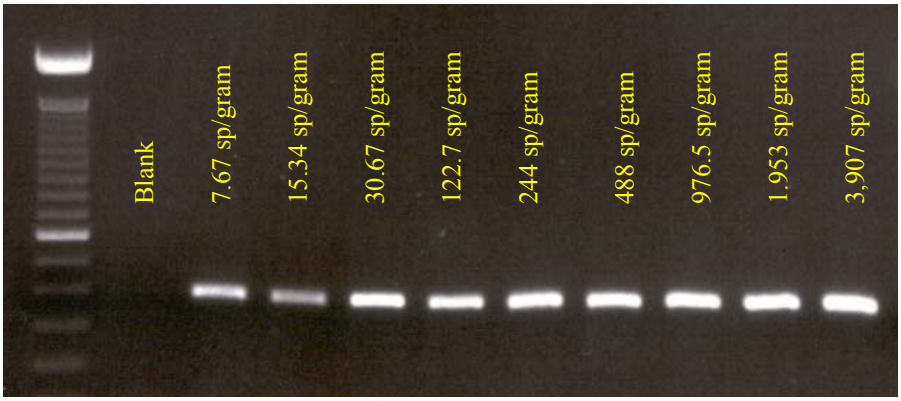


Figure 3-13 Conventional PCR of powdery scab cystorsori from spiked sand.

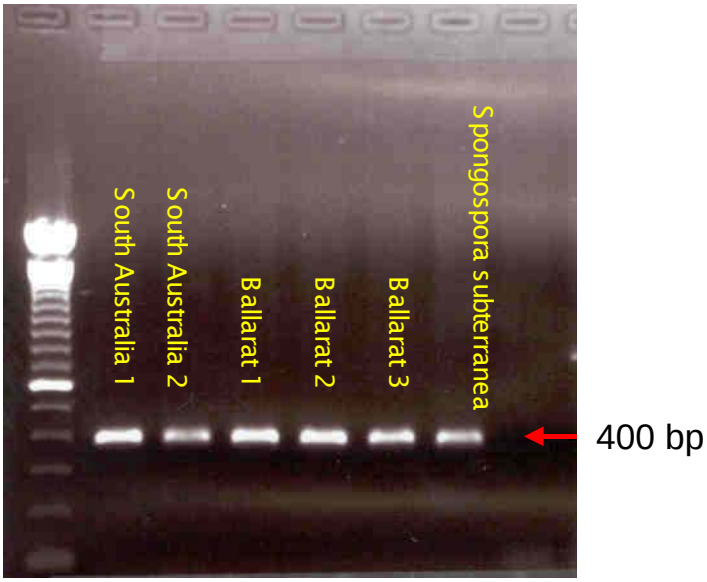


Figure 3-14 Amplification of the ITS region of *S. subterranea* from DNA extracted from soil.

Real time PCR for the detection and quantification of S. subterranea

Preparation of spiked soil with *S. subterranea.* Autoclaved sand was inoculated with cystosori of *S. subterranea* at 3906 spores/g of sand. A dilution series was prepared by the addition of 1 g of sterile sand to 1 g of inoculated sand to give samples with final concentrations of 7.67, 15, 30, 122, 244, 488, 976, 1953 and 3906 spores / g of sand from which DNA was extracted.

Real time quantitative (TaqMan) PCR was performed in 0.2 mL tubes in a Rotor Gene 3000 machine (Corbett Research). The 25 µL reaction mix included 2.5 µL template DNA, 1x Universal QPCR Master Mix (Invitrogen), 0.1 µM of each primer SsTQF1/SsTQR1 (Appendix III) and 0.2 µM of the TaqMan probe SsTQP1 (Appendix III). The thermal cycle protocol was 50ºC for 2 min, 95ºC for 10 min. and 45 cycles of 95ºC for 15 sec and 60ºC for 60 secs. A non-template control of water instead of DNA was always included in each run. A range of standards containing different amounts of pathogen DNA was included in the quantitative PCR assay. This protocol was standardised for all pathogen detection assays.

Assay sensitivity and specificity. The standards of *S. subterranea* DNA was amplified consistently in the quantitative PCR assay with SCRI designed primers (SsTQF1 and SsTQR1) and probe (SsTQP1). However, DNA equivalent to 7.67 sp/g cystosori was not consistently amplified (Figure 3-15). As expected the non-template control had a Ct value > 45 cycles.

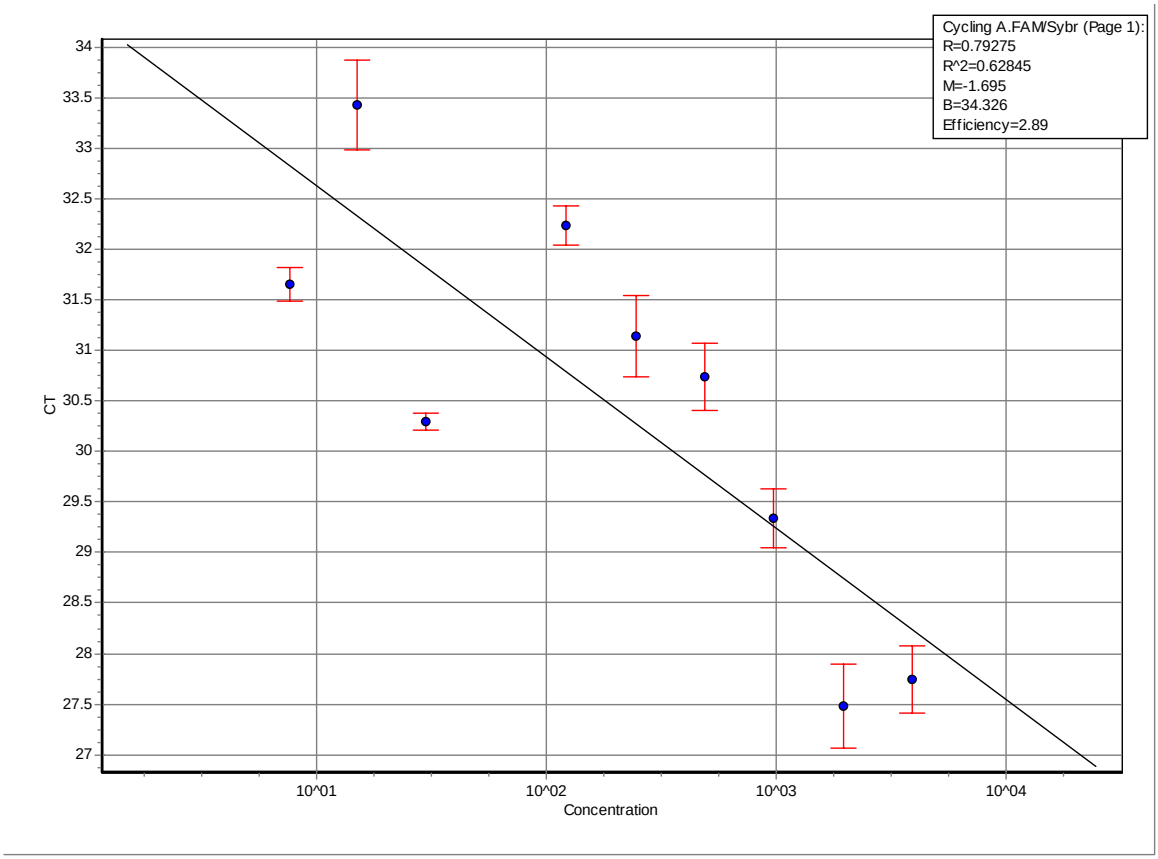


Figure 3-15 Standard curve for the quantification of *S. subterranea* from spiked soil.

3. *Rhizoctonia solani*

Semi-selective agar for Rhizoctonia solani

A range of semi selective medium have been reported in the literature, the purpose of this work was to identify the best media (Appendix II) for isolating *R. solani* from Australian potato soils. Representative isolates of *R. solani* collected from potato were grown on each media. Each media was inoculated in the centre of the plate with single a 5 mm diameter plug of mycelium from a 10 day old culture grown on PDA. For comparison, a PDA plates was also inoculated. Three plates of each agar were inoculated and growth recorded 5 days after inoculation.

All isolates recorded growth on PDA indicating that the isolates were all viable (Table 3-3). The Ko and Hora media appeared as the best agar to culture all anastomosis groups of *R. solani*. Only one isolate (Ro1) did not grow on Ko and Hora plus and KG media.

Table 3-3 Growth of various isolates of *R. solani* on three selective agars

Isolate	AG	PDA	Ko and Hora	Ko and Hora +	KG
M43	1-1	+++	++-	++-	+++
Ro1	2-1	+++	+++	---	---
RCO2	2-1	+++	+++	+++	+++
07-36-4	2-2	+++	+++	+++	+++
Ro Rh165	2-2	+++	+++	+++	+++
88-40-1	2-2	+++	+++	+++	+++
F56L	3	+++	+++	+++	+++
ST-11-6	3	+++	+++	+++	+++
SR-11-6	3	+++	+++	+++	+++
Rh165	4	+++	+++	+++	+++
GM10	5	+++	+++	+++	+++
OT2-1	6	+++	+++	+++	+++
K369	10	+++	+++	+++	+++
Roth 26	11	+++	+++	+++	+++

Bioassays for the detection of Rhizoctonia from soil

Three bioassays were evaluated for the ability to detect and quantify *R. solani* in soil. These bioassays were a soil plug method, a beet seedling method and a beet seed method. The soil plug method (Henis *et al.* 1978), using the Ko and Hora semi selective medium, was found to be too-time consuming and subject to high levels of competition from other organisms in the soil (data not shown). The method also may not be sensitive enough to detect hyphae in soil (Neate and Benger 1995).

The beet seedling assay (Lazarovits *pers. comm.*) detected *Rhizoctonia solani* in soil samples. However, this method detects all *R. solani* that are pathogenic to the beet seedlings and therefore, this test may not give a good indication of disease potential relating to strains of the fungus that attack potatoes. Many isolates belonging to different anastomosis groups were able to cause disease on the beet seedlings (data not shown). In fact, more severe disease symptoms were noted for some isolates belonging to AG2 than with disease caused by AG3 isolates.

The beet seed method was adapted from the method used by Kyritsis (2003), the original method was first described by Papavizas *et al*. (1975).

Red beet seeds of the cultivar "Detroit dark red" which had not been treated with fungicides were soaked over night in sterile distilled water and autoclaved at 121°C for one hour on each of three consecutive days (after the method of McDonald and Rovira (1985)). They were then air-dried in a laminar flow cabinet and stored in a sealed container at 4°C before use.

Field soil was collected from a potato field in the Ballarat region that had a history of Rhizoctonia diseases. The soil was air-dried and passed through a 2mm sieve and stored at 4°C. Four concentrations of field soil made (100%, 50%, 25% and 0% w/w) by blending appropriate amounts of autoclaved soil to field soil. Water was added to the soil 20% (w/v). For each concentration, a total of 75 g of soil was weighed into a 9 cm diameter petri dish. Each concentration was replicated 8 times. The soil was then incubated at 25° for 2 days. After which, ten sterilised beet seeds were added to each dish (a total of 80 seeds for each concentration of each replicate). The dishes were then re-sealed and incubated at 25°C for 48 hours. The seeds were removed using at 2 mm sieve and were washed under running tap water for 5 minutes. Seeds were blotted dry and 40 from each group were plated onto modified Ko and Hora's media and incubated at 25°C. The plates were observed after 24 and 48 hours.

The percentage of seeds colonised by *Rhizoctonia solani* was determined by microscopic examination of the resulting colonies (Figure 3-16). When possible, isolates were sub-cultured onto

PDA. DNA was extracted as previously described. The number of isolates of AG3 isolates was determined by using the PCR method of Lees *et al.* (2002).

The beet seed method was successful in detecting *Rhizoctonia* spp. in soil. The amount of *Rhizoctonia solani* declined with decreasing amounts of field soil (Figure 3-16). The *R. solani* collected from the beet seeds were all identified as AG3 using the Q-PCR method.

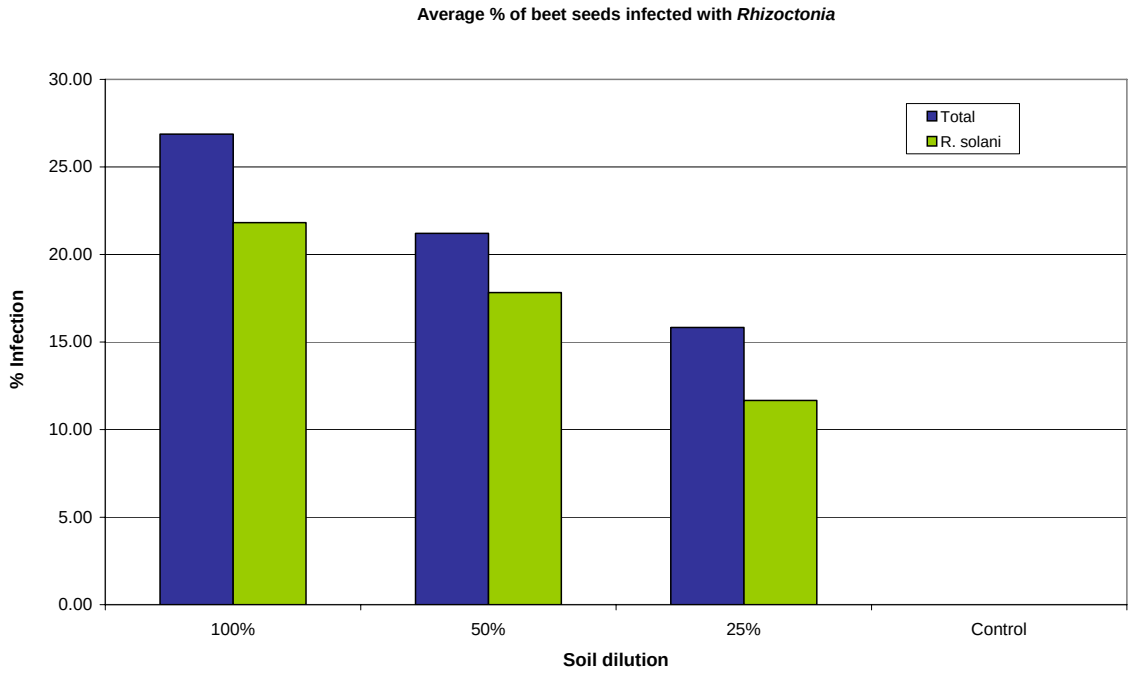


Figure 3-16 Detection of *Rhizoctonia solani* from naturally infested field soil using sterile beet seed assay.

PCR amplification of R. solani ITS region

Fungal mycelium was grown in clarified V8 broth (pH 5.6) for 1 week and then freeze dried. DNA was extracted from 100 mg of freeze dried mycelium following the protocol in Appendix V.

PCR amplification of samples using primers ITS1 (TCC GTA GGT GAA CCT GCG G) and ITS4 (TCC TCC GCT TAT TGA TAT GC) was based on a standard set of conditions [initial denaturation at 95ºC for 3 min., followed by 45 cycles of 95°C for 30, 50ºC for 30 and 72ºC for 30 in a reaction volume of 25 µL. The reaction contained the following components, 1 x reaction buffer, 200 µM dNTPs, 3 mM $MgCl_2$, 0.1 µM of each primer, 2 Units of Taq polymerase and 40 ηg of DNA template. The PCR product is 600 bp. PCR products were separated in 1.0% agarose gels and visualised using ethidium bromide staining.

PCR products were cleaned up using QIAGEN 'QIAquick PCR Purification Kit' according to the manufacturers instructions.

Direct PCR product sequencing was carried out on an Applied Biosystems Automated Sequencer using the ABI PRISM Dye terminator Cycle Sequencing Ready Reaction Kit at Monash University. The Big Dye reaction contained 5 µL of PCR product (20 ηg DNA), 1 x Big Dye Buffer, 1 x Big Dye reaction mix and 15 ηM of one primer in a 20 µL reaction volume. Big dye products were precipitated with 2 µL of 3M NaAce (pH 7.5) and 50 µL of 95% EtOH and incubated at room temperature for 1 h, tubes were centrifuged for 20 min. at 13 000 rpm and rinsed in 100 µL of 70% EtOH and air dried on the bench for 1h or until dry.

The ITS sequence can be used to differentiate isolates of *R. solani* belonging to different anastomosis groups (Figure 3-17). Isolates belonging to AG2-1 had a more conserved ITS sequence (Figure 3-18) than AG2-2 isolates (Figure 3-19).

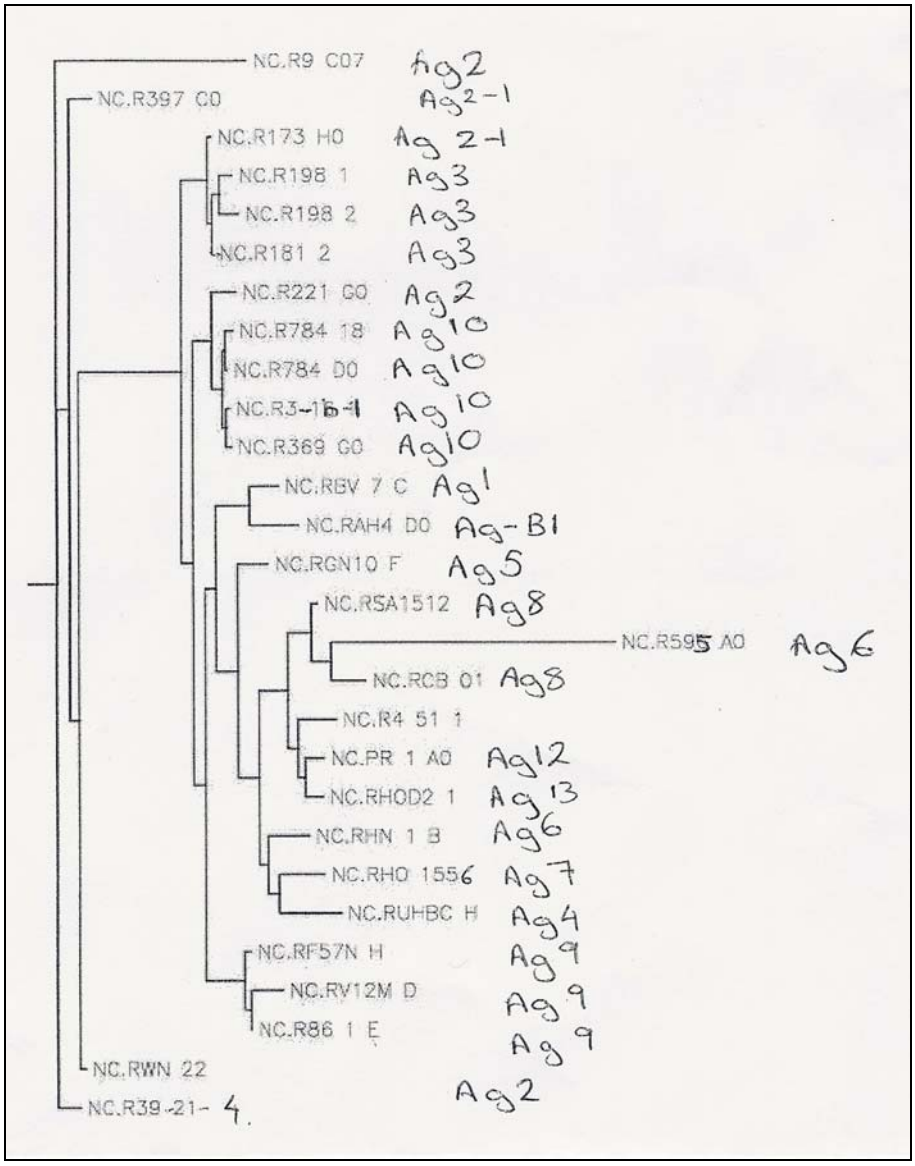


Figure 3-17 Dendrogram of *R. solani* from various anastomosis groups based on ITS sequences

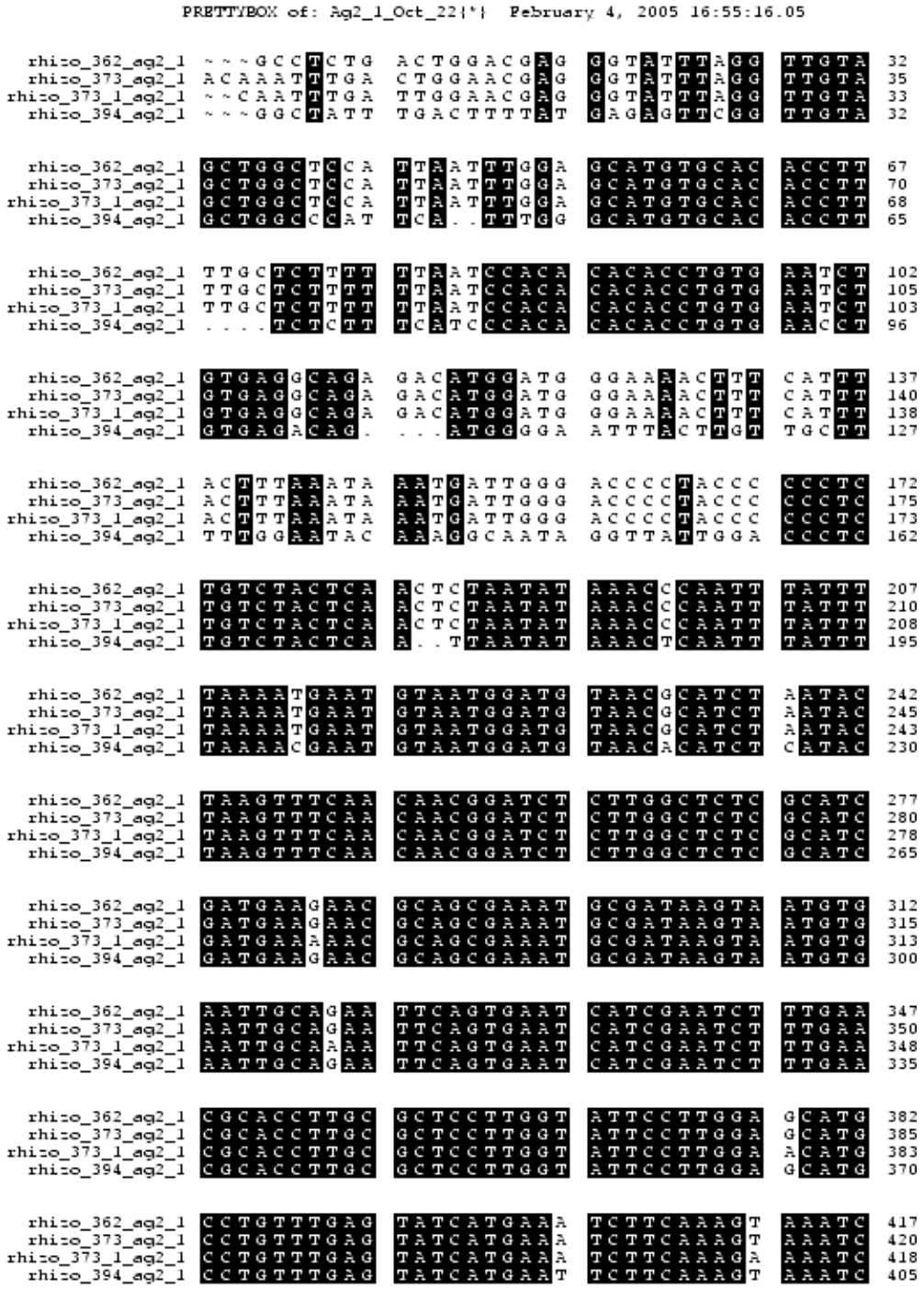


Figure 3-18 Pretty box of ITS sequences of *Rhizoctonia* AG2-1

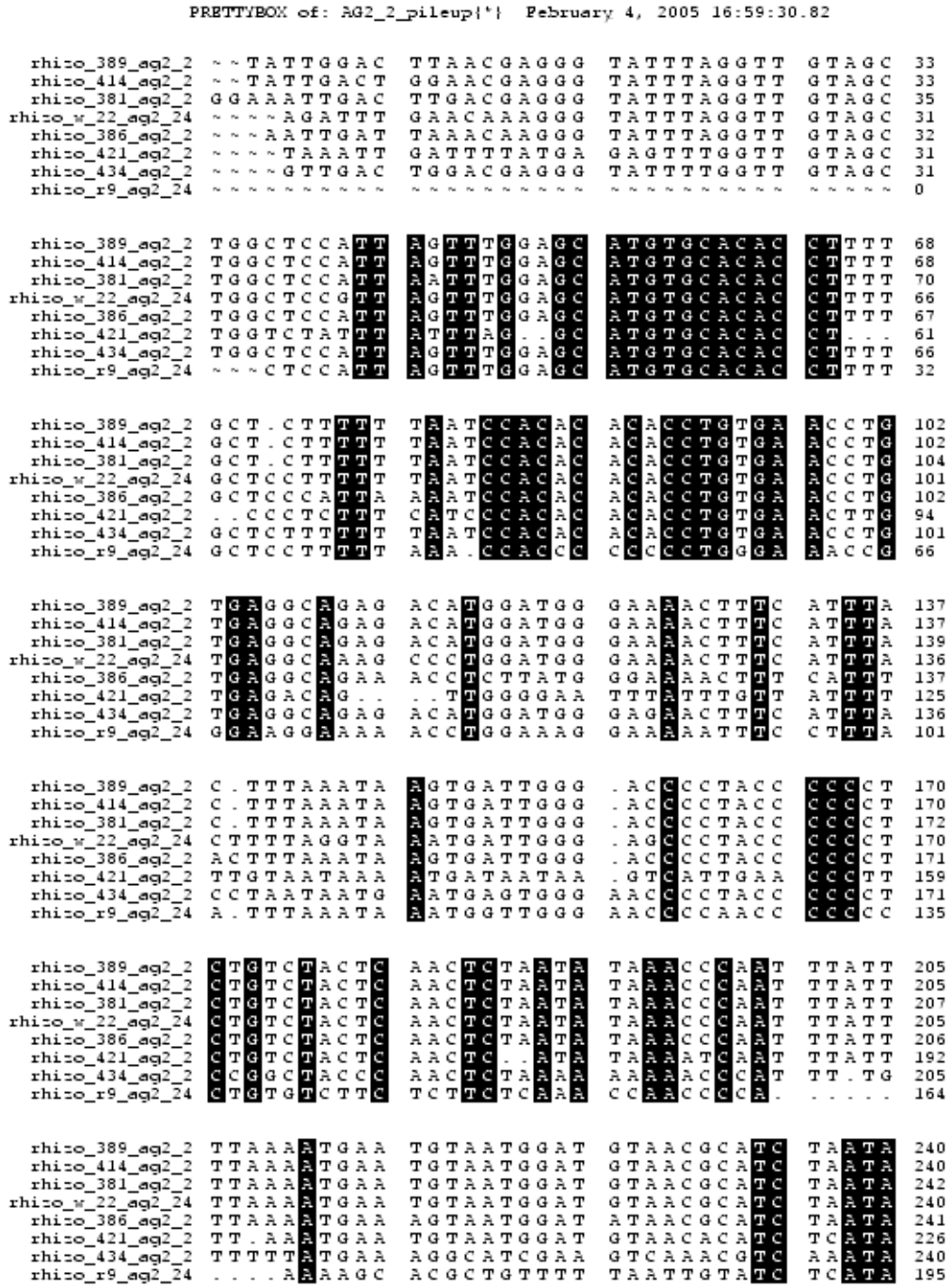


Figure 3-19 Pretty box of ITS sequence of *R. solani* AG2-2

Identification of R. solani AG3 using a SCAR marker

The SCAR (sequence characterised amplified marker) specific to *R. solani* AG3 was developed by Bounou and coworkers (1999).

Fungal mycelium was grown in clarified V8 broth (pH 5.6) for 1 week and then freeze dried. DNA was extracted from 100 mg of freeze dried mycelium following the protocol in Appendix V.

PCR amplification was conducted in a total volume of 50 µL which consisted of 0.1 µM primers SBU-177 and SBL-336 (Appendix III), 1 x reaction buffer, 200 µM dNTPs, 3 mM $MgCl_2$, 2 Units of Taq polymerase and 40 ηg of DNA template. Reaction was based on a standard set of conditions [initial denaturation at 95ºC for 3 min., followed by 45 cycles of 95°C for 30, 38ºC for 1 min. and 72ºC for 1 min. The PCR product is 179 bp. PCR products (15 µL) were visualised in 2.0% agarose

gel using ethidium bromide staining. The remaining PCR products were digested using the endonuclease *Xho* I (New England) according to the manufacturers directions. Briefly, the products were digested at 37°C for 2 hours. DNA fragments were resolved on a 4 % agarose gel stained with ethidium bromide.

Isolates belonging to AG2, 3, 5 and 8 were all amplified using the primers SBU-177 and SBL-336. Following digestion with Xho I only isolates belonging to AG3 were digested producing two fragments 127 bp and 52 bp in size which is consistent with the original work conducted by Bounou and coworkers (1999). In comparison to the original method for anastomosis classification (Ogoshi 1987), the SCAR method provides a rapid method to determine isolates of *R. solani* that belong to AG3. Bounou *et al.* (1999) used this method to detect *R. solani* AG3 in soil and plant material but did not show how this method could be used to quantify *R. solani*.

AG3 specific PCR and quantitative PCR

Based on the method of Lees *et al*. (2002) real time quantitative (TaqMan) PCR was performed in 0.2 mL tubes in a Rotor Gene 3000 machine (Corbett Research). The 25 µL reaction mix included 2.5 µL template DNA, 1x Universal QPCR Master Mix (Invitrogen), 0.1 µM of each primer RsTQF1 and RsTQR1 (Appendix III) and 0.2 µM of the TaqMan probe RsTQP1 (Appendix III). The thermal cycle protocol was 50ºC for 2 min., 95ºC for 10 min. and 45 cycles of 95ºC for 15 sec and 60ºC for 60 secs. A non-template control of water instead of DNA was always included in each run. Isolates *R. solani* AG3 collected from Australian potato fields were amplified consistently in the quantitative PCR assay with SCRI designed primers (RsTQF1 and RsTQR1) and probe (RsTQP1). As expected the non-template control had a Ct value > 45 cycles. Non AG3 isolates were not amplified using this method.

4. ***Colletotrichum coccodes***

Colletotrichum coccodes quantitative PCR assay sensitivity

Micro-sclerotia of *C. coccodes* were harvested from cultures grown on ¼PDA. To prepare spiked soil standards with known levels of *C. coccodes*, varying numbers of micro-sclerotia (1, 10, 50 and 100) were added to 0.5g of autoclaved soil. This was replicated 3 times and DNA extracted from soil using the MoBio method previously described.

The PCR method was based on previous work done by Cullen *et al.* (2002). Real time quantitative (TaqMan) PCR was performed in 0.2 mL tubes in a Rotor Gene 3000 machine (Corbett Research). The 25 µL reaction mix included 2.5 µL template DNA, 1x Universal QPCR Master Mix (Invitrogen), 0.1 µM of each primer CcTQF1 and CcTQR1 (Appendix III) and 0.2 µM of the TaqMan probe CcTQP1 (Appendix III). The thermal cycle protocol was 50ºC for 2 min., 95ºC for 10 min. and 45 cycles of 95ºC for 15 sec and 60ºC for 60 secs. A non-template control of water instead of DNA was always included in each run. A range of standards containing different amounts of pathogen DNA was included in the quantitative PCR assay.

The standards of *C. coccodes* DNA was amplified consistently in the quantitative PCR assay with SCRI designed primers (CcTQF1 and CcTQR1) and probe (CcTQP1) (Figure 3-20). DNA equivalent to 1 micro-sclerotia per gram of soil was consistently amplified. As expected the non-template control had a Ct value > 45 cycles.

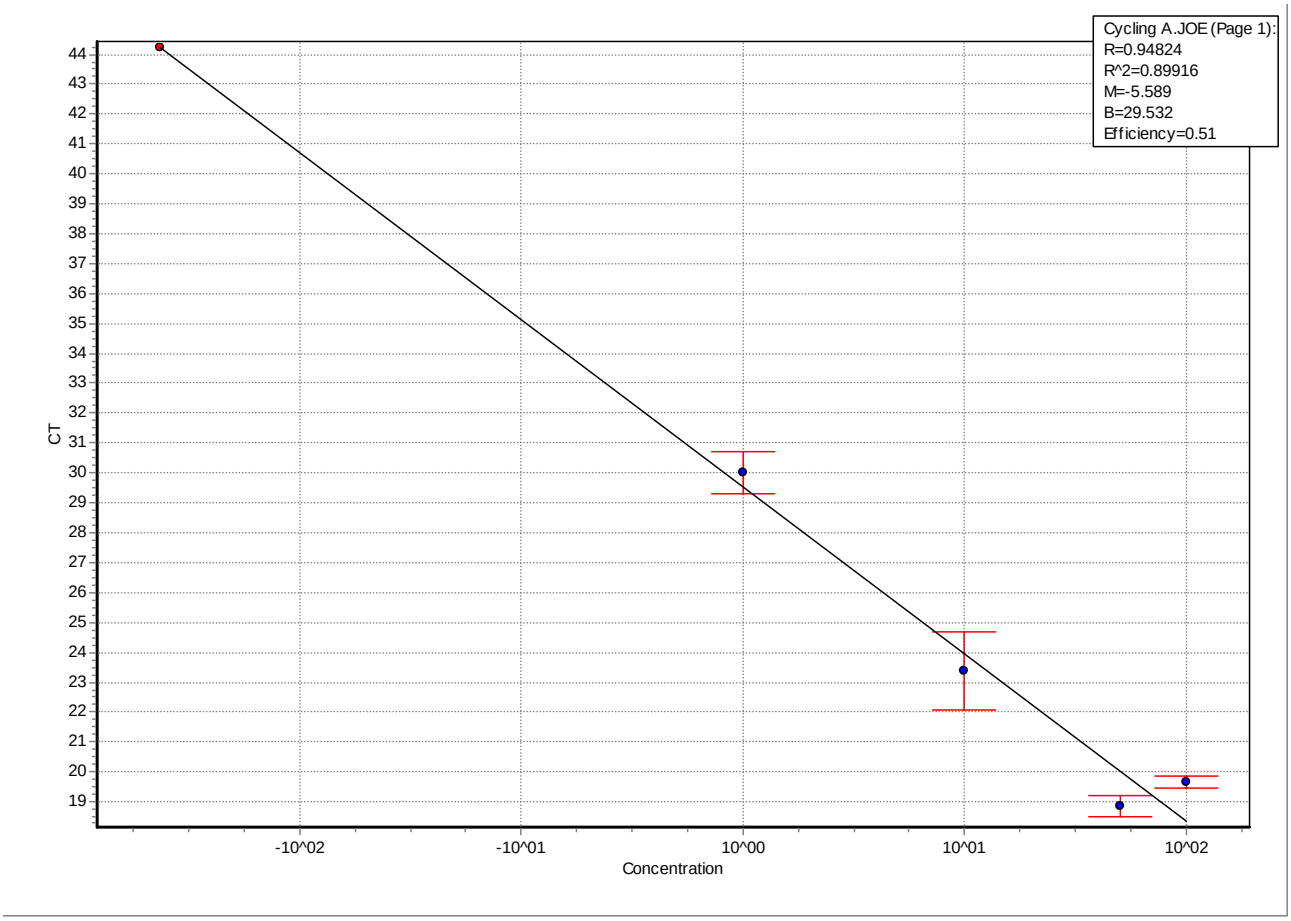


Figure 3-20 Standard curve used in the QPCR assay for the quantification of *C. coccodes* in DNA samples extracted from soil

5. ***Verticillium dahliae***

The conventional PCR method used was adapted from Robb and coworkers (1994). Primers specific for *Verticillium dahliae* (VD1 and VD2 Appendix III) were used to amplify pure cultures of *V. dahliae* collected from potato. PCR was performed in 0.2 mL tubes in a theromcycler (Corbett Research). The 25 µL reaction mix included 2.5 µL DNA, 1 x G buffer, 3 mM $MgCl_2$, 0.3 µM of each primer, 0.2 µM of each dNTP, 0.1% DMSO, 160 µg/mL BSA and 2 units Taq polymerase. The thermal cycle protocol was 95ºC for 3 min. and 45 cycles of 95ºC for 55 sec, 60ºC for 55 secs and 72°C for 60 sec followed by a single cycle of 72°C for 7 min. A non-template control of water instead of DNA was always included in each run.

All ten isolates of *V. dahliae* amplified a 350 bp fragment (data not shown) which is consistent with the outcomes of Robb and coworkers (1994). Two isolates of *V. albo-atrum* did not amplify. The PCR test was then used on soil samples collected from potato fields.

V. dahliae could be detected using PCR in soils from fields showing symptoms of wilting (Vd +Robynvale) and soils from fields not showing symptoms of disease (Figure 3-21). The results were confirmed by the isolation from potato of petioles collected from each site using semi-selective media SPT. The detection of *V. dahliae* using PCR was much more rapid than the conventional methods of soil plating and requires no expertise in pathogen identification. However, as the PCR can only detect at the species level and not the strain (race) level, therefore, some caution must be applied in interpreting the results in terms of identifying the potential disease risk. The PCR test for the detection of *Verticillium* can be used in further research to determine the distribution and significance of *Verticillium* spp. in Australian potato production.

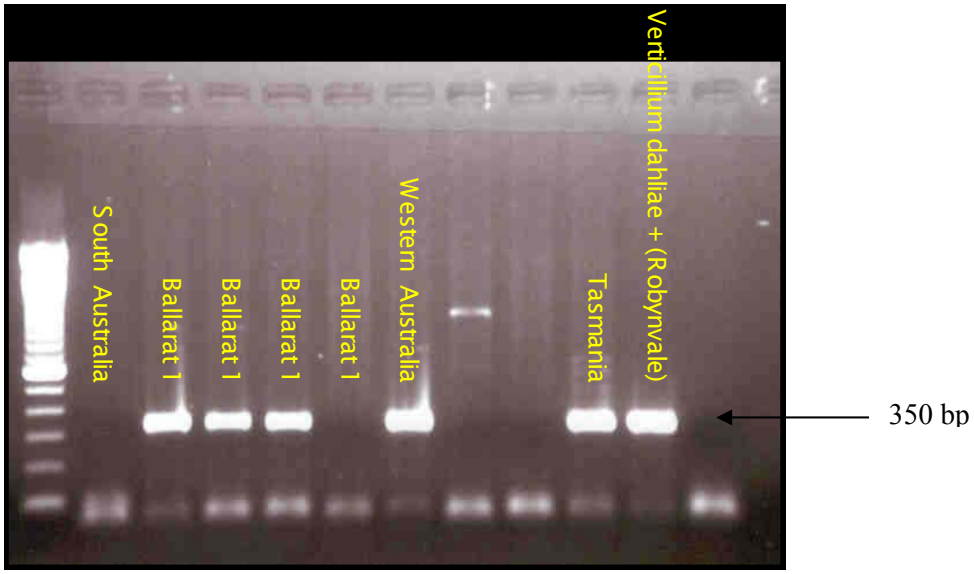


Figure 3-21 Detection of *V. dahliae* from soils. Lane 1 100bp ladder (Invitrogen), lane 2 soil from South Australia, lane 3 -6 soil from Ballarat, lane 6 soil from Western Australia, lane 7 soil from Ballarat, lane 9 soil from Tasmania, lane 10 soil from Victoria and lane 11 negative control (no DNA added).

6. ***Detection of fungicide resistance in Helminthosporium solani***

Adapted from the method of McKay and Cooke (1997). PCR amplification of the β tublin gene using specific primers for *H. solani*. PCR was performed in 0.2 mL tubes in a theromcycler (Corbett Research). The 50 µL reaction mix included 2.5 µL DNA, 1 x buffer (Invitrogen), 1.5 mM $MgCl_2$, 0.2 µM of each primer (SS-rev and SS-for Appendix III) and 0.2 µM of each dNTP, and 2 units Taq polymerase. The thermal cycle protocol was 95ºC for 3 min. and 30 cycles of 95ºC for 15 sec, 54ºC for 20 secs and 72°C for 20 sec followed by a single cycle of 72°C for 7 min. A non-template control of water instead of DNA was always included in each run. The PCR product was digested using the restriction enzyme *Bsa* I (New England) in accordance with the manufacturers directions. Digested PCR products were visualised on a 2 % agarose gel and stained using ethidium bromide.

Isolates that were sensitive to thiabenzadole produced fragments, 420, 390 and 62 bp in size whereas isolates resistant to the fungicide produced fragments of 482 and 390 bp (Figure 3-22). This was consistent with the outcomes of the work done by McKay and Cooke (1997) and provides evidence that resistance to TBZ occurs in Australian population of *H. solani.*

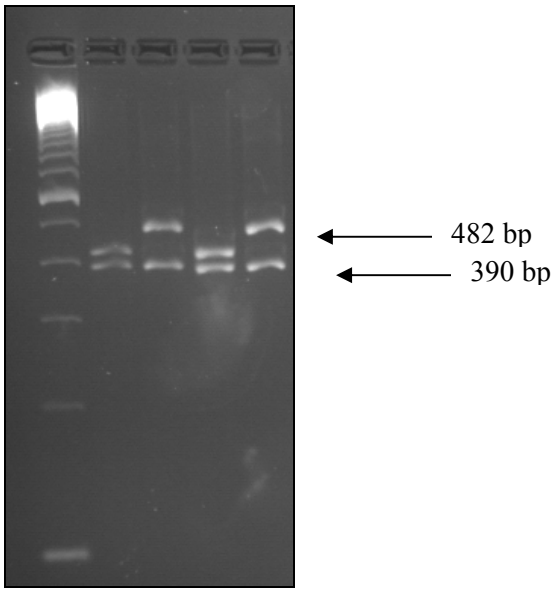


Figure 3-22 Digestion of the PCR product with the restriction enzyme Bsa I. Lane 1 DNA ladder, Lanes 2 and 4 TBZ susceptible isolate of *H. solani*, Lanes 3 and 5 TBZ resistant isolate of *H. solani*

Summary/conclusions

- PCR assays that are specific to *Streptomyces* spp., *S. subterranea*, *R. solani* and *C. coccodes* were developed. These assays are a rapid and reliable method for the detection and quantification of these pathogens in soil. The use of these methods in research programs will help to significantly improve our knowledge of pathogen ecology through the monitoring of pathogen populations in different cropping systems and management regimes. Furthermore, the PCR assays can be used to as a tool for researchers, providing results within days as opposed to weeks or months using conventional methods of pathogen detection and quantification.
- The success of any PCR based assay is dependent on the efficient extraction of target DNA from a soil sample. In this study, a commercial kit for extracting DNA from soil was routinely used. This kit is readily available, enabling other researchers to use the PCR tests described in this study.
- Conventional tests have been used to identify thiabendazole resistant strains of *H. solani* (R.F. de Boer, pers. comm.). These tests are labour intensive, time consuming and not suitable for routine diagnostics. The PCR adapted in this study allows for the rapid detection of thiabendazole resistant isolates of the silver scurf pathogen and makes the routine screening of isolates feasible.

Chapter 4 Linking PCR soil tests with disease in the field

Introduction

Chapter 3 reports on the development of DNA based tests for the detection and quantification of populations of several potato pathogens in soil. The ultimate use of these tests is to be able to obtain a reliable measure of 'inoculum' and to predict the 'disease risk ' or 'disease potential' associated with this inoculum for different potato cultivars growing in a range of soil types and conditions. This chapter reports on the results of three-years of field trials in which the results of DNA tests of field soil were correlated with disease incidence and severity on potato tubers grown in that soil.

Materials and Methods

The method employed here was to replace a 10 m length of row of potato tubers in a commercial potato crop (10 m 'micro-plot'), around the time of planting, with seed tubers of a disease susceptible variety. The potato cultivar Desireé was chosen because its tubers are rated as being highly susceptible to common scab and moderately susceptible to powdery scab, and quantities of seed tubers were readily available. This cultivar was used to 'bioassay' the 10 m micro-plot for disease incidence and severity. The occurrence of disease was compared with the results of real-time DNA test results of the soil sample taken from the strip at the time of planting seed tubers.

Micro-plots were established in 16 to 21 potato fields during the 2001/02, 2003/03 and 2003/04 seasons at different locations around Victoria. Micro-plots were located in the Thorpdale district, South Gippsland, the Koo-Wee-Rup swamp, the Central Highlands (Ballarat), the potato production areas south-east of Colac, the Otway Ranges, the Heytsbery Plains and Portland. Fields selection was based on their common scab history (either a high risk or no history of common scab) and the willingness of growers to participate in the study. A list of sites sampled is presented in Table 4.6.

Soil sampling and disease assessments

A bulked soil sample (one kilogram) was taken from each micro-plot prior to planting or replanting with seed tubers of *cv.* Desireé. The bulked sample was made up of 10 individual sub samples taken at 1 m intervals to a depth of 15 cm from along length of the plot. Bulked samples were air-dried to constant weight and stored at 1ºC prior to conducting DNA extraction. All tubers from each micro-plot were harvested at the end of the season and a sub-sample of 50 tubers assessed for the incidence and severity of common scab, powdery scab and black dot. Disease incidence was recorded as the proportion of tubers with a particular disease. Disease severity was recorded as a severity score based on the proportion of the tuber surface affected by particular symptoms on a scale of 0-4 (0 = no disease, 1 = <2% of tuber surface affected, 2 = 3-10% surface affected, 3 = 11-25% surface affected and 4 = >25% surface affected).

Real-time PCR tests of soil samples

DNA was extracted from the soil samples using the commercial kit (MoBio) as previously described (Chapter 3). Real-time quantitative (TaqMan) PCR was performed in 0.2 mL tubes in a Rotor Gene 3000 machine (Corbett Research). The 25 µL reaction mix included 2.5 µL template DNA, 1 x Universal QPCR Master Mix (Invitrogen), 0.1 µM of each primer and 0.2 µM of the TaqMan probe. The thermal cycle protocol was 50ºC for 2 min., 95ºC for 10 min. and 45 cycles of 95ºC for 15 sec and 60ºC for 60 secs. A non-template control of water instead of DNA was always included in each run. A range of standards containing different amounts of DNA from each pathogen DNA was included in the quantitative PCR assay. This protocol was standardised for all pathogen detection assays.

Results and Discussion

The results of disease assessments and PCR analysis for each season are presented in Table 4-2, Table 4-3 and Table 4-4. The most common diseases observed were black dot, common scab and powdery scab. The incidence of each disease varied considerably ranging from 0-100% for common scab, 0-96% for powdery scab and 0-96% tubers affected for black dot.

DNA data and disease incidence and severity were poorly correlated. Table 4-5 shows comparisons between the detection of a pathogen, either positive or negative (PCR result), against detection of disease on tubers in the micro-plots. On average, PCR and disease results matched in 58%, 75% and 59% of plots for common scab, powdery scab and black dot, respectively (averaged over the three years). A positive PCR result but no disease recorded was less common (12%, 10%, 2% of plots, respectively). In comparison, a positive disease result against a negative PCR result was more common (average of 30%, 15% and 39%, respectively).

There is clearly more work needed before we have confidence in using these DNA tests as a measure of disease potential. Experience shows that the development of common scab and powdery scab is very dependent on soil temperature and moisture conditions at the critical time of tuber initiation, these conditions being relatively warm and dry and cool and wet, respectively. The development of black dot, on the other hand, is not constrained by very narrow environmental parameters. Thus outbreaks of common and powdery scab are seasonal dependent or can be influenced by the use of irrigation for instance. This factor accounts for some of the poor correlation between PCR results and disease incidence.

It is often assumed that there is a linear relationship between amount of inoculum and disease incidence. However, with powdery scab for instance, no such relationship has been found. Relatively high levels of disease have been recorded against low levels of inoculum (Burnett 1990; van de Graaf *et al.* 2005)). There is more research needed to define sampling strategies, DNA extraction efficiency and more clearly define the relationship between inoculum and disease incidence and severity. The relationship depends not only on amount of inoculum but also on soil properties (eg, pH, nutrients, organic content, and biological activity) which affect both the pathogen, the host and hence disease. Other factors such as cropping history, crop husbandry, seed inoculum and disease history should also be taken into account as essential influences on disease development. The integration of these factors into a decision-support-management system, combined with the ability to measure inoculum using DNA tests, would ultimately provide a powerful tool in the prediction of disease risk.

Table 4-1 Codes and origins of soil samples from 10 m micro-plots in commercial fields around Victoria.

Year	Soil number	Location
2001/2002	001	Gippsland
	002	Gippsland
	003	Gippsland
	004	Thorpdale
	005	Thorpdale
	006	Thorpdale
	007	Portland
	008	Portland
	009	Portland
	010	Ballarat
	011	Ballarat
	012	Ballarat
	013	Ballarat
	014	Otways
	015	Otways
	016	Otways
2002/2003	101	Gippsland
	102	Gippsland
	103	Gippsland
	104	Thorpdale
	105	Thorpdale
	106	Thorpdale
	017	Ballarat
	018	Ballarat
	019	Ballarat
	020	Ballarat
	021	Ballarat
	022	Otways
	023	Otways
	400	Portland
	401	Portland
	402	Portland
	403	Portland
	404	Portland
	500	Kooweerup
	600	Kooweerup
	700	Kooweerup
2003/2004	301	Gippsland
	302	Gippsland
	303	Thorpdale
	304	Otways
	305	Otways
	306	Otways
	307	Otways
	308	Otways
	309	Ballarat
	310	Ballarat
	311	Ballarat
	312	Gippsland
	313	Thorpdale
	314	Thorpdale
	315	Koroit
	316	Portland
	317	Portland
	318	Portland
	319	Ballarat
	320	Ballarat

Table 4-2 Real-time PCR results and the incidence and severity of diseases on tubers of cv. Desireé in 10 m micro plots in commercial fields around Victoria 2001/02

Year	Soil #	Disease on tubers						PCR Quantification		
		Incidence			*Severity*			units/0.5 g soil		
		Black Dot	Powdery Scab	Common Scab	Black Dot	Powdery Scab	Common Scab	*C.c*	*S.s*	*S. spp*
2001/02	001	0	0	2	0	0	0.02	0	0	3044
	002	0	4	14	0	0.04	0.14	0	0	2816
	003	0	0	26	0	0	0.34	0.06	0	1100
	004	54	74	82	0.74	0.94	1	0.03	351	651
	005	0	24	0	1.2	0.32	0	0.01	10	371
	006	52	0	14	0.9	0	0.18	0	0.4	398
	007	26	0	80	0.32	0	0.94	0	0	75
	008	Na	Na	Na	Na	Na	Na	0	0	48
	009	22	0	100	0.28	0	3.94	0	0	8596
	010	0	2	0	0	0.06	0	0	0	550
	011	Na	Na	Na	Na	Na	Na	0.1	0	18823
	012	0	96	0	0	1.78	0	0.04	1013	651
	013	0	62	6	0	1.24	0.12	0	0	599
	014	0	0	6	0	0	0.06	0	0	1302
	015	0	0	22	0	0	0.22	0	0	622
	016	12	0	32	0.14	0	0.46	0	0	4368

Na = sample not assessed for disease.

Table 4-3 Real-time PCR results and the incidence and severity of diseases on tubers of cv. Desireé in 10 m micro plots in commercial fields around Victoria 2002/03

Year	Soil #	Disease on tubers						PCR Quantification		
		Incidence			*Severity*			units/0.5 g soil		
		Black Dot	Powdery Scab	Common Scab	Black Dot	Powdery Scab	Common Scab	*C.c*	*S.s*	*S. spp*
2002/03	101	0	0	6	0	0	0.08	0	0	0
	102	0	0	16	0	0	0.28	0	0	6613
	103	0	0	54	0	0	1.24	0	0	20064
	104	8	0	16	0.12	0	0.16	0	0	21423
	105	54	0	44	0.9	0	0.68	0	0	8783
	106	0	4	2	0	0.04	0.02	0	11.98	12556
	017	48	0	84	1.24	0	1.96	0	0	0
	018	Na	Na	Na	Na	Na	Na	0	0	3283
	019	96	12	8	2.68	0.12	0.08	0	0	11113
	020	80	36	6	2.12	0.48	0.06	979	74.14	0
	021	88	0	80	1.98	0	1.18	10	0	6782
	022	Na	Na	Na	Na	Na	Na	0	0	0
	023	0	0	18	0	0	0.16	0	0	0
	400	Na	Na	Na	Na	Na	Na	0	0	0
	401	0	0	100	0	0	3.8	0	0	10701
	402	2	0	4	0.02	0	0.14	0	0	2309
	403	0	0	12	0	0	0.14	0	0	0
	404	0	0	28	0	0	0.66	0	0	0
	500	12	2	12	0.16	0.02	0.14	0	0	0
	600	16	0	36	0.22	0	0.54	0	52.3	18673
	700	Na	Na	Na	Na	Na	Na	0	0.07	0

Na = sample not assessed for disease.

Table 4-4 Real-time PCR results and the incidence and severity of diseases on tubers of cv. Desireé in 10 m micro plots in commercial fields around Victoria 2003/04

Year	Soil #	Disease on tubers						PCR Quantification		
		Incidence			*Severity*			units/0.5 g soil		
		Black Dot	Powdery Scab	Common Scab	Black Dot	Powdery Scab	Common Scab	*C.c*	*S.s*	*S. spp*
2003/04	301	0	0	2	0	0	0.02	0	0	0
	302	10	0	100	0.22	0	1.94	0	0	0
	303	28	0	4	0.38	0	0.04	0	0	0
	304	12	0	0	0.12	0	0	0	0	0
	305	8	0	0	0.1	0	0	0	0	0
	306	12	4	0	0.24	0.04	0	0	0.08	0
	307	Na	Na	Na	Na	Na	Na	0	0	0
	308	6	10	4	0.06	0.12	0.04	0.01	0.46	1.84
	309	38	0	0	0.4	0	0	0.02	0	65.8
	310	22	64	0	0.22	0.76	0	0.26	5.89	0.1
	311	94	0	2	1.4	0	0.06	0	1.11	0
	312	0	0	2	0	0	0.06	0	0	6.5
	313	78	0	4	1.14	0	0.04	0.1	0.01	0
	314	24	10	6	0.32	0.16	0.2	0.19	0	5.5
	315	2	0	6	0.02	0	0.14	0	0	0
	316	0	0	8	0	0	0.1	0	0	0
	317	6	0	10	0.06	0	0.12	0	0	0
	318	11	0	98	0.22	0	1.62	0	0	0
	319	36	0	0	0.54	0	0	0.03	0.05	1.26
	320	86	76	0	1.3	1.1	0	0.02	2.99	0

Na = sample not assessed for disease.

Table 4-5 A comparison of DNA tests results with tuber disease incidence from 10 m micro plots.

	Common scab				*Powdery scab*				*Black dot*				All pathogens all years
	01/002	02/03	03/04	Average	01/002	02/03	03/04	Average	01/002	02/03	03/04	Average	
	Percent Disease				Percent Disease				Percent Disease				
PCR+Disease+	79%	59%	37%	58%	64%	82%	79%	75%	64%	59%	53%	59%	63%
PCR+Disease0	21%	0%	16%	12%	7%	6%	16%	10%	7%	0%	0%	2%	8%
PCR0 Disease+	0%	41%	47%	30%	29%	12%	5%	15%	29%	41%	47%	39%	29%
TOTAL SITES	**14**	**17**	**19**		**14**	**17**	**19**		**14**	**17**	**19**		**150**

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Technical Transfer

Aspects of the project have been published in various industry articles including:

- "Common scab - lessons from Canada" Eyes on potatoes March 2002 Vol 15 p.10.
- "Knowing more about potato diseases" National Marketplace News March 2002
- "Prediction and molecular detection of soilborne pathogens of potato". Potato Australia September 2002.
- Article published in the "Weekly Times" 2004
- Good Fruit and Vegetables 2005

Communication through various grower workshops including:

- Grower workshops in Colac, Portland and Ballarat in Victoria, and in Devonport and Scottsdale, Tasmania during the winter/spring period in 2002.
- Central Highlands Potato Growers - demonstration farm - "New tools for the diagnostics of potato pathogens" 2003, 2004
- Common scab workshops in Victoria and Tasmania 2003
- Victorian Seed Potato Conference in Portland 2003

Scientific Publications

Wiechel, TJ, Crump, N.S de Boer, R F. (2003) Characterisation of Victorian isolates of *Streptomyces* causing scab on potato. 13th International Symposium on the Biology of Actinomyces Melbourne 1-5 December 2003.

Crump, NS., Conn, K.L., Squire, S., Lazarovits, G. (2003) Quantification of pathogenic *Streptomyces* spp. in field soils. 13th International Symposium on the Biology of Actinomyces Melbourne 1-5 December 2003.

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Appendices

Appendix I

MOBIO SOIL DNA PROTOCOL – WITH MODIFICATIONS

1. To the 2-mL Bead Solution tubes provided, add **0.5-g** of air-dried soil sample.
2. Gently vortex to mix.
3. **(Check Solution S1).** If precipitated, heat to 60°C until dissolved.
4. **Add 60-µl of Solution S1** and invert several times or vortex briefly.
5. **Add 200-µl of Solution IRS** (Inhibitor Removal Solution). Only required if DNA is to be used for PCR.
6. Secure bead tubes into Fastprep™ machine with speed of **5.5 for 30 sec**. Pulse centrifuge, then incubate at room temp for 10 minutes.
7. Make sure the 2-mL tubes rotate freely in your centrifuge without rubbing. Centrifuge tubes at 13,000 rpm for 1 min. **CAUTION:** Be sure not to exceed 13,000 rpm or tubes may break.
8. Transfer the supernatant to a clean microcentrifuge tube (provided). **Take as much as possible**.

   ***Note**: With 0.25gm of soil and depending upon soil type, expect between 400 to 450 µl of supernatant. Supernatant may still contain some soil particles.*
9. **Add 250 µl of Solution S2** and vortex for 5 sec. Incubate 4°C for 5 min.
10. Centrifuge the tubes for 1 minute at 13,000 rpm.
11. Avoiding the pellet, **transfer 450-µl[†] of supernatant** to a clean microcentrifuge tube (provided).
12. **Add 900µl of Solution S3** to the supernatant and vortex for 5 seconds.
13. Load approximately 700 µl onto a spin filter and centrifuge at 13,000 rpm for 1 minute. Discard the flow through and add the remaining supernatant to the spin filter and centrifuge at 13,000 rpm for 1 minute. ***Note**: A total of two loads for each sample processed are required.*
14. **Add 300 µl of Solution S4** and centrifuge for 1 minute at 13,000 rpm.
15. Discard the flow through.
16. Centrifuge again for 1 minute.
17. Carefully place spin filter in a new clean tube (provided). Avoid splashing any Solution S4 onto the spin filter.
18. **Add 100 µl of Solution S5** to the centre of the white filter membrane.
19. Centrifuge for 1 minute.
20. Discard the spin filter. DNA in the tube is now application ready. No further steps are required. We recommend storing DNA frozen (-20°C). Solution S5 contains no EDTA.

[†] NB. Only take 450 µL of liquid

Appendix II

Media

A semi-selective medium for *Verticillium dahliae* (SPT)

1.5 g	KH_2PO_4
4.0 g	K_2HPO_4
2.0 g	polygalacturonic acid
25.0 mL	soil extract (see soil extract procedure)
1.0 L	dd H_2O
15.0 g	granulated agar

- Stir until dissolved.
- Adjust pH to 7.0 with 5M KOH before adding agar.
- Autoclave for 30 minutes, with a bung and tinfoil over mouth of flask.
- ***When cool,*** add 1.0 ml of Antibiotic Solution (see antibiotic solution) then pour.

Soil Extract

Mix 1.0 kg of field soil 2.0 L of tap water. Steam 30 minutes; decant; filter through cheesecloth; centrifuge at 12 K for 20 minutes (8500 for 15 minutes). Distribute filtered solution to universal bottles, 25mls each.

Antibiotic Solution

50.0 mg	chloramphenicol
50.0 mg	streptomycin
0.5 mL	Tergitol-NPX
1.0 mL	95% C_2H_5OH

STR (Streptomyces Selective Medium)

Ingredient	500 mL	1000 mL
Soluble potato starch (Sigma)	2.5 g	5.0 g
Yeast extract	2.0 g	4.0 g
K_2HPO_4	0.5 g	1.0 g
$MgSO_4*7H_2O$	0.25 g	0.5 g
Bacto peptone (Difco)	0.3 g	0.6 g
Proteose peptone (Difco)	0.3 g	0.6 g
NaCl (20 g NaCl for STR-M)	5.0 g	10.0 g
Agar	8.5 g	17.0 g
salt stock solution	0.5 mL	1.0 mL
Water	500 mL	1 L

- Heat on hot plate until bubbles just start to form on the bottom of the flask.
- Autoclave standard liquid cycle.

- Cool to 55°C add:

Antibiotics	STR 500 mL	STR 1L
Rifampicin	24 uL stock	48 µL stock solution
Naladixic acid	75 uL stock	150 µL stock
Nystatin	1.25 mL stock	2.5 ml stock
Cycloheximide	500 uL stock	1.0 ml stock

Stock Solutions

Antibiotics

Rifampicin	12.5 mg/mL MEOH
naladixic acid	0.1 g/mL 0.1M NaOH
Nystatin	0.022 g/10 mL 95% EtOH
Cycloheximide	0.2 g/mL 70% EtOH

Salt Stock Solution (per 100 mL H_2O)

1.8 g	$FeSO_4*7H_2O$
0.18 g	$ZnSO_4*7H_2O$
0.13 g	$MnCl_2*4H_2O$

Autoclave standard liquid cycle and store at 4°C

Oatmeal Media for Thaxtomin production in Streptomyces

40 g	dry oatmeal
600 ml	deionised H_2O
2 ml	$ZnSO_4*7H_2O$ stock solution (1 g / 100 mL dH_2O)
10 g	Technical agar

Add dry oatmeal to 600 mL water in 1L container

Microwave for 6 minutes or until boiling

Filter through cheesecloth to get 400 ml of residual volume

Make up to 1 L with deionised H_2O

Add $ZnSO_4$ and adjust pH to 7.2

Autoclave standard liquid cycle

Oatmeal Broth for production of Thaxtomin A and B standards

Follow Oatmeal Medium instructions but don't add agar.

Pour 100 ml of broth into 250 mL flasks

If isolates are added, make sure they were grown in Oatmeal Broth as well and add 20 ml of growth to the 100 mL volume.

Ko & Hora medium for growing *R. solani* from soil

(From: Ko & Hora, 1971, *Phytopathol.* **61:** 707-710)

K_2HPO_4	1.0 g
$MgSO_4.7H_2O$	0.5 g
KCl	0.5 g
$FeSO_4.7H_2O$	10 mg
Agar	20 g
Distilled water	1000 mL

Autoclave standard liquid cycle

Cool to 50°C

then add:

- Metalaxyl (Ridomyl) 90 mg

(or *Dexon:* fenaminosulf – original citation)

- chloramphenicol (Calbiochem) 50 mg
- streptomycin sulfate (Calbiochem) 50 mg
- Gallic acid 0.2 g
- $NaNO_2$ 0.4 g

K-HP medium (II) (Cited in Basic Plant Pathology)

(ref: *Phytopathology*, **78:** 1287)

This is the *Ko and Hora medium*, amended with 5µg/ml of prochloraz to permit growth of slow growing isolates (AG-3 type).

KG Medium - (Rhizoctonia selective medium IV) (Cited in Basic Plant Pathology)

(ref: *Mycological Res*., **92:** 458)

K_2HPO_4	1 g
KCl	0.5 g
$NaNO_2$	0.2 g
$MgSO_4.7H_2O$	0.5 g
$FeSO_4.7H_2O$	10 mg
Agar	20 g
Distilled water	1000 mL

Autoclave, cool and add microbial inhibitors:

Gallic acid	0.4 g
Metalaxyl (Ridomil) (or Fenaminosulf)	90 mg
Chloramphenicol	50 mg
Streptomycin	50 mg

Fosetyl-A1 250 mg/L

(add this to inhibit *Macrophomina phaseolina*)

Imazalil 2-5 mg/L

(add this also to inhibit R. cerealis & isolate R. solani)

Triadimefon 2.5-5 mg/L

Pencycuron 25 – 50 mg/L

(add these two in place of Imazalil to isolate R. cerealis & inhibit R. solani)

Appendix III

Primers used for quantitative PCR

Pathogen	Forward primer	Reverse primer	Taqman Probe	Reference
Rhizoctonia. Solani	RsTqF1 AAGAGTTTGGTT GTAGCTGGTCTA TTT	RsTqR1 AATTCCCCAACGT CTCACAAGTT	RQP1 TTTAGGCATGTGCAC ACCTCCCTCTTTC	(Lees *et al.* 2002)
Spongospora Subterranea	SsTQF1 CCGGCAGACCC AAAACC	SsTQR1 CGGGCGTCACCTT CA	SsTQP1 CAGACAACGCACCCA GGTTCTCATG	(van de Graaf *et al.* 2003)
Colletotrichum coccodes	CcTqF1 TCTATAACCCTT TGTGAACATAC CTAACTG	CcTqR1 CACTCAGAAGAA ACGTCGTTAAAAT AGAG	CcTqP1 CGCAGGCGGCACCCC CT	(Cullen *et al.* 2002)
Streptomyces scabies	TxtAQ1	TxtAQ2	TxtATQ	This study (sequence available on request)

Primers used for conventional PCR

Pathogen	Forward primer 5'-3'	Reverse primer 5'-3'	PCR product size bp	Reference
All	ITS1 TCCGTAGGTGAAC CTGCGG	ITS4 TCCTCCGCTTATTGATA TGC		Brun
S. scabies	Nf ATGAGCGCGAAC GGAAGCCCCGGA	Nr GCAGGTCGTCACGAAG GATCG	720	(Bukhalid *et al.* 1998) *et al* 1998
	PA AGAGTTTGATCCT GGCTCAG	PH AAGGAGGTGATCCAGC CGCA	1514	(Edwards *et al.* 1989)
	Scab1 CAACACTCTCGGG CATCCGA	ScabII TTCGACAGCTCCCTCCT TAC	1278	(Lehtonen *et al.* 2004)
	TStxtAF1	TstxtAR1	230	This study (sequence available on request)
	TStxtBF1	TStxtBR1		This study (sequence available on request)
	NosF1	NosR1	150	This study (sequence available on request)
H. solani	SS-for	SS-rev		(McKay and Cooke 1997)

Appendix IV

5 x Gitschier Buffer (Kogan *et al.* 1987)

Prepare the following stock solutions and autoclave them separately:

1 M $(NH_4)_2SO_4$
1 M Tris-HCl (pH 8.8)
1 M $MgCl_2$
0.5 M EDTA (pH 8.8)

To prepare 200 mL of 5 x Gitschier **(33.3 mM $MgCl_2$)** combine;

16.6 mL of 1M $(NH_4)_2SO_4$
67 mL of 1 M Tris-HCl (pH 8.8)
6.7 mL of 1 M $MgCl_2$
1.3 mL of a 1: 100 dilution of 0.5 M EDTA (pH 8.8)
2.08 mL of a 14.4 M commercial stock of β-mercapto-ethanol, stored at 4°C

Adjust finally to 200 mL with approximately 106 mL water and mix. Filter sterilise. Store at -20°C in 1 mL aliquots.

Appendix V

DNA extraction fungal

Fill a 2.0 mL centrifuge tube one-third up the conical portion with lyophilised mycelium (20-60 mg dry) or fresh mycelium (0.1 to 0.3 g wet)

Grind mycelium using liquid nitrogen and pestle

Add 600 µL lysis buffer, mix with pestle so that the mixture is homogeneous.

Incubate at 65°c for 1 hour briefly vortex. Careful lids do not come off

Add 600 µL chloroform: isoamyl alcohol (24:1)

Centrifuge at 13,000 rpm for 15 minutes at room temperature or until aqueous (top) phase is clear

Remove 550 µL of the aqueous (top) phase containing the DNA to a new tube. Be Careful not to take any cellular debris from the interface. Add 550 µL chloroform: isoamyl alcohol (24:1)

Centrifuge at 13,000 rpm for 10 minutes at room temperature or until aqueous (top) phase is clear.

Remove 500 µL of the aqueous (top) phase containing the DNA to a new tube. Be careful not to take any cellular debris from the interface.

Add 50 µL or (0.1 volumes) of 3 M NaOAc to the aqueous phase and 500 µL or (0.54 volumes) of isopropanol. Invert gently to mix. DNA clots that precipitate may or may not be visible depending on the amount of starting material

Centrifuge at 13,000 rpm for 5 minutes at room temperature. Pour off the supernatant. Add 500 µL 70% ethanol. Invert tubes and centrifuge for 2 minutes. Discard liquid and allow tubes to dry on paper towel

Re-suspend DNA pellet in 100 µL of TE or distilled water