



Know-how for Horticulture™

**Enhanced detection of
potato cyst nematode
and bacterial wilt to
improve market
access for the
Australia and New
Zealand Potato
Industries**

Robert Faggian
Agriculture Victoria

Project Number: PT01031

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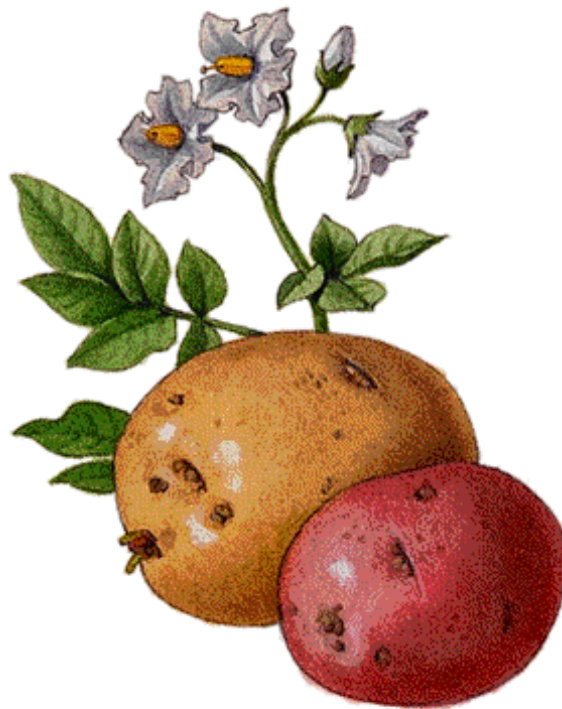
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Project: HAL PT01031

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Purpose of project:

This project report details the outcomes of a 3 year study to develop new diagnostic tests and investigate sampling regimes for bacterial wilt and potato cyst nematode, with the aim of improving market access for the Australian and New Zealand potato industries.

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30th June 2004

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Contents

Media Summary		2
Technical Summary		3
Section 1	General Introduction	5
Section 2	Bacterial Wilt	7
	Introduction	7
	Part 1. Field sites and monitoring protocols	9
	Materials and Methods	9
	Visual symptoms	9
	Isolation of <i>R. solanacearum</i> from plant tissue	9
	Isolation of <i>R. solanacearum</i> from soil	10
	Hypersensitivity Response in Tobacco	10
	Part 2. DNA-based Detection	11
	Isolate collection and strain variation	11
	DNA extraction methods	11
	Pre-enrichment for PCR	11
	PCR Protocols	12
	PCR Reaction conditions	12
	Sensitivity of test procedures	14
	Results and Discussion	14
	Part 3. Field Surveys	18
	Materials and Methods	18
	Soil Sampling	18
	Water Sampling	18
	Results	18
	Discussion	31
	Field Surveys and Sampling	32
	Local Watercourses	33
	Part 4. Concluding Remarks	34
	References	35
Section 3	Potato Cyst Nematode	38
	Introduction	38
	Part 1. Methods- Plot trial and probe development	41
	Plot Trial	41
	Development of duplex primers for quantifying PCN	41
	Results and Discussion	42
	Nematode Responses	42
	Mapping of PCN infestations	44
	Development of duplex primers	45
	Part 2. Sampling Strategies	48
	Discussion	63
	Summary	67
	References	68
Section 4	Conclusions	69
	Technology Transfer	70

Media Summary

Bacterial wilt (brown rot) caused by the bacteria *Ralstonia solanacearum* and damage caused by the potato cyst nematodes *Globodera rostochiensis* and *G. pallida* are two of the most destructive diseases of potatoes worldwide. Both are subject to quarantine restrictions in Australia and New Zealand and outbreaks of either disease can severely limit trade in potatoes across state and international boundaries resulting in millions of dollars of unrealised income.

A collaborative research project between the Department of Primary Industries, Victoria and Crop & Food Research, New Zealand, has addressed this issue by developing a series of forensic-style DNA tests, together with improved sampling strategies. A range of diagnostic tools were developed that allow the quick and reliable detection of these exotic pathogens in plant material, potato tubers, soil and water. New diagnostic procedures for bacterial wilt and PCN are quicker, more sensitive and more specific than traditional testing methods. These procedures are also amenable to automation, which will reduce the cost of routine testing significantly for growers.

A new DNA test developed in this project proved to be indispensable in identifying species of the potato cyst nematode in a recent outbreak of disease caused by the nematode in Koo-Wee-Rup in Victoria. The species of PCN was identified within 72 hours of receiving suspect PCN cysts. Other laboratories, including established international testing facilities, were unable to determine the species, even after several months. The type of response initiated by the government authorities, charged with managing the outbreak, depended on the species of nematode involved.

Effective sampling procedures and the ability to rapidly, accurately and reliably detect the presence or absence of exotic pathogens in plants, tubers, soil and water, ultimately help improve market access for the Australian and New Zealand potato industries.

Technical Summary

The potato industries in Australia and New Zealand are worth approximately \$600 million. Internationally, the two most destructive diseases and most significant impediments to production are bacterial wilt and potato cyst nematode.

PCN only occurs in Australia sporadically and in contained zones while New Zealand is free of bacterial wilt. This situation allowed collaborative research to be conducted that was of mutual benefit. Both countries urgently needed new technology to support contingencies for outbreaks of the target pathogens.

Current testing procedures, using classical methods in plant pathology and nematology (such as selective media and elutriation), are slow, cumbersome and in some cases inaccurate. This project therefore developed sensitive DNA technologies that are important for enhanced detection and to prevent the spread of disease.

The project has delivered DNA-based testing procedures for bacterial wilt and PCN that are more sensitive and robust than traditional methods, as well as improved soil sampling regimes to increase the likelihood of field detection.

DNA-based tests, particularly PCR, are specific, sensitive and robust, and therefore appropriate for improving field survey protocols. The new PCR testing procedures will

- 1) enable proper assessment of the disease-status of export produce
- 2) 2) allow industry to keep pace with the phytosanitary requirements of trading partners,
- 3) 3) give national regulatory bodies sensitive detection tools to enhance quarantine and biosecurity and
- 4) 4) enable scientifically sound declarations of area freedom.

Summary of Project Outcomes:

Bacterial Wilt:

- New laboratory-based testing procedures were established for the detection of *R. solanacearum* in soil and water. Traditional diagnostic tests (selective plating and visual symptom assessments) and new DNA-based procedures were evaluated and integrated into disease monitoring protocols for bacterial wilt. DNA-based tests were faster and significantly more sensitive than traditional testing methods. However, disease-monitoring efforts should be founded on a suite of tests, not just DNA-based procedures.
- *R. solanacearum* populations in crops and soil were monitored over time using both the traditional and DNA-based methods. The pathogen population declined to undetectable levels after approximately two years. Growers can therefore potentially eradicate bacterial wilt from their paddocks by rotation with a non-host crop for at least 2 years, provided all external sources of inoculum are removed (such as latently infected seed).
- *R. solanacearum* was detected in a local watercourse within the Koo Wee Rup potato production region, but the source of inoculum remains unknown. Given that many growers in the Koo Wee Rup region irrigate their potato crops with water from rivers and streams, this is a potentially serious issue. However, similar situations in Europe have resulted in successful eradication programs. For example, *R. solanacearum* was found to overwinter in river-

inhabiting weeds in Sweden. The pathogen was eradicated from Sweden by removing the weed host.

PCN:

- A species-specific detection assay and the first-ever quantitative detection system were developed, based on conventional and real-time PCR methodology with Taqman chemistry. The new techniques enable the detection of the species of PCN in soil and the relative numbers of eggs present in the sample. This relationship has been established for both *Globodera rostochiensis* and *G. pallida* and will enable sensitive detection for quarantine purposes.
- Using the new quantitative DNA tools, the relationship between initial level of PCN infection (eggs /ml) and the host response, expressed as changes in tuber weight, was established. This will enable pre-planting disease prediction and therefore assist growers to make production and disease management decisions.
- A highly sophisticated mathematical and computer model to develop a soil sampling regime to improve the chances of detecting PCN, was established through a collaborative link with Plant Research International (the Netherlands). The model enabled the assignment of percentage detection probability for all common sampling regimes. Using these data and interactive models, the efficiency of sampling regimes was assessed and the best options identified for the Australian and New Zealand potato industries. The optimisation of sampling regimes will enhance PCN management and phytosanitary declarations.

Section 1.

General Introduction

Summary

Bacterial wilt and PCN are two of the most destructive diseases of potatoes and two of the greatest impediments to production when established. Traditional methods of detecting these pathogens are slow, cumbersome and not always accurate. Speed and sensitivity are vital to prevent the spread of disease and to reduce the time between contamination by a pathogen and symptom development in the crop. Molecular biology-based tests, particularly PCR, are specific, sensitive and robust. Further development of more accurate and rapid PCR testing in this study will lead to;

- 1) More accurate assessments of the disease-status of export produce;*
- 2) Industry keeping pace with the phytosanitary requirements of trade partners;*
- 3) Provision of national regulatory bodies with sensitive detection tools to enhance quarantine and biosecurity and*
- 4) Better evidence to support scientifically sound declarations of area freedom in Australia and New Zealand. This will benefit both countries if testing methods are uniform, since the present lack of harmonisation may cause market access difficulties.*

Introduction

Bacterial wilt (brown rot) caused by the bacteria *Ralstonia solanacearum* and damage caused by the potato cyst nematodes *Globodera rostochiensis* and *G. pallida* are two of the most destructive diseases of potatoes worldwide. PCN (*G. rostochiensis*) only occurs sporadically in contained zones in Australia. However, PCN (*G. rostochiensis* and *G. pallida*) is endemic in New Zealand, where researchers are learning to detect and manage the problem. In contrast, bacterial wilt is not present in New Zealand, but does occur sporadically in Australia. Both diseases are subject to quarantine restrictions in Australia and New Zealand and outbreaks of either disease can severely limit trade in potatoes across state and international boundaries, resulting in millions of dollars of unrealised income.

Current testing-procedures for these pathogens are slow, cumbersome and in some cases inaccurate. For instance, one of the most reliable methods for the detection of *R. solanacearum* relies on exhaustive dilution tests on a semi-selective medium, followed up by bacterial characterisation tests and pathogenicity studies. The bacterium is generally isolated from symptomatic tubers but the method can also be used for pre-emptive testing of latently infected seed potatoes. However, the semi-selective plating requires a trained eye since a range of non-pathogenic and visually similar strains can also be isolated. Also, the method is not widely used for soil testing to assess the disease-status of production areas because it is labour-intensive, time-consuming and the presence of the pathogen can be masked by soil saprophytes. Testing is also time consuming and potentially unreliable for PCN. In New Zealand, fork-testing is the accepted testing procedure for PCN. Growing crops are fork-sampled at the stage when immature female nematodes protrude from potato roots and are still pale cream in colour, and thus distinguishable

from roots, soil and other debris. This narrow window of opportunity for testing rapidly closes because the females soon turn brown and become very difficult to see.

This project therefore evaluated sensitive DNA technologies for enhanced detection and to prevent the spread of disease, with the aim of having the tests available in Australian and New Zealand diagnostic laboratories. Molecular biology-based tests, specifically PCR tests, are many times more sensitive than existing microbiological or antibody tests. PCR test results can be available in less than a day and the tests are amenable to automation and high-throughput processing. Extensive sample preparation is not required and the results do not need to be interpreted by a highly skilled diagnostician. The tests can be repeated a number of times and results are operator and laboratory independent. Also, with automation and robotics, the cost of PCR is comparable to that of the cheapest traditional tests.

The project also considered protocols for soil or crop sampling (e.g. field surveys) to assist detection of PCN and bacterial wilt so that outbreaks could be detected much earlier than can presently be achieved by assessment of visual symptoms. International trade in produce relies on a complex set of agreements, assurances and audits to certify that export produce complies with the importing country's phytosanitary requirements. Many of the procedures in place around the world have evolved from tradition and convention, which have subsequently been enshrined in regulation. The use of field surveys is an example of this, given that, often, the parts of plants examined are not the parts traded as product (e.g. potato tops for trade in tubers or pea leaves and pea pods for trade in pea seed). Similarly, the sampling regimes and testing procedures that form the backbone of field surveys are not always based on sound scientific methodology.

In addition, existing methods of field survey and diagnosis tend to be biased towards locating symptomatic material and then identifying diseases. Sampling a field without reference to the incidence of symptoms would more accurately indicate the level of pathogens in soil or in the crop. That is, grid sampling without reference to diseases is a statistically valid technique for detecting disease levels in comparison to the current system. A more appropriate focus would therefore be to directly detect or measure pathogens to eliminate the lag period between incursion and symptom development. Again, success hinges on reliable and highly sensitive testing procedures.

It is important to commit to PCR technology because our trading partners are already doing so to protect their own plant-based industries from exotic diseases. This presents the possibility that such tests will be used routinely by other countries and then used against us to hinder market access. It is therefore vital that we pro-actively seek to determine the disease status of our export material using the most sensitive and accurate testing procedures available. Australian and New Zealand regulators will also have the tools at their disposal to test the phytosanitary-status of imports for improved quarantine and biosecurity.

It is important that Australasia and New Zealand moves towards a harmonised system based on a scientifically sound field and soil sampling system coupled with well-founded principles of laboratory diagnosis, to replace testing for diseases in the field. Bacterial wilt and potato cyst nematode (PCN) provide a unique opportunity to accomplish this.

Section 2.

Bacterial wilt

Robert Faggian, DPI Knoxfield

Summary

- Traditional diagnostic tests (selective plating and visual symptom assessments) and new DNA-based procedures were evaluated and integrated into disease monitoring protocols for bacterial wilt. DNA-based tests were faster and significantly more sensitive than traditional testing methods. However, disease monitoring efforts should be founded on a suite of tests, not just DNA-based procedures.
- *R. solanacearum* populations in crops and soil were monitored over time using both the traditional and DNA-based methods. The pathogen population declined to undetectable levels after approximately two years. Growers can therefore potentially eradicate bacterial wilt from their paddocks by rotation with a non-host crop, provided all external sources of inoculum are removed (such as latently infected seed).
- *R. solanacearum* was detected in a local watercourse within the Koo Wee Rup potato production region, but the source of inoculum remains unknown. Given that many growers in the Koo Wee Rup region irrigate their potato crops with water from rivers and streams, this is a potentially serious issue. However, similar situations in Europe have resulted in successful eradication programs. For example, *R. solanacearum* was found to overwinter in river-inhabiting weeds in Sweden. The pathogen was eradicated from Sweden by removing the weed host.

Introduction

Bacterial wilt (or brown rot) is caused by the soil-borne bacterium *Ralstonia solanacearum* (syn. *Pseudomonas solanacearum* E. F. Smith) (Yabuuchi *et al.*, 1995). It is one of the most important and widespread bacterial diseases of crops and it causes great economic losses world-wide (Hayward, 1991). The pathogen invades xylem vessels and causes dysfunction in water transport. Control measures are limited and consist of the use of resistant cultivars and cultural practices. The pathogen has been reported on more than 200 species representing 50 families, and includes economically important crops such as potato, tomato and tobacco (Schell, 2000). Five races of *R. solanacearum* have been described according to the hosts they affect (Buddenhagen *et al.*, 1962; He *et al.*, 1983; Pegg & Moffett, 1971), as well as six biovars (1, 2, N2, 3, 4, 5) according to their ability to utilise various hexose alcohols and disaccharides (Hayward, 1964; Hayward *et al.*, 1990). Also, based on restriction fragment length polymorphisms (RFLP) (Cook *et al.*, 1989; 1991; Cook and Sequeira, 1994) and 16S rDNA gene sequences (Li *et al.*, 1993; Taghavi *et al.*, 1996; Seal *et al.*, 1993), *R. solanacearum* has been split into two distinct divisions, which relate to the geographical origin of strains (Asian and American).

Race 1 (biovars 1, 3 and 4) has a very wide host range that includes potato, but it is restricted to tropical areas. Race 3 (biovar 2) has a narrow host range, infecting mainly potato and tomato, and occurs in cool upland areas of the tropics because it has a lower optimum temperature. It is for

this reason that race 3 / biovar 2 is considered the major threat to potato production in the temperate regions of the world.

R. solanacearum can be spread within and between countries by the movement of soil, water and latently infected planting material (Hayward, 1991). In fact, the pathogen has been spread around the world on latently infected potato tubers, and in the 1990's was found in the Netherlands, one of the world's major seed producing nations. International trade in seed potatoes is based on a zero-tolerance approach to bacterial wilt infection. In countries affected by the disease the costs of surveillance and eradication are significant (Hayward, 2000). Therefore, highly sensitive and rapid diagnostic methods are required for quarantine to help limit the spread of *R. solanacearum*.

The success of quarantine relies on a rapid and accurate response, which is facilitated by rapid and specific diagnostic tools. Traditionally diagnosis for *R. solanacearum* has relied on a series of biochemical tests on pure cultures followed by confirmation of pathogenicity with a bioassay. The whole process is very time-consuming and therefore not practical for quarantine or other diagnostic laboratory purposes. However, in the absence of more suitable tests, these methods have been used in Victoria.

The aims of this component of the project were to improve the quality of bacterial wilt surveying by developing the tools required to monitor disease outbreaks and prevent their spread. This was to be achieved by:

- 1) Developing accurate and sensitive DNA-based detection methods
- 2) Validating the results of the DNA-based tests using traditional testing methods
- 3) Developing improved sampling regimes

Part 1 – Identification of field sites and establishment of monitoring protocols

Field sites for this study were identified with the assistance of local growers in the Koo Wee Rup potato-growing region of Victoria. This growing region is a reclaimed swamp that produces predominantly crisping potatoes. Bacterial wilt was first reported in Koo Wee Rup from 1911 to 1914 (Hayward, 1998), and has occurred sporadically ever since.

The sporadic nature of disease incidence meant that the identification of field sites was difficult, especially when coupled with the reluctance of local growers to admit to having the disease in their crops. Despite this, five sites were sampled extensively because they either had a currently infected crop or a recent crop (within the last rotation period) that had been infected (locations have been withheld to protect the confidentiality of the growers involved). The aims of sampling were to 1) assess traditional and DNA-based detection methods in field soil, 2) develop an understanding of pathogen ecology in Koo wee rup and identify possible sources of inoculum. All sites were irrigated using water from local watercourses, and since irrigation water is a source of inoculum in Europe, such water was also sampled regularly.

Disease monitoring protocols were established to assess the disease status of field sites. In the initial stages of the study, the monitoring protocols consisted of non-molecular means of detection: 1) visual assessment of symptoms and 2) isolation from soil and plant tissue using semi-selective media. Both methods are used routinely around the world and are widely accepted for the diagnosis of bacterial wilt infection.

Materials and Methods

Visual symptoms of bacterial wilt infection in potato plants and in tubers.

Plant: Early field infections are characterised by wilting of the leaves towards the top of the plant during the day, particularly at high temperatures. Although the plant may recover at night, over time the wilting becomes irreversible and the plant dies. The vascular tissue of cut stems usually appears brown with a milky bacterial exudate from the cut surface. When the cut stem is placed vertically in water, oozing threads of bacteria will stream from the vascular bundles.

Tuber: Symptoms in tubers can be seen by making a transverse cut close to the stolon end or a longitudinal cut over the stolon end. The early stage of infection is characterised by a glassy yellow discolouration of the vascular ring, from which a cream-coloured bacterial exudate oozes either spontaneously or when pressure is applied to the tuber skin near the cut surface. Later, the vascular discolouration becomes brown as necrosis extends into the parenchymatous tissue. In advanced stages, infection breaks outwards from the heel of the tuber as well as the eyes, from which bacteria ooze, causing soil particles to adhere (and thus giving rise to one of the common names for the disease, ‘jammy eye’). Secondary infection of fungal and bacterial soft rots is common in the advanced stages of the disease.

In this study, a tentative positive diagnosis was made with regard to visual symptoms only when an infected tuber, cut transversely close to the stolon end, showed the characteristic brown ring and bacterial ooze.

Isolation of *R. solanacearum* from plant tissue

Suspected bacterial wilt was isolated from affected plants by removing ooze or sections of discoloured tissue from the vascular ring in the potato tuber, or from the vascular strands in

stems. The ooze was suspended in a small volume (50 µl) of sterile distilled water which was streaked onto agar plates of either Kings B or SMSA (see below), depending on the cleanliness of the original sample. If the original sample was a tuber or stem in the early stages of infection, from which clean ooze could be obtained, King's B was used. If, however, the original sample was rotting or mixed with soil, SMSA was used. The plates were incubated for 4 to 6 days at 28 °C.

On the King's B media, virulent isolates of *R. solanacearum* develop pearly cream, flat, irregular and fluidal colonies often with characteristic whorls in the centre with a transparent brown halo in the media. Avirulent forms of *Ralstonia solanacearum* form small round non-fluidal, butyrous colonies that are entirely white with no brown halo.

On SMSA media, the whorls are blood red in colour. Avirulent forms of *R. solanacearum* form small round non-fluidal, butyrous colonies that are entirely deep red.

Isolation of *R. solanacearum* from soil

R. solanacearum was isolated from soil using the semi-selective medium SMSA described by Engelbrecht (1994) and modified by Elphinstone *et al.* (1996).

Soil was sampled by taking up to 500 g of soil to a depth of 20 cm and mixing them thoroughly to ensure any soil-clumps were broken down. Sub-samples of 10 g were further dispersed by vigorous shaking in 150 ml extraction buffer (50 mM phosphate buffer, pH 7.0) and a 100 µl aliquot was used to prepare a serial 10-fold dilution down to 10⁻⁶. A 100 µl sample of suspension from each dilution was spread onto agar plates of SMSA and incubated for 4 to 6 days at 28 °C.

The suspensions were maintained at 4 °C for subsequent pre-enrichment steps in liquid SMSA for DNA extraction purposes.

In this study, a positive diagnosis was made only when characteristic colonies were observed on SMSA media, whether isolated from plant tissue or soil.

Hypersensitivity Response in Tobacco

To determine whether isolates of *R. solanacearum* were virulent a hypersensitivity reaction was carried out on a tobacco host.

Preparation of inoculum: Bacterial cells from a single colony were transferred to 5 mL of Luria-Bertani broth and incubated at 28°C for 24 hours. A 1 mL aliquot of the resulting bacterial suspension was pelleted by centrifugation, washed once in sterile distilled water and then resuspended in 1 mL of sterile distilled water.

Inoculation of tobacco: A section of tobacco interveinal leaf tissue from plants in pots was injured using a small gauge needle. Bacterial inoculum was drawn into a syringe (minus needle) and injected into the wound site until it infiltrated the leaf to the point where inoculum spread was restricted by adjacent veins. Inoculation was repeated with sterile distilled water and reference isolates of biovars 2 and 3 to act as negative and positive controls. Inoculated plants were kept at room temperature for up to 10 days.

Biovar 2 induces chlorosis in the inoculated leaf somewhere between 2 and 8 days, with no further symptom development. Biovars 1, 3 and 4 also induce chlorosis, which is followed by wilting.

Part 2 – DNA-based Detection

Isolate collection and strain variation

A number of PCR-based diagnostic tests have been developed for the detection of *R. solanacearum*, some of which have also been validated with respect to inter-laboratory standardisation of protocols. The development of new detection methods and the assessment of genetic variation of local isolates were therefore deemed unnecessary provided the existing tests were able to detect all local isolates of the pathogen and could achieve a greater level of sensitivity than traditional tests.

All publicly available PCR-based tests were therefore assessed for their ability to detect Australian isolates and sensitivity of detection in soil and water.

Nine Victorian isolates were obtained from infected tubers showing obvious signs of bacterial wilt infection at sites A and B (see below). Suspected *R. solanacearum* was isolated using the standard isolation techniques described above (and later the collection was expanded with soil isolates). Reference isolates of *R. solanacearum* biovar 2 (ref#0158, isolated from potato) and biovar 3 (ref#0170 and ref#0171 isolated from tobacco and eggplant respectively) were also obtained from Dr. Chris Hayward at the University of Queensland. Local isolates were stored by suspending a loopful of bacterial cells in 50 mL sterile distilled water, which was kept in the dark at room temperature. Reference isolates were aliquoted and kept at -70°C in a quarantine-approved facility.

To test whether local isolates were in fact virulent strains of *R. solanacearum*, colony morphology on King's B and SMSA, hypersensitivity reaction in tobacco leaves, sequencing of the 16S rDNA region and PCR with Division 1 and 2 –specific primers were all used and compared to the biovar 2 reference strain #0158.

DNA extraction methods

For PCR testing of sub-cultures, DNA was extracted directly from colonies on King's B media. Bacterial cells were transferred to 500 μL sterile distilled water in a 1.5 mL Eppendorf tube using a sterile loop. The cells were vortexed briefly then boiled for 5 minutes at 99°C in a heating block. One or two μL of the resulting lysate was used as template DNA in PCR reactions, without purification. After being used successfully in PCR, the DNA was discarded. Larger amounts of pure DNA was extracted from broth cultures using the DNeasy mini kit (Qiagen), and this DNA was stored at -20°C and used as positive controls in PCR experiments.

Soil DNA was extracted using the FastPrep Soil extraction kit (Q-Biogene) either directly from 0.5-1.0 g of soil, or from pre-enriched soil suspensions (see below).

Soil DNA was also extracted from 500 g samples using a propriety method at the South Australian Research and Development Institute (SARDI).

Pre-enrichment for PCR

A 100 μL sample of soil suspension (from the soil isolation protocol) was transferred to an Eppendorf tube and 1.4 mL of SMSA media was added (minus Bacto-agar and 2,3,5-tetrazolium chloride). The soil suspensions were incubated at 28°C for 24 hours with shaking, then pelleted by centrifugation. The pellet was washed once with sterile distilled water then resuspended in 980

μL of phosphate buffer (the first solution used in the FastPrep Soil extraction kit). The resulting solution was then subjected to DNA extraction according to the manufacturers protocol.

PCR Protocols

Thirteen published PCR protocols for the detection of *R. solanacearum* (table 1) were assessed for 1) sensitivity of detection, 2) specificity for biovar 2 and 3) ease of use in terms time, cost and technical complexity.

PCR Reaction conditions

PCR temperature cycling regimes and reagent concentrations were as described by the authors of the original protocol. However, local reagents were used, which included HotStarTaq DNA Polymerase (Qiagen), dNTP's (Promega) and oligonucleotide primers (MicroMon) in a final reaction volume of 50 μL. All PCR reactions were carried out in a Hybaid Sprint thermocycler.

Two of the protocols used are briefly described below.

1. PCR protocol of Boudazin *et al.* (1999).

Temperature cycling regime:

Step i)	3 minutes at 95°C	- 1 cycle
Step ii).	1 minute at 95°C	\
	1 minute at 60°C	- 35 cycles
	1 minute at 72°C	/
Step iii)	10 minutes at 72°C.	- 1 cycle

Reaction conditions: 0.5 Units of enzyme, 2.5mM MgCl₂, 0.2mM dNTP's and 1μM of each primer.

2. Protocol of Opina *et al.* (1997)

Temperature cycling regime:

Step i)	3 minutes at 94°C	\
	1 minute at 53°C	- 1 cycle
	1.5 minutes at 72°C	/
Step ii).	1 minute at 92°C	\
	1 minute at 60°C	- 35 cycles
	1 minute at 72°C	/
Step iii)	10 minutes at 72°C.	- 1 cycle

Reaction conditions: 0.5 Units of enzyme, 1.5mM MgCl₂, 0.05mM dNTP's and 1μM of each primer.

Table 1. PCR protocols assessed for detection of *R. solanacearum*.

#	Primer	Primer Sequence	Gene	Biovar	Size	Reference
1	759f 760r	5'-GTC GCC AAC TCA CTT TCC-3' 5'-GTC GCC AGC AAT GCG GAA TCG-3'	unknown	1,2	281bp	Opina <i>et al.</i> , 1997
2	OL1I Y2	5'-GGG GGT AGC TTG CTA CCT GCC-3' 5'-CCC ACT GCT GCC TCC CGT AGG AGT-3'	16S	1,2,N2	288	Seals <i>et al.</i> , 1993 Niepold, 1999
3	OL1I BV345	5'-GGG GGT AGC TTG CTA CCT GCC-3' 5'-CGT CAT CCA CAC CAG GTA TTA ACC AGT-3'	16S	3, 4, 5	409bp	Seal <i>et al.</i> , 1999
4	DIV2F ITSR	5'-CGC TTC GGT TAA TAC CTG GAG-3' 5'-GCA GAG ACT TCC ACC TCC A-3'	16S	1,2 & N2	1246bp	Seal <i>et al.</i> , 1999
5	D2 Primer B	5'-GTC CGG AAA GAA ATC GCT TC-3' 5'-GCA CAC TTC ATC CAG CGA-3'	16S	1,2 & N2		Seal <i>et al.</i> , 1999 Boudazin <i>et al.</i> , 1999
6	OL1I Primer Z	5'-GGG GGT AGC TTG CTA CCT GCC-3' 5'-CCA CTC CAG GTA TTA ACC GAA-3'	16S	1,2 & N2	403 bp	Boudazin <i>et al.</i> , 1999
7	D1 Primer B	5'-GTC CGG AAA GAA ATC GCA CT-3' 5'-GCA CAC TTC ATC CAG CGA-3'	16S	3, 4, 5		Boudazin <i>et al.</i> , 1999
8	PS-1 PS-2	5'-AGT CGA ACG GCA GCG GGG G-3' 5'-GGG GAT TTC ACA TCG GTC TTG CA-3'	16S	All - RFLP	553 bp	Pastrik and Maiss, 2000
9	RS30 RS31	5'-GAA GAG GAA CGA CCG AAA GC-3' 5'-CGA ACA GCC CAC AGA CAA GA-3'	<i>Hrp</i>	All		Poussier and Luisetti, 2000
	RS30a RS31a	5'-GGC GCT GGC GGT GAA CAT GG-3' 5'-CAA CAT CCT GGC CCG CAT CGT G-3'	<i>Hrp</i>	RFLP		Poussier and Luisetti, 2000
	RS30b RS31b	5'-TCT TGC GCT CGC CCT TGA TGT G-3' 5'-CGA CAG CAG CCG GCA CCC-3'	<i>Hrp</i>	RFLP		Poussier and Luisetti, 2000
10	Rs-1-F Rs-1-R	5'-ACT AAC GAA GCA GAG ATG CAT TA-3' 5'-CCC AGT CAC GGC AGA GAC T-3'		1,2	718 bp	Pastrik <i>et al.</i> , 2002
11	DIV2F DIV2R	5'-CGC TTC GGT TAA TAC CTG GAG-3' 5'-CTG CCG TGG TAA TCG CCC CCC-3'	16S	1,2, N2	1019	Seal <i>et al.</i> , 1999
12	DIV1F DIV1R	5'-CGC ACT GGT TAA TAC CTG GTG-3' 5'-CTA CCG TGG TAA TCG CCC TCC-3'	16S	3, 4, 5	1019	Seal <i>et al.</i> , 1999
13	BP4-R BP4-L	5'-GAC GAC ATC ATT TCC ACC GGG CG-3' 5'-GGG TGA GAT CGA TTG TCT CCT TG-3'	SCAR	All	1102	Lee and Wang, 2000

Sensitivity of test procedures

“*R. solanacearum*-free” soil was collected from a residential property in Koo Wee Rup that was isolated from potato growing activity and which had no known history of potato production. The “*R. solanacearum*-free” status of the soil was checked by attempting to isolate the bacterium using the soil isolation technique described earlier. No *R. solanacearum* could be isolated and so the soil was assumed to be free of the pathogen. The soil was autoclaved and then divided into 10 g sub-samples. Each of sub-sample was spiked with an aliquot of *R. solanacearum* biovar 2 solution previously diluted 10-fold to contain between 10^8 - 10^2 cells. The spiked soil samples were then subjected to the soil isolation method to assess the percentage recovery on SMSA media. Aliquots of the soil solution produced during the isolation procedure were then subjected to DNA extraction, with and without a pre-enrichment step, as described previously.

Results & Discussion

Identification of nine Victorian isolates to race/biovar level

The nine isolates collected from infected tubers were confirmed as *R. solanacearum* and more specifically race 3/biovar 2 by:

- 1) the production of characteristic red-whorled colonies on SMSA media
- 2) the induction of the characteristic hypersensitivity response in tobacco
- 3) 100% sequence similarity with the *R. solanacearum* 16S region
- 4) positive test result in Division 2-specific PCR (i.e. protocol of Opina *et al.*, 1997) and negative in Division 1-specific PCR (i.e. protocol of Seal *et al.*, 1999)
- 5) the fact that they originated from tubers with classic bacterial wilt symptoms.

The above set of results were mirrored by those of the race 3/biovar 2 reference isolate #0158, providing further evidence for the classification of the locally collected isolates as *R. solanacearum* race 3/biovar 2.

Additionally, according to Hayward (1998), there is no evidence to suggest that strains other than race 3/biovar 2 occur in southern Australia. Also, as far as is known, southern Australian strains are uniform in genotype and phenotype.

Suspected *R. solanacearum* were later isolated from field soil and again testing indicated they belonged to race 3/biovar 2.

PCR Protocol selection

Of the thirteen PCR protocols examined, numbers 3, 7 and 12 (Table 1) were specific for Division 1 (that is, races 3, 4, and 5) and were therefore disregarded. These protocols did however amplify DNA fragments of the expected sizes from the biovar 3 reference isolates #0170 and #0171, but not from the biovar 2 reference isolate #0158.

Protocol number 13 was non-strain specific and so was also disregarded.

Of the remaining protocols, four required a post-PCR restriction digest step to produce unique and division-specific RFLP patterns, while one was a nested PCR protocol. These protocols were

therefore technically more demanding and time-consuming. As sensitivity for quarantine was the priority, all protocols were included in experiments to determine sensitivity.

Protocols 1, 2, 4, 5, 6, 8, 9, 10 and 11 were all able to amplify DNA of the expected size from the locally collected isolates. Some sample gel photos have been included (Fig 1 and 2).

Also of note was an attempt to apply in-house PCR reaction conditions to one of the protocols, rather than those specified by the author (Fig. 3). The in-house conditions allow reliable and sensitive PCR detection of *Plasmodiophora brassicae*, a soil-borne pathogen of brassicas, but the same conditions resulted in extremely poor amplification of crude *R. solanacearum* DNA. This demonstrates the high level of optimisation and validation that the *R. solanacearum* protocols have undergone and that the specified conditions should be strictly adhered to.

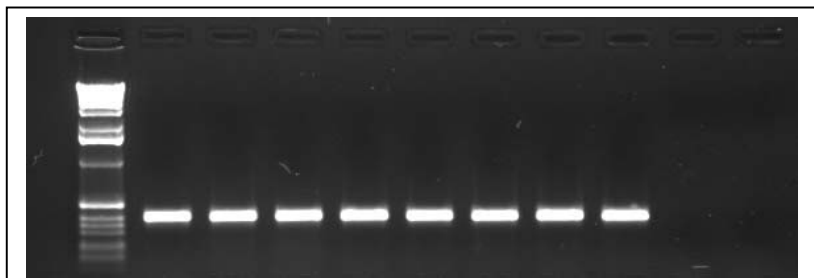


Fig. 1. PCR Amplification of DNA from eight local *R. solanacearum* isolates (lanes 1-8) with primers OLI1/PrimerZ, and two negative controls (lanes 9 & 10).

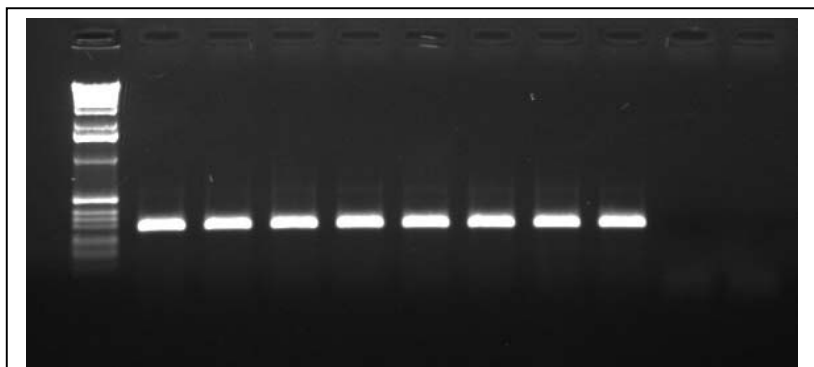


Fig. 2. PCR Amplification of DNA from eight local *R. solanacearum* isolates (lanes 1-8) with primers OLI1/Y2, and two negative controls (lanes 9 & 10).

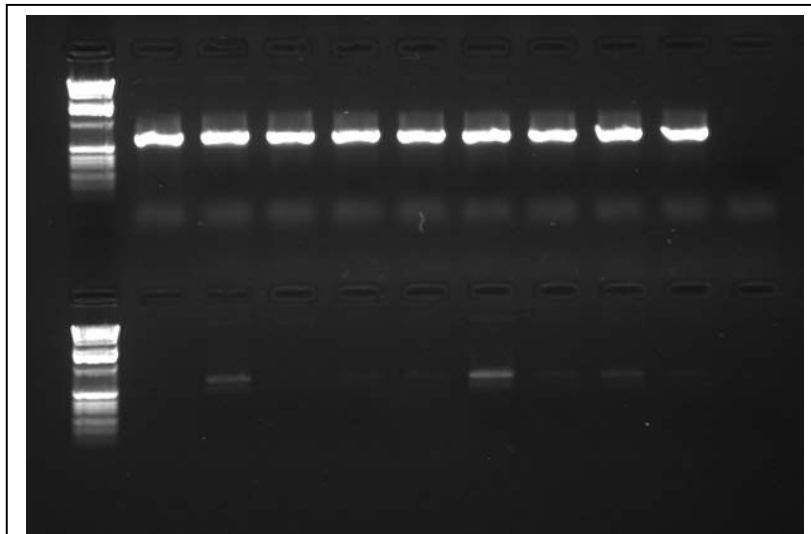


Fig. 3. PCR amplification of DNA from nine Victorian *R. solanacearum* isolates (lanes 1-9) with primers D2/B, and one negative control (lane 10) using the authors reaction conditions (top row) and in-house reaction conditions for another soil-borne pathogen (bottom row).

Sensitivity of detection methods

R. solanacearum was recovered on SMSA media from artificially inoculated soil from all but the lowest dilution (10^1) (Table 2). This indicated that populations as low as 100 colony-forming units (CFU) could be detected using the semi-selective medium. Also, the recovery efficiency was high (approximately 95%) for the artificially inoculated soil, suggesting that the quantification estimate gained through plating is accurate, at least under artificial conditions.

Table 2. Recovery of *R. solanacearum* on SMSA from artificially inoculated soil

CFU per gram soil	CFU recovered per mL soil suspension	Recovery efficiency (%)
10^7	9.4×10^5	94
10^6	9.7×10^4	97
10^5	9.7×10^3	97
10^4	9.5×10^2	95
10^3	9.2×10^1	92
10^2	8.7×10^0	87
10^1	-	

PCR detection methods were able to detect *R. solanacearum* down to the 10^2 CFU per gram of soil level (Table 3). All protocols achieved a level of sensitivity of 10^6 CFU per gram of soil (Table 3). If the soil suspensions were enriched for 24 hours prior to DNA extraction, detection sensitivity increased 10-fold as a result of the multiplication of the target pathogen. Four protocols achieved the highest detection sensitivity of 10^2 CFU per gram of soil, at which point enrichment no longer had an effect. Interestingly, one of the first protocols to fail was the nested protocol (number 9). This may be due to the fact that it targets the *hrp* gene, which is single copy, compared with the other protocols that target the 16S rRNA region, which is multicopy. Also, as the concentration of spiked inoculum decreased, so too did the amount of amplification product generated (as expected). However, when close to the limits of sensitivity, those protocols relying on restriction digestion of the product become useless because there is enough amplicon.

Protocols 1, 6, 10 and 11 were the most sensitive for detection of *R. solanacearum* in soil. All were technically straightforward and performed similarly. However, protocols 1 and 6 were selected for subsequent field surveys because both have undergone substantial validation in Europe and form part of the European Union's harmonised set of protocols for the detection of *R. solanacearum*.

Table 3. Sensitivity of various PCR protocols for the detection of *R. solanacearum* in spiked soil, with and without a pre-enrichment step.

CFU per g soil	Successful PCR detection without enrichment step	Successful PCR detection with enrichment step
10^7	1, 2, 4, 5, 6, 8, 9, 10 and 11	1, 2, 4, 5, 6, 8, 9, 10 and 11
10^6	1, 2, 4, 5, 6, 8, 9, 10 and 11	1, 2, 4, 5, 6, 8, 9, 10 and 11
10^5	1, 2, 4, 5, 6, 8, 10 and 11	1, 2, 4, 5, 6, 8, 9, 10 and 11
10^4	1, 2, 4, 5, 6, 8, 10 and 11	1, 2, 4, 5, 6, 8, 10 and 11
10^3	1, 6, 8, 10 and 11	1, 4, 5, 6, 10 and 11
10^2	1, 6, 10 and 11	1, 6, 10 and 11
10^1	-	-

Part 3 – Field Surveys

The monitoring protocols (visual symptoms and SMSA isolation) and PCR protocols outlined above were used to map the presence of *R. solanacearum* at 5 field sites in the Koo Wee Rup region. Field sites were repeatedly sampled at 6-monthly intervals when *R. solanacearum* was discovered, in order to analyse the pathogens persistence in the environment. Also, water from the local watercourses was tested on a fortnightly basis over a 12-month period from June 2002 to June 2003.

Material and Methods

Soil sampling

Sampling of field soil was carried out using standard nematode protocols on either a 4m x 4m grid pattern or an 8m x 8m grid pattern. At each grid position, a number of small soil samples were collected to a depth of 20 cm to a total of approximately 500 g of soil. Samples were collected in heavy gauge, airtight polyethylene bags, transported to the laboratory at ambient temperature and then stored at 4°C prior to testing. Generally soil-processing for testing was completed over a two-week period after sample collection.

At each grid sampling point, visual disease symptoms were assessed, *R. solanacearum* isolation was carried out from soil, and PCR detection was carried out using one of the two selected PCR protocols. Approximately one in ten positive isolations was randomly re-tested with the other PCR protocol to confirm the presence of *R. solanacearum*. Similarly, the DNA from one in ten negative grid positions was tested using generic eukaryote PCR primers to ensure the presence of amplifiable DNA.

Water sampling

Water was sampled from five points along a local watercourse, from within the potato production area to three kilometres upstream beyond the limit of potato production. Water was collected in 500 mL Schott bottles from 2 m from the riverbank by suspending the bottles on rope from bridges. The water was transported at ambient temperature to the laboratory and processed for testing immediately. Sub-samples of 50 mL were pelleted by centrifugation and tested using SMSA media and PCR.

Results

Site A

Site A was a section of crop containing plants with suspected bacterial wilt. The grower invited project staff onto the property 2 weeks before harvest which is when the first set of samples were taken. Visual inspection of tubers revealed typical bacterial wilt symptoms and symptomatic plants were concentrated around an irrigation hydrant. Systematic sampling and testing using visual, SMSA isolation and PCR revealed a foci of infection that was larger in area than visual symptoms alone indicated (Fig. 4). Wherever visual symptoms were found, *R. solanacearum* was also isolated from the soil and detected in the soil with PCR. Where only SMSA-isolated *R.*

solanacearum was found, PCR tests were also positive. This overlapping pattern of test results was common through all the field surveys.

The site was re-sampled after 6 months (Fig. 5), 12 months (Fig. 6) and 18 months (Fig. 7). As no crop was present after the initial sampling event, visual symptoms could not be assessed. The area of both SMSA-isolated *R. solanacearum* and PCR-detectable *R. solanacearum* decreased markedly after 6 months and 12 months. *R. solanacearum* was not detectable at the 18 month sampling period.

Sample numbers were decreased from the 12-month sample onwards as it became clear that the pathogen distribution was shrinking around the hydrant and therefore the initial grid size was excessive.

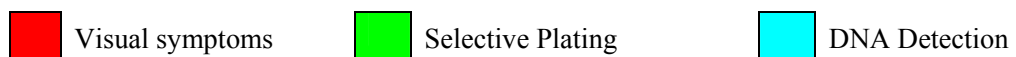
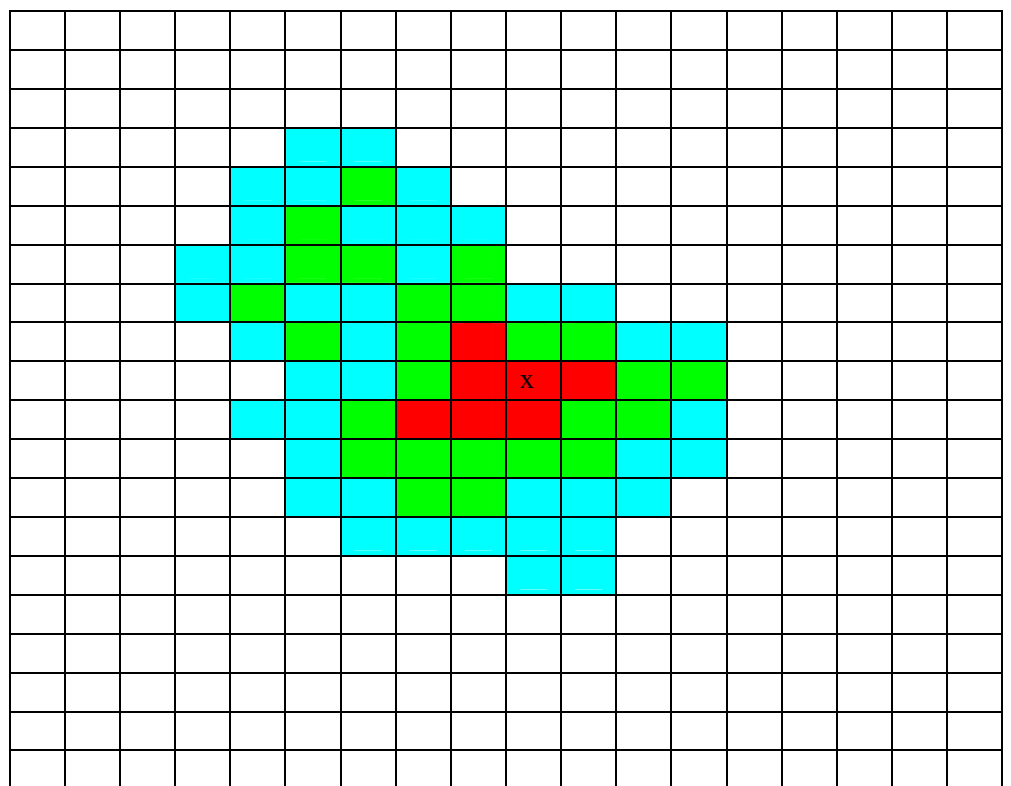
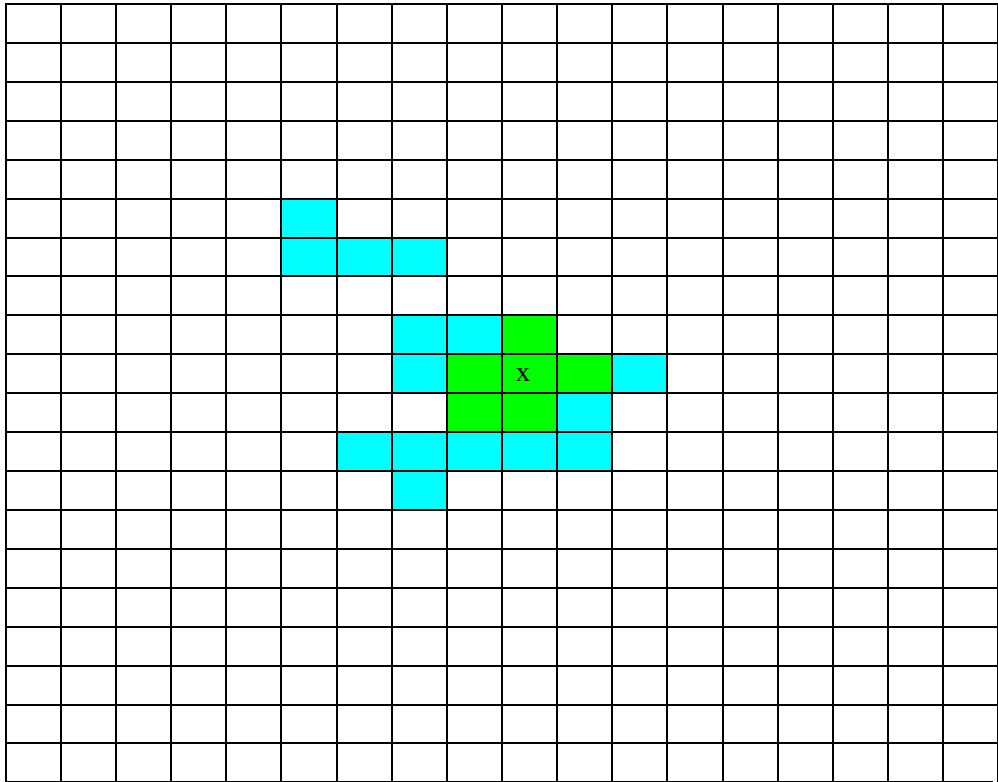


Figure 4. Site A 4mx4m detection map 2 weeks prior to crop harvest ('x' indicates position of irrigation hydrant)






 Visual symptoms  Selective Plating  DNA Detection

Figure 5. Site A 4mx4m detection map 6 months after crop harvest.

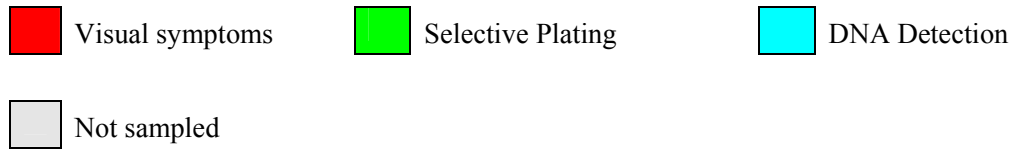
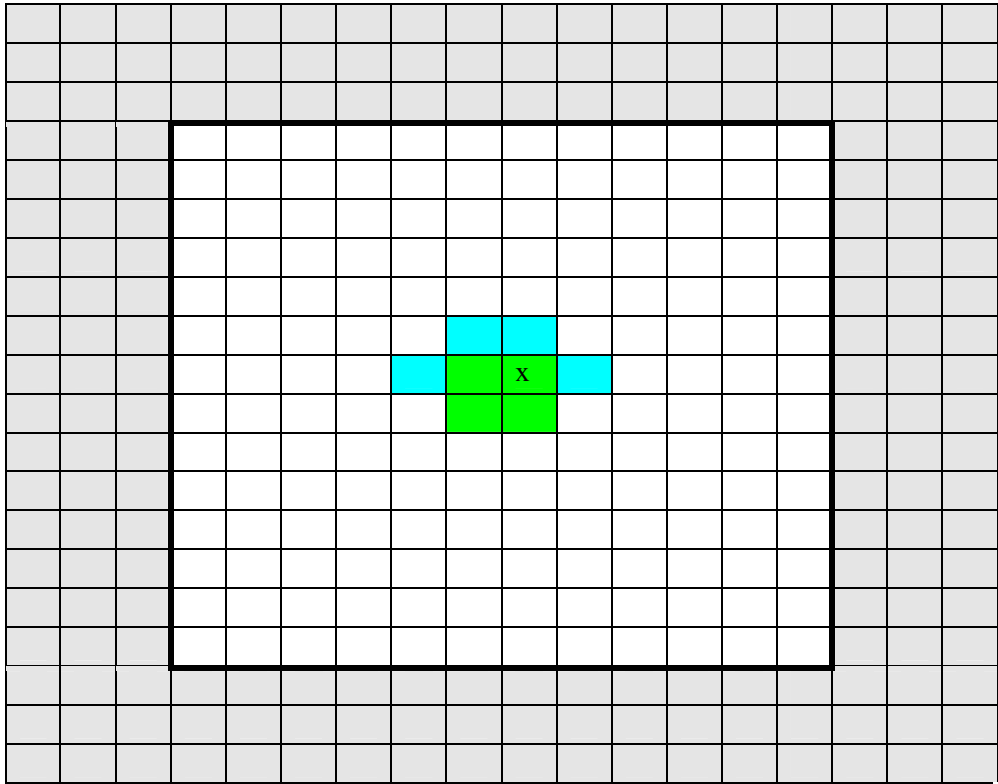


Figure 6. Site A 4mx4m detection map 12 months after crop harvest.

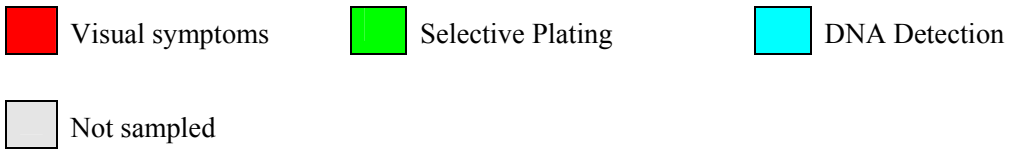
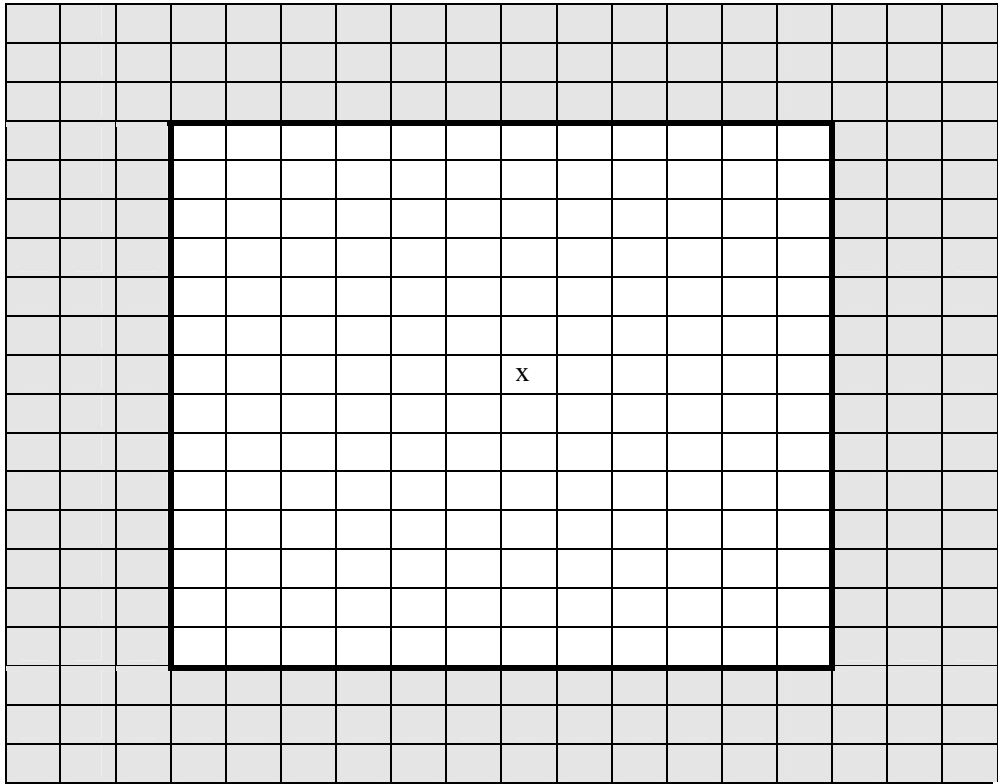
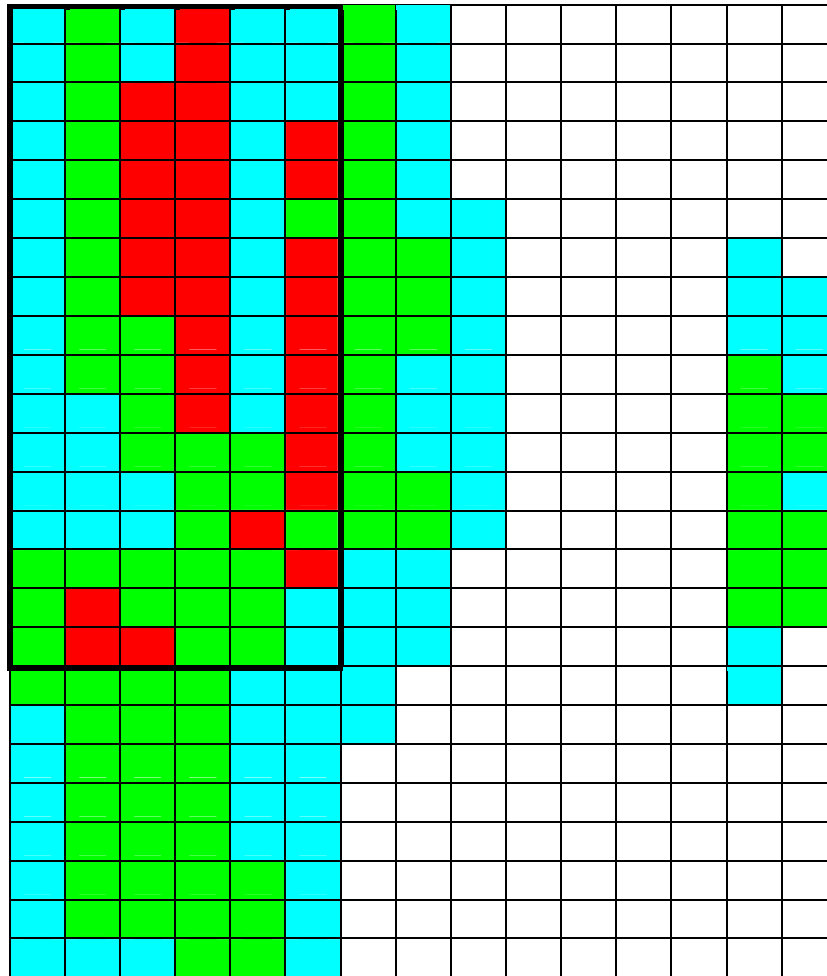


Figure 7. Site A 4mx4m detection map 18 months after crop harvest.

Site B

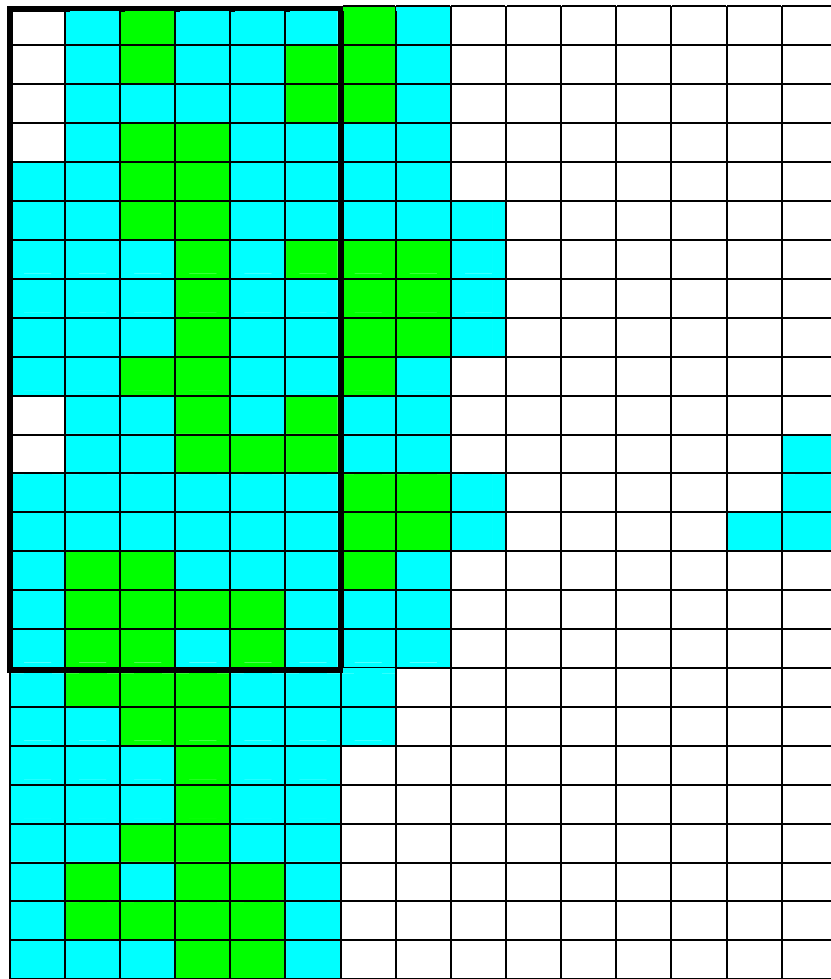
Site B was a section of crop partially harvested 2 weeks prior to the first sampling. At harvest it became evident to the grower that the crop was heavily infected with bacterial wilt, so part of the crop was left to rot (indicated by bold border on Figure 8 –10). As such, visual symptoms could only be recorded for a portion of the crop. Visual symptoms (Fig. 8) appeared to correlate with natural depressions in the paddock where water could accumulate. In fact, the west side of the sampling grid was characterised by a paddock-length depression, with other smaller depressions dotted throughout the crop (only one of which fell within the sampling grid, on the east side). SMSA-positive and PCR positive soil test results surrounded the visually positive plants and extended the full length of the western side of the grid. Another smaller SMSA- and PCR-positive test area was found on the east side of the grid, which coincided with the topographic depression mentioned earlier.

Again, over time (Figs 9 and 10), both the SMSA- and PCR-positive areas decreased in size. Unfortunately, this paddock was on a short-term lease that ended before the sampling period could determine the time-end-point for *R. solanacearum* persistence.



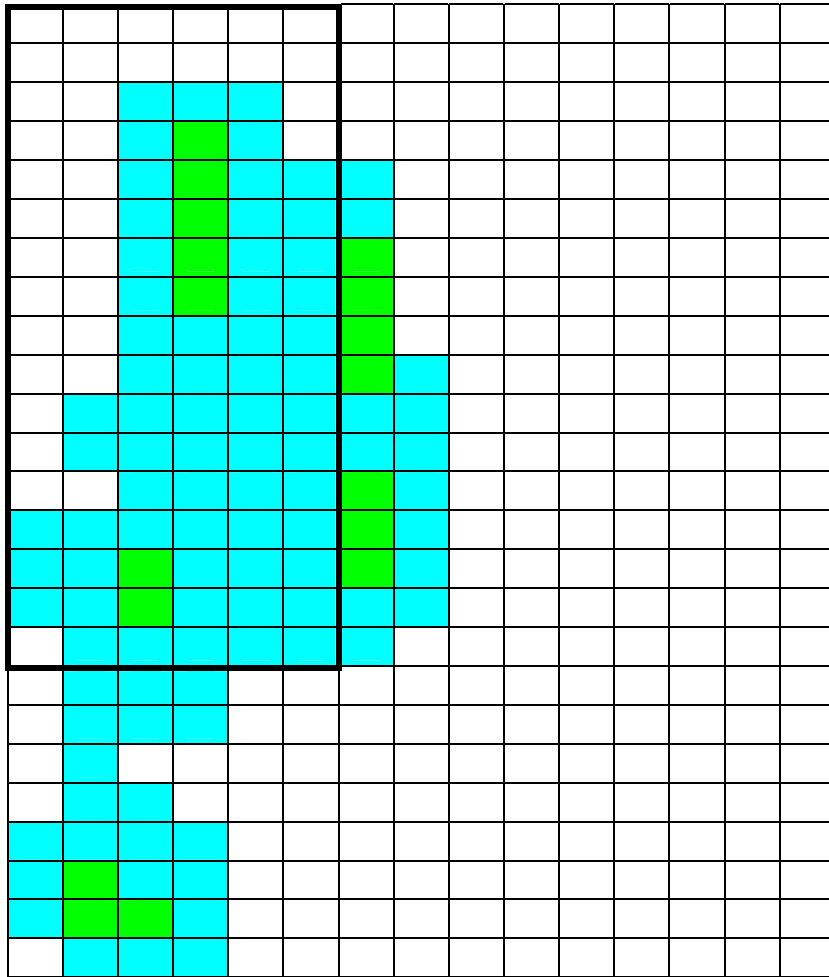
Visual symptoms
 Selective Plating
 DNA Detection

Figure 8. Site B 4m x 4m detection map 2 weeks after crop harvest (bold highlighted area was not harvested).



Visual symptoms
 Selective Plating
 DNA Detection

Figure 9. Site B 4mx4m detection map 6 months after crop harvest (bold highlighted area was not harvested).



Visual symptoms
 Selective Plating
 DNA Detection

Figure 10. Site B 4mx4m detection map 12 months after crop harvest (bold highlighted area was not harvested).

Site C

Site C was first sampled twelve months after crop harvest, on an 8m x 8m grid. According to the grower, the crop was heavily infected with bacterial wilt approximately 30 metres from the eastern edge of the sampling grid. Infection apparently ran the length of the paddock from North to South and coincided with the path of the irrigating equipment. This site was sampled during the early stages of the project and initially only SMSA-isolation was available (i.e. at first sampling). SMSA-isolation indicated the presence of *R. solanacearum* at several points (Fig. 11) along the irrigator tracks, as indicated by the grower. Further samples were taken 18 months after harvest, including a 4m x 4m sampling grid surrounding the previous detections (Fig. 12). On this occasion samples were tested both with SMSA and PCR, but no positives were found.

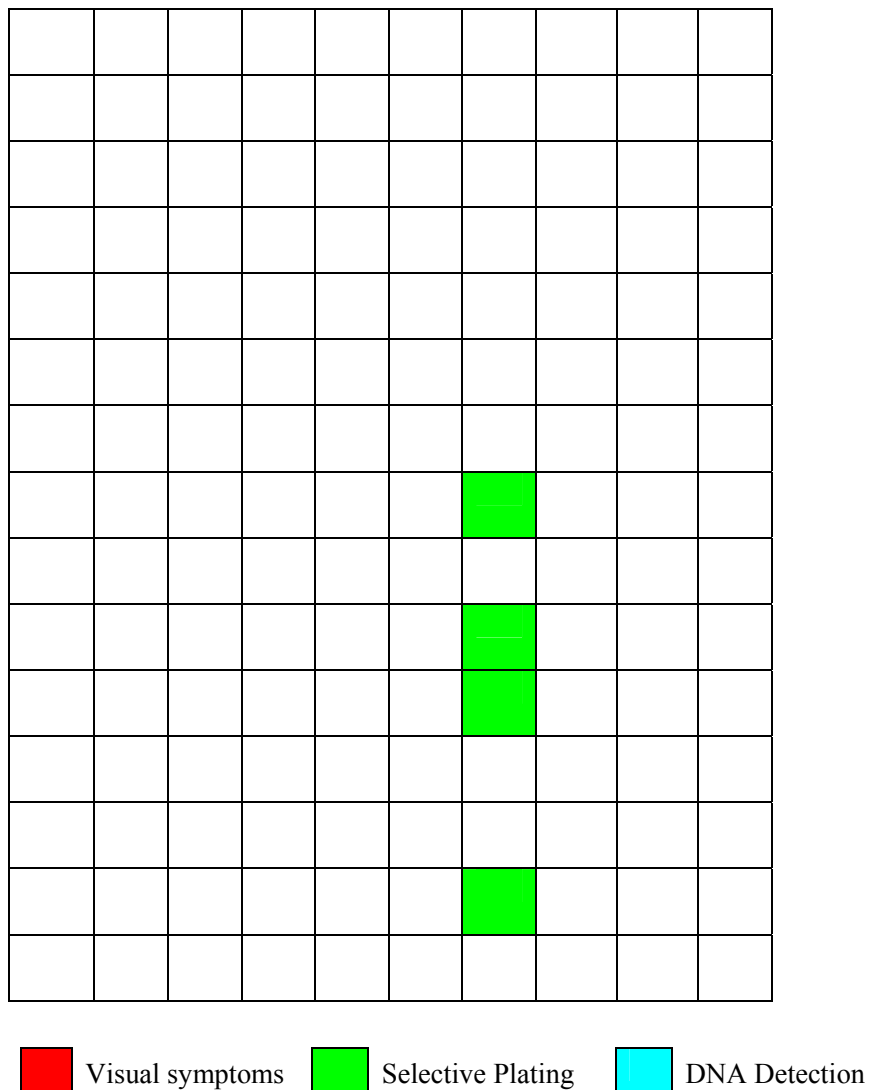


Fig. 11. Site C 8mx8m detection map 12 months after crop harvest.

Site C. Detection map 18 months after crop harvest.

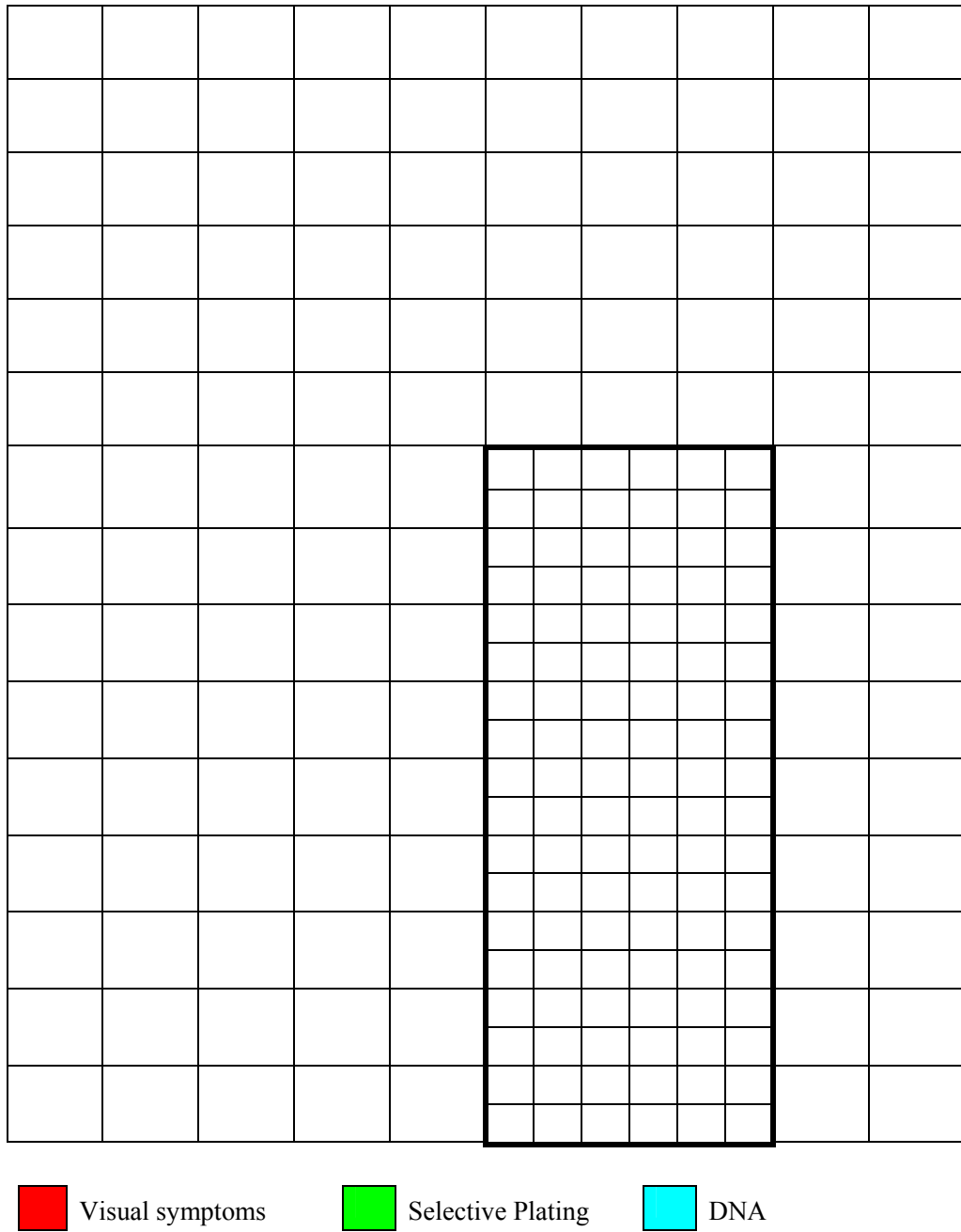


Fig. 12. Site C 8mx8m detection map 18 months after crop harvest (with embedded 4mx4m grid surrounding area of previous positive test results).

Site D

Site D was sampled 2 years after harvest. According to the grower at this property, the crop was heavily infected with bacterial wilt only where irrigation from the two adjacent paddocks overlapped, on the east side of the boundary fence. The site was tested using only SMSA but no positives were found (Fig. 13).

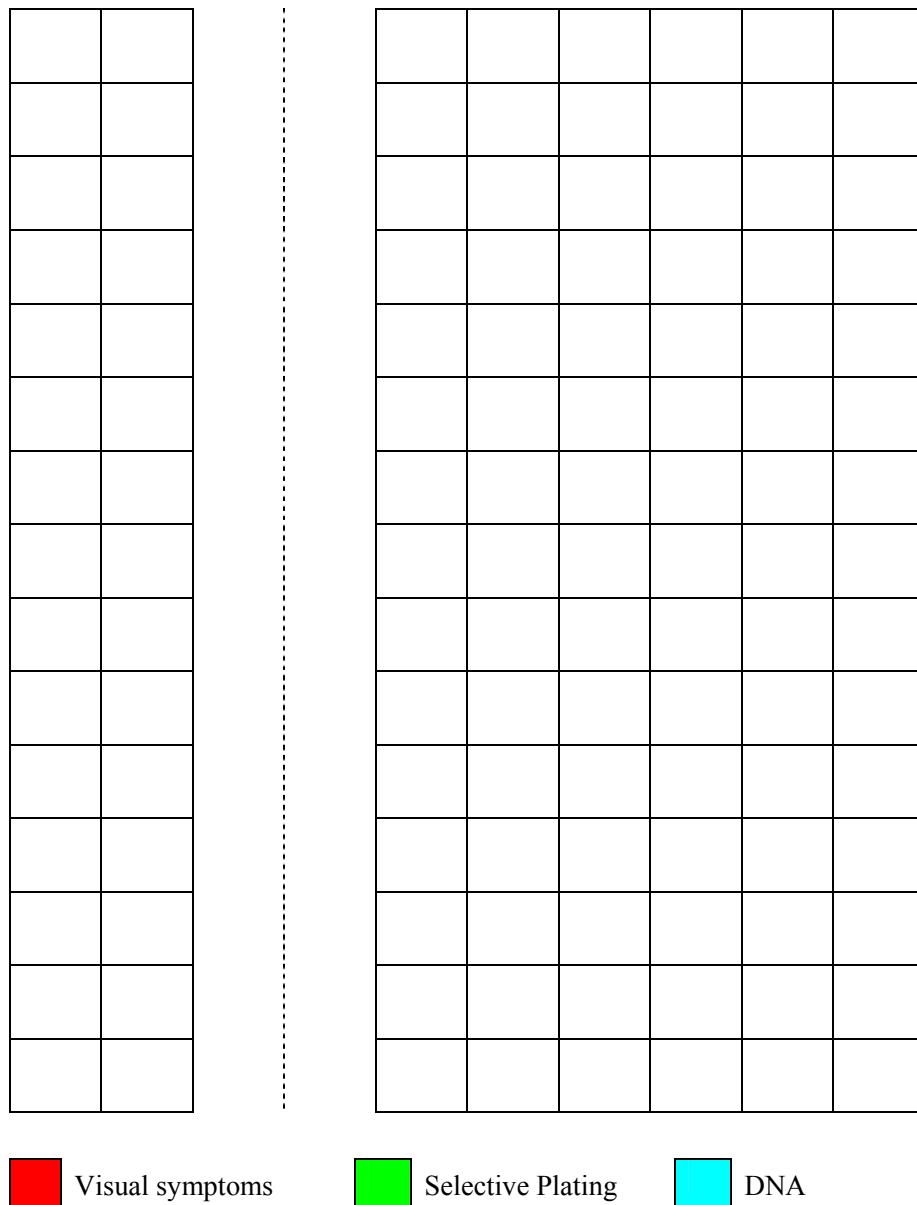
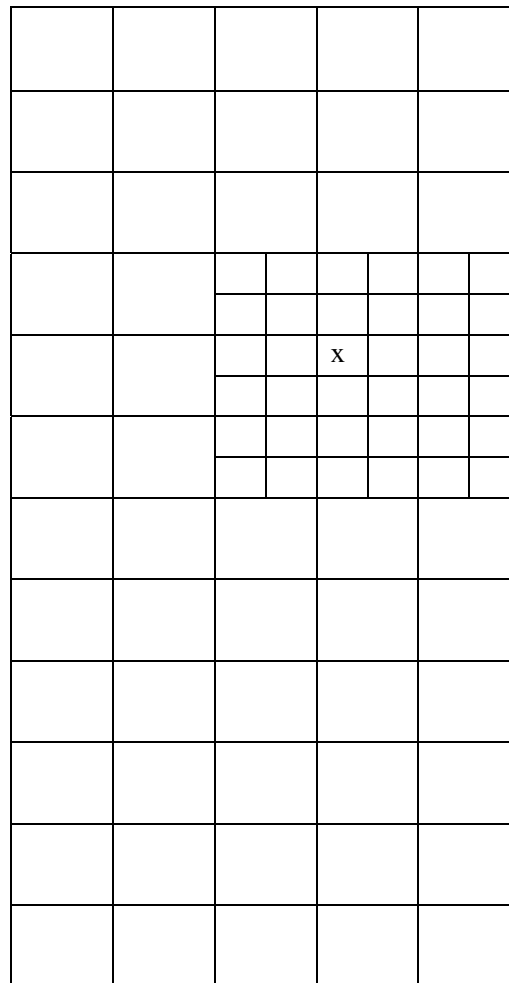


Figure 13. Site D 8mx8m detection map 2 years after crop harvest. Grid spans two properties which are separated by a boundary fence (dashed north-south line).

Site E

Site E was sampled 4 years after crop harvest. According to the grower the crop had been heavily infested in a small area immediately surrounding an irrigation hydrant, with minor spot infection dotted throughout the crop. Soil samples were tested with both SMSA and PCR but no positives were found.






 Visual symptoms  Selective Plating  DNA

Figure 14. Site E 8m x 8m detection map 4 years after crop harvest (with embedded 4m x 4m sampling grid around suspected infection site and 'x' marks hydrant position).

Water Sampling

Three positive water samples were recovered from a local watercourse. One positive sample was recovered in January 2003 and two positive samples in March 2003.

Discussion

Although essential in validating PCR results, the use of selective media was problematic. The results of isolations on SMSA media from field-collected soil were initially difficult to interpret because of an abundance of *R. solanacearum*-like colonies. For instance, it was not always a straightforward task to determine whether a colony should be classified as sufficiently 'fluidal' or whether the characteristic 'blood-red whorl' constituted more of the colony area than was appropriate. As a result, many of these suspect colonies were found not to be *R. solanacearum* when examined further with PCR.

However, with experience, the accuracy with which colonies were correctly classified as *R. solanacearum* correctly increased. A level of confidence in the technique could be achieved such that confirmatory PCR tests were carried out only occasionally, and as a matter of procedure rather than necessity.

Another problem was the abundance of colonies of any type, not just the *R. solanacearum*-like colonies. SMSA is only semi-selective and it was quickly evident that dozens of species could be isolated from soil using this media. In some cases unknown species covered the plates in 48 hours, possibly masking the presence of the slow-growing *R. solanacearum*. This was overcome by plating out dilution series of each soil extract, in order to provide better colony separation and therefore increase the probability of visible *R. solanacearum* growth.

Other disadvantages of the soil-isolation method as a monitoring tool were the time required to obtain a result, the laboratory space required to process samples and incubate plates and the cost of reagents. Generally, plates could only be assessed after 4-5 days. When additional dilutions had to be analysed or colony-identity confirmed with PCR, it could take up to 2 weeks to obtain a result. Logistical problems were also a concern. In order to ensure maximum consistency between tests, it was important to process soil as soon after collection as possible, and also conduct the isolation in a timely manner. Since bacteria cannot be easily encouraged to stop growing, there were no steps in the procedure that could be delayed or stored for continuation later. And although soil-solutions were kept at 4°C for occasions where additional isolations had to be repeated, there is the possibility of a decrease in *R. solanacearum* viability or an increase in the numbers of contaminating rhizosphere bacteria. The resultant build-up of test plates therefore required technically efficient laboratory staff and considerable incubator space.

The soil-isolation procedure was also quite expensive to carry out because of the high cost of antibiotics and other substrates. *R. solanacearum* grew in 3-5 days on Bacto-agar, which is expensive, compared to 5-7 days on the cheaper Oxoid agar.

PCR on the other hand was a relatively simple and straightforward procedure that gave unambiguous results. DNA could be extracted from soil extracts to capture the population structure within the sample at a particular point in time. The DNA could then be frozen and stored for long periods without compromising the results of future testing. It is also possible to limit the amount of user input during all stages of the PCR testing procedure by introducing automation. DNA extraction, PCR set-up and PCR product analysis can all be carried out by laboratory robotics equipment. In the latter stages of this study, real-time PCR was evaluated as a method of automating the product analysis stages of sample testing. PCR reactions were simply carried out in a Corbett RotorGene real-time PCR instrument with the addition of SYBR-Green to the reaction mix. Because the chosen protocols generate a single product with no non-specific amplification, they could be used with the non-specific reporter dye, SYBR-Green, which binds to double stranded DNA and fluoresces. In the real-time format, both protocols detected product

in samples known to be positive and did not detect product in samples known to be negative. Automated PCR set-up was also trialed on Corbett Research CAS1200 robotic equipment, but time would only be saved when processing large numbers of samples. The other advantage of automating the PCR procedure is that user-error is eliminated and labour costs are decreased due to minimal manual handling.

An alternative, propriety DNA extraction procedure from SARDI was also used on soil from site A. The results from PCR using DNA from pre-enriched soil solutions and DNA extracted directly from soil by SARDI were very similar. The only difference was in the intensity of PCR product generated, where the SARDI DNA tended to give marginally stronger bands at the lower limits of detection.

It must be assumed that under certain situations the large sample size processed by SARDI produces a more representative DNA sample. This would be a significant advantage over other methods, particularly when dealing with obligate pathogens such as PCN. In this case however, it appears that the ability to multiply *R. solanacearum* prior to testing obviates the need for large sample sizes.

On the other hand, a disadvantage of using the SARDI method is the commercially confidential nature of the procedure. If a sample produces a false-negative result because the extraction procedure fails, there is no way to trace the problem or be sure that measures have been put in place to prevent a repeat failure.

Field Surveys and Sampling

The results of the field survey at sites A and B indicate that of the monitoring protocols used, PCR-based detection was the most sensitive. The detection maps reveal that the area of soil contamination with *R. solanacearum* extends well beyond the limit of visual symptoms. This is at odds with the results of detection sensitivity in spiked soil, where both PCR and SMSA-plating could detect as few as 10^2 CFU per gram soil. However, It has been reported that reliable isolation of *R. solanacearum* on SMSA from field soils in Nepal only occurred when the pathogen population exceeded that of saprophytic soil bacteria (Pradhanang *et al.*, 2000). The authors also report similar results in SMSA broth that artificially inoculated with both *R. solanacearum* and an unidentified soil saprophyte, where excessive overgrowth of the saprophyte masked detection of the pathogen. Another possible explanation is that the PCR is detecting non-viable cells or viable-but-non-culturable cells, which would not be isolated on SMSA but would return positive PCR results.

The distribution of pathogen populations in soil, as well as anecdotal evidence from growers, indicated that *R. solanacearum* could often be found wherever soil moisture was high, such as immediately around irrigation hydrants or depressions in the landscape where water collects. EU directives recommend that soil sampling for *R. solanacearum* should follow standard nematode regimes (i.e. 4m x 5m grids), but this recommendation comes with the proviso that generally, soil detection is not sufficiently sensitive to detect low populations or irregularly dispersed populations. It would therefore seem sensible to target sampling to the parts of the environment that the pathogen likes to inhabit, such as moist soil. This would decrease the likelihood of false negatives, particularly in soil where the pathogen is not established.

The results that could be garnered from the few infected sites identified during this study also indicate that populations of *R. solanacearum*, decline over time and fall below detectable levels

somewhere between 12 months and 24 months after initial infection of a crop. This agrees with other studies, which generally report that *R. solanacearum* persists in the environment for up to 18 months (for example, Pradhanang, 1998). This is by no means a definitive time-frame, since the pathogen's survival in the environment is one of the few aspects of its biology that are not well understood. Persistence in the absence of a host could be due to the bacterium surviving amongst the debris of infected plants or with symptomless carrier plants. For instance, studies in New South Wales (Graham & Lloyd, 1979; Graham *et al.*, 1979) demonstrated that *R. solanacearum* can survive between successive seasonal potato crops, or for up to 2 years, primarily in host debris and latently infected volunteer potatoes, but also in free soil and in the deeper layers of soil. Also, in Europe, contamination of watercourses used for irrigation was occurring through the release of *R. solanacearum* from the roots of latently-infected *Solanum dulcamara* (Elphinstone *et al.*, 1998).

Another possibility is that the bacterium enters a dormancy state that has been termed 'viable-but-not-culturable' (Grey and Steck, 2001), which may enable it to survive for extended periods without a host.

Local Watercourses

River water in Europe is often the source of *R. solanacearum* contamination, through irrigation. The weed host of *R. solanacearum*, *S. dulcamara* grows on riverbanks and its roots trail into watercourses, where they can release contaminating bacterial cells. Campaigns to eliminate the weed in Scotland and Sweden have eradicated *R. solanacearum* from local potato growing regions. In Sweden, potato crops being irrigated with river water became infected with bacterial wilt over several seasons (Olsson, 1976). It was later found that *R. solanacearum* was overwintering in *S. dulcamara*, which was growing upstream of potato production areas. *R. solanacearum* was later eradicated in Sweden by removing the weed host (Persson, 1998).

In Scotland, the eradication of *R. solanacearum* from the Tay river system in Perthshire began in 2001 (BPC Potato Newsletter, December 2001). This has included a three-year ban on irrigation from affected rivers while removal of *S. dulcamara* takes place.

Although *S. dulcamara* does not occur in Australia (except in Tasmania, which is the only state where bacterial wilt has not been reported), its story provides encouragement that bacterial wilt could be eradicated from the Koo Wee Rup swamp if the source of contamination could be identified. Given that the pathogen has been a problem in Koo Wee Rup for nearly a century, and that it is unlikely to persist in soil for more than 2 years, it could be surviving on an alternative host.

A range of other possibilities also exist, such as contaminated wash-water from upstream potato washing plants, infected volunteer potatoes, which can be found on the river banks and contaminated run-off from infected crops. In this case it is impossible to identify a source for the contamination, but a further complicating factor was that during the sampling period, local watercourses were undergoing major construction works to straighten and widen banks. The disruption to the waterways course and associated works could have temporarily introduced the pathogen into the water by many routes.

Still, bacterial wilt seems to be consistently associated with growers who irrigate from the local watercourses, so it is necessary to expand the search for alternative hosts and sources of contamination into the water.

Part 4 – Concluding Remarks

The results of this study indicate that PCR-detection of *R. solanacearum* for the purposes of monitoring field infections is a better option than traditional methods. However, it would be unwise to rely solely on PCR when the results are being used to inform quarantine decisions, as it is impossible to be certain of the specificity of a primer set. For instance, most of the primers tested in this study that claimed specificity for race 3/biovar 2, also amplified DNA from the Blood Disease Bacterium. Although it can be argued that the likelihood of encountering this organism is negligible, the point remains that 100% confidence in PCR primers is not possible. Standard testing protocols should therefore include two different PCR protocols, as well as other tests such as SMSA-isolation. The distinction should also be made between ‘presumptive’ testing and diagnostic testing. Visual symptoms give a presumptive diagnosis, because bacterial wilt symptoms look very similar to ring rot symptoms (*Clavibacter michiganensis* subsp. *Sepidonicus* (which is not present in Australia), and therefore further testing is required.

Also, although the focus of this study was *R. solanacearum* race 3/biovar 2, new and potentially important strains of the pathogen continue to be reported, such as those causing disease on Eucalyptus, casuarina and mulberry (Hayward, 1985; He, 1985; Dianese *et al.*, 1990). It would therefore be wise to consider the inclusion of a generic *R. solanacearum* test in any widespread surveillance protocol or standard testing protocol, to maximise the chances of intercepting new exotic strains.

In the event of an incursion, the suite of tests trialed in this study would provide the means to confirm the identity of a *R. solanacearum* strain and assess its pathogenicity, map its distribution in the environment and then monitor its survival over time. Further mapping of the distribution of this pathogen may lead to a clearer understanding of infection sources in Koo Wee Rup. Eradication of the pathogen in the Koo Wee Rup region is a possibility given the experience of European nations, and would require the elimination of all sources of contamination followed by rotation with a non-host species for more than 2 years.

References

- Boudazin, G., A.C. Le Roux, K. Josi, P. Labarre and B. Jouan. 1999. Design of division specific primers of *Ralstonia solanacearum* and application to the identification of European isolates. *European Journal of Plant Pathology* 105; 373-380.
- Buddenhagen, I., Sequeira, L. and Kelman, A. 1962. Designation of races in *Pseudomonas solanacearum*. *Phytopathology*, 52:726
- Cook, D., Barlow, E. and Sequeira, L. 1989. Genetic diversity of *Pseudomonas solanacearum*: detection of restriction fragment length polymorphisms with DNA probes that specify virulence and the hypersensitive response. *Mol. Plant-Microbe Interact.*, 2:113-121.
- Cook, D., Barlow, E. and Sequeira, L. 1991. DNA probes as tools for the study of host-pathogen evolution: the example of *Pseudomonas solanacearum*. In: Henneke, H. and Verma, D. P. S. (eds) *Advances in Molecular Genetics of Plant-Microbe Interactions*. Vol I (pp 103-108). Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Cook, D. and Sequeira, L. 1994. Strain differentiation of *Pseudomonas solanacearum* by molecular genetics methods. In Hayward A. C. and Hartman, G. L. (eds) *Bacterial Wilt: The disease and its causative agent, Pseudomonas solanacearum*. (pp 77-93). CAB International, Wallingford, United Kingdom.
- Dianese, J. C., Dristig, M. C. G. and Cruz, A. P. 1990. Susceptibility to wilt associated with *Pseudomonas solanacearum* among six species of *Eucalyptus* growing in equatorial Brazil. *Australasian Plant Pathology*, 19: 71-76.
- Elphinstone, J. G., Hennessy, J., Wilson, J. K. and Stead, D. 1996. Sensitivity of different methods for the detection of *Ralstonia solanacearum* in potato tuber extracts. *Bulletin OEPP/EPPO* Bulletin, 26:663-678.
- Engelbrecht, M. C. 1994. Modification of a selective medium for the isolation and quantification of *Pseudomonas solanacearum*. *ACIAR Bacterial Newsletter*, 10:3-5.
- Graham, J., Jones, D. A. and Lloyd, A. B. 1979. Survival of *Pseudomonas solanacearum* race 3 in plant debris and in latently infected potato tubers. *Phytopathol*, 69:1100-1103.
- Graham, J. and Lloyd, A. B. 1979. Survival of potato strain (race 3) of *Pseudomonas solanacearum* in the deeper soil layers. *Aus J Agric Res*, 30:489-496.
- Grey, B. E. and Steck, T. R. 2001. The viable but nonculturable state of *Ralstonia solanacearum* may be involved in long-term survival and plant infection. *Appl. Environ. Microbiol.* 67: 3866-3872.
- Hayward, A. C. 1964. Characteristics of *Pseudomonas solanacearum*. *J Appl Bacteriol*, 27:265-277.
- Hayward, A. C. 1985. Bacterial wilt caused by *Pseudomonas solanacearum* in Asia and Australia: an overview. In: Persley G. J., ed. *Bacterial Wilt Diseases in Asia and the South Pacific*. Canberra, Australia: ACIAR Proceedings No. 13, 15-24.

- Hayward, A. C. 1991. Biology and epidemiology of a bacterial wilt caused by *Pseudomonas solanacearum*. *Ann. Rev. Phytopath.* 29: 65-87.
- Hayward, A. C., El-Nashaar, H. M., Nydegger, U. and De Lindo, L. 1990. Variation in nitrate metabolism in biovars of *Pseudomonas solanacearum*. *J Appl. Bacteriol.*, 69:269-280.
- He, L. Y. 1985. Bacterial wilt in the Peoples Republic of China. In: Persley G. J., ed. *Bacterial Wilt Diseases in Asia and the South Pacific*. Canberra, Australia: ACIAR Proceedings No. 13, 40-48.
- He, L. Y., Sequeira, L. and Kelman, A. 1983. Characteristics of strains of *Pseudomonas solanacearum*. *Plant Dis.*, 67:1357-1361.
- Lee, Y.- A. and Wang, C.- C. 2000. The design of specific primers for the detection of *Ralstonia solanacearum* in soil samples by polymerase chain reaction. *Bot. Bull. Acad. Sin.*, 41:121-128.
- Li, X., Dorsch, M., Del Dot, T., Sly, L. I., Stackebrandt, E. and Hayward, A. C. 1993. Phylogenetic studies of the rRNA group II pseudomonads based on 16S rRNA gene sequences, *J Appl Bacteriol*, 74:324-329
- McCarter, S. M., Dukes, P. D. and Jaworski, C. A. 1969. Vertical distribution of *Pseudomonas solanacearum* in several soils. *Phytopathology*, 59: 1675-1677.
- Opina, N., F. Tavner, G. Holloway, J.-F Wang, T.-H Li, R. Maghirang, M. Fegan, A. C. Hayward, V. Krishnapillai, W.F. Hong, B.W. Holloway, J.N.Timmis. 1997. A novel method for development of species and strain-specific DNA probes and PCR primers for identifying *Burkholderia solanacearum* (formerly *Pseudomonas solanacearum*). *As Pac. J. Mol. Biol. Biotechnol.* 5; 19-33.
- Pastrik, K.H. and Maiss, E. 2000. Detection of *Ralstonia solanacearum* in potato tubers by polymerase chain reaction. *J. Phytopathology* 148; 619-626.
- Pastrik, K. H., Elphinstone, J.G., Pukall, R. 2001. Differentiation and identification of *Ralstonia solanacearum* biovars by RAPD-PCR and analysis of amplified 16S-23S ribosomal intergenic spacer region. *Applied and Environmental Microbiology*
- Pastrik, K. -H., Elphinstone, J. G. Pukall, R. 2002. Sequence Analysis and Detection of *Ralstonia solanacearum* by Multiplex PCR Amplification of 16S-23S Ribosomal Intergenic Spacer Region with Internal Positive Control. *European Journal of Plant Pathology*, 108: 831 - 842
- Pegg, K. and Moffett, M. 1971. Host range of the ginger strain of *Pseudomonas solanacearum* in Queensland. *Aust J Exp Agric Anim Husb*, 11:696-698.
- Persson, P. 1998. Successful eradication of *Ralstonia solanacearum* from Sweden. *Bulletin OEPP/EPPO*, 28:113-119.
- Pradhanang, P. M. 1998. Bacterial wilt of potato caused by *Ralstonia solanacearum* biovar 2: a study of the ecology and taxonomy of the pathogen in Nepal. PhD Thesis, University of Reading, United Kingdom.

Pradhanang, P. M., Elphinstone, J. G. and Fox, R. T. V. 2000. Sensitive detection of *Ralstonia solanacearum* in soil: a comparison of different detection techniques. *Plant Pathology*, 49:414-422

Schell, M. A. 2000. Control of virulence and pathogenicity genes of *Ralstonia solanacearum* by an elaborate sensory network. *Annu. Rev. Phytopathol*, 38:263-292.

Seal, S.E., L.A. Jackson, J.P.W.Young, and M.J.Daniels. 1993. Detection of *Pseudomonas solanacearum*, *Pseudomonas syzygii*, *Pseudomonas pickettii* and Blood Disease Bacterium by partial 16S rRNA sequencing: construction of oligonucleotide primers for sensitive detection by polymerase chain reaction. *J. Gen. Microbiol.* 139: 1587-1594.

Taghavi, M., Hayward, A. C., Sly, L. I. And Fegan, M. 1996. Analysis of the phylogenetic relationships of strains of *Burkholderia solanacearum*, *Pseudomonas syzygii*, and the Blood Disease Bacterium of banana based on 16S rRNA gene sequences. *Int J Syst Bacteriol*, 46:10-15.

Yabuuchi, E., Kosako, Y., Yano, I., Hotta, H. and Nishiuchi, Y. 1995. Transfer of two *Burkholderia* and an *Alcaligenes* species to *Ralstonia* gen. Nov.: Proposal of *Ralstonia pickettii* (Ralston, Palleroni and Doudoroff 1973) comb. Nov., *Ralstonia solanacearum* (Smith 1986) comb. Nov. and *Ralstonia eutropha* (Davis 1969) comb. Nov. *Microbiol. Immunol*, 39:897-904.

Section 3.

Potato Cyst Nematode (PCN)

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Summary

- A quantitative detection system was developed, based on conventional and real-time PCR methodology with Taqman chemistry. The new technique enables the detection of the species of PCN in soil and the relative numbers of eggs present in the sample. This relationship has been established for both *Globodera rostochiensis* and *G. pallida* and will enable sensitive detection for quarantine purposes.
- Using the new quantitative DNA tools, the relationship between initial level of PCN infection (eggs /ml) and the host response, expressed as changes in tuber weight, was established. This will enable pre-planting disease prediction and therefore assist growers to make production and disease management decisions.
- A highly sophisticated mathematical and computer model was established through a collaborative link with Plant Research International (the Netherlands). The model enabled the assignment of percentage detection probability for all common sampling regimes. Using these data and interactive models, the efficiency of sampling regimes was assessed and the best options identified for the Australian and New Zealand potato industries. The optimisation of sampling regimes will enhance PCN management and phytosanitary declarations.
- Considerable discussion has taken place between VegFed and MAF policy in New Zealand, as well as with the designated organisations contracted to carry out regulatory inspections. A set of draft regulations are being developed in New Zealand that will allow for DNA based detection systems to be an acceptable method for declaration of assurance of freedom from PCN. Similar discussions are occurring in Australia, particularly surrounding the introduction of DNA-based testing to help manage the new PCN outbreak in Koo Wee Rup. In fact, the DNA-based tests developed in this study provided the only means of rapid species differentiation during the recent outbreak of PCN in Victoria, Australia, whereas traditional methods failed.

Introduction

New Zealand regulations require that a crop of potatoes destined for export is inspected for the presence of PCN. This is done by fork sampling of growing crops, at the stage when immature female nematodes protrude from potato roots and are still pale cream in colour. This window of opportunity for sampling and identifying PCN is limited as the females rapidly turn brown and are difficult to see. After a crop has been inspected and declared free of PCN, the potatoes can be exported, provided all the other necessary pre-conditions have been met.

Most countries accept this procedure and the survey system is well established. However, attempts to remove the import prohibition imposed by Korea and Taiwan have not been successful as both countries require additional assurance that inspected crops have not been

subsequently contaminated by chance spread from an undetected infestation in adjacent fields, even if these fields have not been used to grow potatoes. The concept of PCN freedom for cropping districts is also in doubt. At present, there is no other approved method for checking mature crops or bare soil.

In addition, the development of integrated control practices such as crop rotation, effective soil sampling techniques, and the use of resistant cultivars are necessary to ensure that infestations are maintained at as low a level as possible.

This will assist with quality assurance of potato exports, as it will be necessary that export orders can be guaranteed to be free of PCN. If PCN levels are not well managed, it is possible potato production in New Zealand may follow the lead of England and other parts of Europe where PCN is a major factor risking sustainable production.

Effective control of PCN also relies on knowledge of the species and pathotype present in the soil. Physical detection methods are slow and labour intensive as well as sometimes being ineffective and so in recent years molecular biology tools such as Polymerase Chain Reaction (PCR) have been used to examine the PCN species and pathotypes.

PCR has been used to distinguish the two main species of PCN in New Zealand. (Bulman, S. R.; Marshall, J. W. 1997) and were used to locate and speciate populations.

Species-specific qualitative primers

The ribosomal internal transcribed spacer (ITS) region was amplified and sequenced from a number of PCN collections. A low level of sequence variation was found between *Globodera rostochiensis*, *G. pallida*, and a Peruvian PCN collection, but no variation within Australasian collections of species was noted. Polymerase chain reaction (PCR) primers based upon the *G. rostochiensis* - *G. pallida* sequence differences were designed and successfully used to identify mixed PCN species in a single PCR reaction.

Both primers were used as a duplex and could simultaneously identify the species and determine if there was a mixture of the two species.

The need to determine how many nematodes were in sample

Standard PCR detection gives us information on the species present, not the quantity of each species. Fluorescence detection of PCR products using the Perkin-Elmer ABI® 7700 Sequence Detector (TaqMan™), allows for rapid detection and quantification of DNA sequences. This method uses a fluorescent oligonucleotide probe with a 5-prime (5') reporter dye and a downstream 3-prime (3') quencher dye. During PCR, the reporter dye is released and the resultant fluorescence is detected. Relative normalised fluorescence (R_n , (emission intensity of reporter) over (emission intensity of reference)) v. time (PCR cycle number) is plotted to allow real time assessment of the PCR. ΔR_n is the R_n value at any given cycle number after subtracting the baseline R_n setting. The average background fluorescence emission is calculated and the standard deviation derived. Threshold-fluorescence intensity is established at 10 times this standard deviation. Any sample that reaches a fluorescence value exceeding the fluorescence threshold is considered positive and the cycle at which this first occurs is defined as the threshold cycle (C_t). The C_t values are obtained for known quantities of DNA sequence and linear

regression was used to estimate a relationship between Ct value and DNA amount. This allows us to then estimate a quantity for the Ct value of the unknown sample.

The objective of this project was therefore to:

1. Establish a plot trial within a naturally infested paddock to determine the effect of different initial PCN (*Globodera pallida*) population levels on potato yield and multiplication rates of PCN. The effect of resistant potato cultivars on multiplication rates of PCN was also assessed.
2. Develop duplex primers for the quantitative PCR detection of the potato cyst nematodes *G. pallida* and *G. rostochiensis*.

Part 1. Methods – Plot trial and probe development

Plot trial

To ensure a range of initial PCN population levels were included within the main potato plot trial, it was necessary to initially map the existing infestation. A 1.2-hectare research paddock known to contain PCN was intensively sampled using a sampling grid of 4 x 5 metres, where approximately 1 kg of soil was collected from each position. Soil samples were brought back to Crop & Food, Lincoln, for assessment of cyst numbers using standard elutriation procedures. Assessment involved the soil samples being elutriated to recover cysts and then counted under a stereoscopic microscope. From this initial mapping of the infestation, it was possible to establish plots across the full range of initial PCN levels to assess the effect of differing initial population levels on potato yield and PCN multiplication rates ($= Pf/Pi$, where Pf is the final cyst population, determined at crop harvest). Multiplication rates provide a measure of the PCN population dynamics during the potato crop's growing season.

Thirty-two potato plots were established, where each plot consisted of 7 tubers by 5 rows. Soil samples were collected from each plot at the time of planting and analysed in the laboratory to determine the initial (P_i) populations at planting, which ranged from nil to 66 eggs/ml soil. This confirmed that the positioning of the plots for the main trial encompassed a range of initial PCN levels. The cultivar selected for this trial was Ilam Hardy as this particular cultivar is susceptible to PCN. To determine the number of eggs in a sample, the first step involves removing and counting cyst numbers, as described above. The eggs were then released from the cysts by staining with 0.1% w/v new blueR overnight and egg numbers determined in a Doncaster counting cell under a stereoscopic microscope at 30 x magnification.

The entire trial site was managed using standard commercial fertiliser, herbicide, and fungicide programmes.

The plots were later harvested and soil samples collected. To exclude "edge effects", yield assessments for the main trial were based on the middle 5 plants by 3 rows; equivalent to an effective sampling area of 3.4 m² per plot. Tubers were graded into table and seed, and the number and weights of each grade were recorded. Soil samples were collected from all 32 plots and assessed for cyst and egg counts to determine final (P_f) PCN populations.

In addition, map coordinates of each of the 32 potato plots in the main trial were captured using GPS (Global Positioning System) technology to ensure the sites could be accurately found in future.

Development of duplex primers for quantifying PCN

Nematode cysts were ground in Eppendorf tubes, using plastic micro-pestles with 500ul of solution containing 5M guanidine isothiocyanate, 10mM EDTA, 50mM Tris-HCl (pH7.5) and 8% mercaptoethanol. After room temperature incubation for up to 1 hour, the DNA-containing solution was extracted once with equal volumes of phenol and chloroform-isoamyl alcohol (24:1) and once with chloroform-isoamyl alcohol, then precipitated with 0.3M sodium acetate and two volumes of isopropanol. DNA was resuspended in 100ul of H₂O.

Quantitative PCR was performed on the Perkin-Elmer ABI® 7700 Sequence Detector (TaqMan™) using primers PCNTQPr1f and PCNTQPr1r with species specific probes for each of *Globodera pallida* and *G. rostochiensis*.

The primers were designed to amplify the DNA from both *Globodera pallida* and *G. rostochiensis* equally well. The fluorescent probes were designed to be species specific.

All reaction components for quantitative PCR were purchased from Applied Biosystems. For the template, 5µl of DNA was used in a final PCR reaction volume of 25µl. PCR reagents were 1x TaqMan™ universal PCR master mix, 300nM of each primer, 200nM of pallida probe and 100nM of rostochiensis probe. The thermal cycling conditions for the reactions were: a hold step for AmpErase activation at 50°C for 2 min; a second hold step for AmpliTaq Gold activation at 95°C for 10 min; then 40 cycles of a denaturation at 95°C for 15 seconds and an annealing phase at 60°C for 1 minute.

Results and Discussion

Nematode responses - Main trial

The multiplication rates (= Pf/Pi) were approximately x 8 for the range of Pi levels found within the potato plots (Figure 1). To assist with the interpretation of the data, Figure 1 also shows the theoretical x 1 and x 10 multiplication rates. At high Pi levels, a theoretical maximum final population is reached, beyond which a decrease in the population is known to occur, largely because of increasing competition between individuals and decreasing food supplies (Southey 1978; Been et al. 1995). This would be illustrated in Figure 1 as a drop in the multiplication rate below the x 1 line for high Pi levels. Clearly, such conditions did not occur in this plot trial, and, instead, at all Pi levels there was a similar increase in the PCN population.

Potato yields were directly influenced by the PCN infestation. The infestation had a greater impact on the more marketable table grade, with a reduction in tuber numbers and weight being observed. Figure 2 illustrates the substantial decline in table grade yield with increasing Pi. This decline is apparent from Pi levels of 5-eggs/ml soil and greater. It is unlikely that an equilibrium density has been reached, as there appears to be no change in the rate of yield decline. The equilibrium density can be defined as the population at which no further plant response is observed. The highest level of Pi is 66 eggs/ml, at which point yields are approximately 30% of that obtained from the nematode-free plots. Clearly, this is a marked reduction in yield that is likely to affect the marginal profit for a grower.

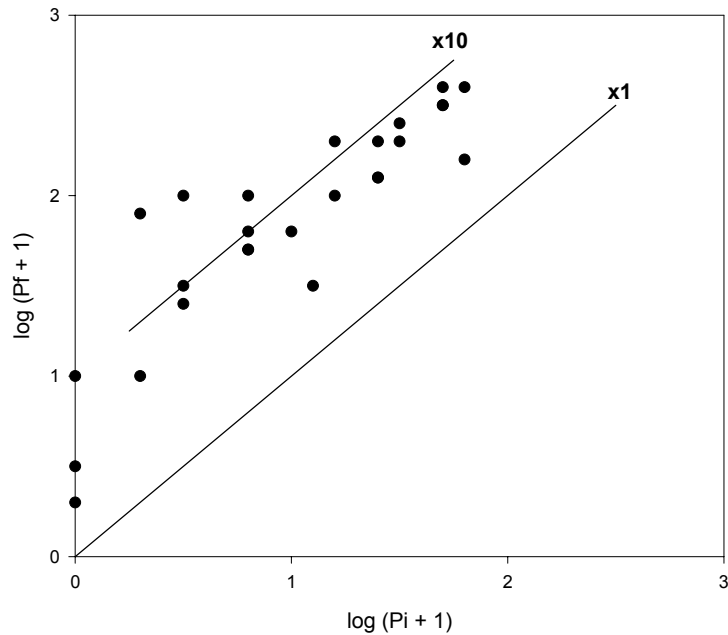


Figure 1. Relationship between the initial PCN population (P_i) and post-harvest populations (P_f), with theoretical x 1 and x 10 multiplication rates also shown.

Results from the main trial clearly illustrate the effect of initial PCN populations on both the final populations and potato yields. To ensure PCN levels remain manageable, it is recommended that crop rotations and effective soil sampling techniques are employed as part of integrated control practices. By taking lightly infested paddocks with no groundkeepers out of potato production for at least 4 years, PCN populations will decline to negligible levels. However, heavily infested paddocks, with groundkeepers present, even at low levels, will require a considerably longer period of time out of potato production before negligible levels of PCN are achieved. Consequently, if potato production is to continue on infested land, it will be necessary to grow more resistant cultivars.

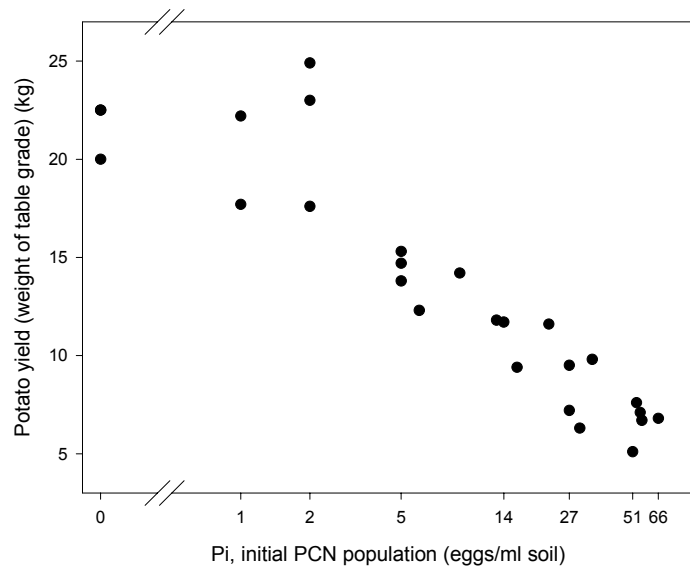


Figure 2. Relationship between yield of Ilam Hardy (weight of table grade) and population density of PCN at planting time.

Soil sampling of paddocks needs to be conducted at such an intensity that the probability of detection is high. Recent Dutch research into soil sampling strategies for PCN detection show that a recommended sampling grid of 5 x 6 m provided a detection probability of 90% (Been & Schomaker 2000). An intensive sampling protocol may be necessary for New Zealand conditions to ensure quality assurance processes satisfy existing and potential export markets. Such sampling procedures in practice may prove to be expensive, therefore the development of simpler sampling strategies, mechanisation of soil sampling techniques or alternative detection methods would be more cost-effective.

Mapping of PCN infestations within a paddock using Global Positioning System (GPS) technology

At the time of potato harvest, the central position of each of the 26 plots used in the main trial was “captured” using a GPS unit. GPS relies on a constellation of 24 satellites that provides worldwide accurate position coordinates. The level of accuracy associated with the GPS coordinates is approximately 1.0 metre. Appendix I contains three maps illustrating the location of the trial site (red markers on Mile Bush Rd, Pukekawa) and the central position of each plot (blue markers). Each potato plot had an effective soil sampling area of approximately 3.4 m² (3 potato rows x 5 potato plants). The GPS unit will allow staff to return to within the effective sampling area of each plot. This will allow the study of PCN infestations within paddocks to be conducted over time with the knowledge that the quality of the data is not being compromised by potential errors in accurately locating field-sampling positions.

Development of duplex primers for quantifying PCN

Various levels of *G. pallida* cysts (100, 75, 50, 25, 10, 5 and 1 cyst) were extracted from spiked soils to obtain the DNA. The resultant DNA was then analysed on the TaqMan™ in triplicate and an amplification plot of the DNA was generated (Figure 3; mean data for each level is presented).

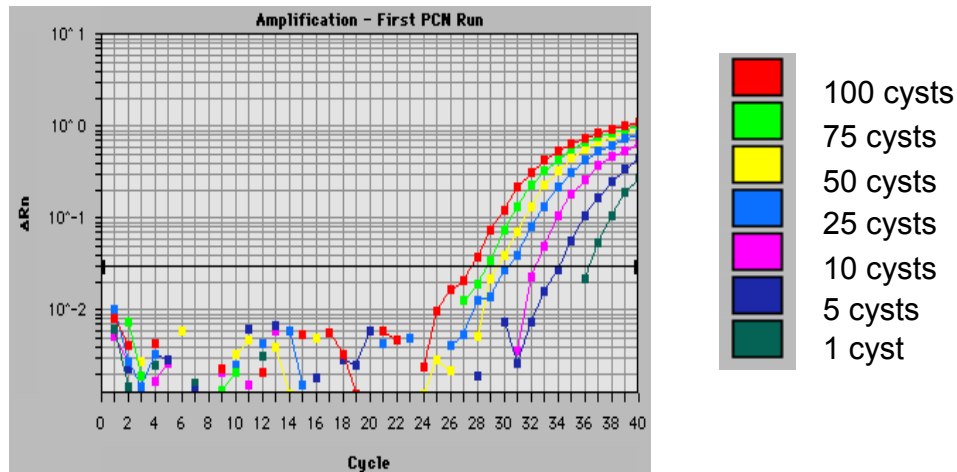


Figure 3: Plot of the fluorescence signal detected as an indication of the amplification of DNA. A low cycle number indicates high concentrations of DNA.

The TaqMan™ Ct value was then plotted against the log number of cysts extracted to allow a regression line to be fitted. The mean data of one run conducted in triplicate is presented in Figure 4. This produced an equation to estimate the relationship between the Ct value and the amount of cysts. This relationship can then be used to estimate the number of cysts contained in an unknown sample. The Ct values of egg content in cysts relating to field populations were determined. This involved counting all the cysts in a given soil sample, taking a portion of the cysts and counting the eggs contained, extracting DNA from remaining cysts and using the eggs contained in a portion of the cysts to obtain the number of eggs contained in extracted cysts. The DNA was put through the TaqMan™ as before and Ct values were subsequently related to the egg counts obtained. A weak relationship was identified between egg content, cyst numbers and Ct values.

The variability in the relationship between Ct values and egg count was caused by large uncontrolled variability in the number of eggs per cyst between samples. Resolution of this problem is ongoing and forms one of the primary objectives in future funded studies.

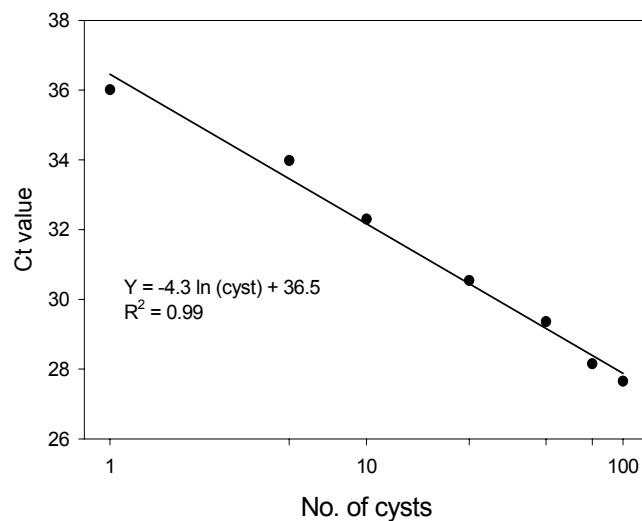


Figure 4. Relationship between Ct value from TaqMan™ and cyst numbers (mean of one run in triplicate). Fitted line with regression equation is shown.

This method of detection has been successful for the individual species of *G. pallida* and *G. rostochiensis*, with a corresponding minimum detection level of 1 cyst per 100g of soil sample.

These primers designed to discriminate between *G. rostochiensis* and *G. pallida* were found to produce some cross fluorescence signal when duplexed, which gave unexpected levels of cysts from both species when in a mixture, therefore the design of the probes and placement on the sequence was re-investigated.

New probes were designed and checked for sequence specificity by comparing for homology to all known sequences in the Genbank database using a BlastN search. This showed that the probes designed would possibly pick up some other species as well, namely *Punctodera punctata* and *Globodera tabacum*. The sequences for these two species of nematode were down-loaded from Genbank and compared with *G. rostochiensis* and *G. pallida* and an area of differences between all four investigated as the site to re-design the probes. Primers were redesigned as well to allow discrimination between all four species. Forward primers were designed to be species specific, reverse primer to amplify the DNA from both *pallida* and *rostochiensis* equally well. The fluorescent probes were designed to be species specific.

G.pa forward primer	5'-confidential -3'
G.ro forward primer	5'-confidential -3'
PCN reverse primer	5'-confidential -3'
G.pa MGB probe	5'-confidential -3'
G.ro MGB probe	5'-confidential -3'

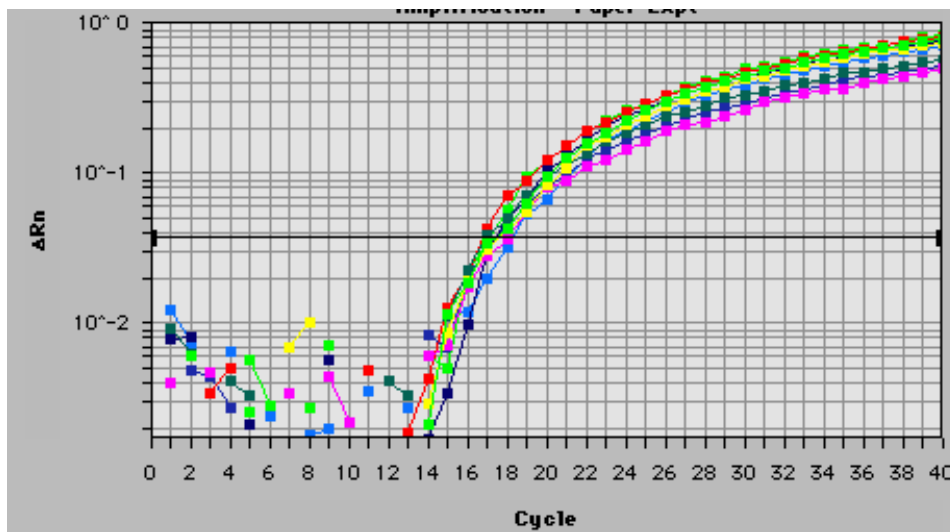


Figure 5: Amplification plot for *rostochiensis* with / without *pallida*. There is no interference from *pallida*.

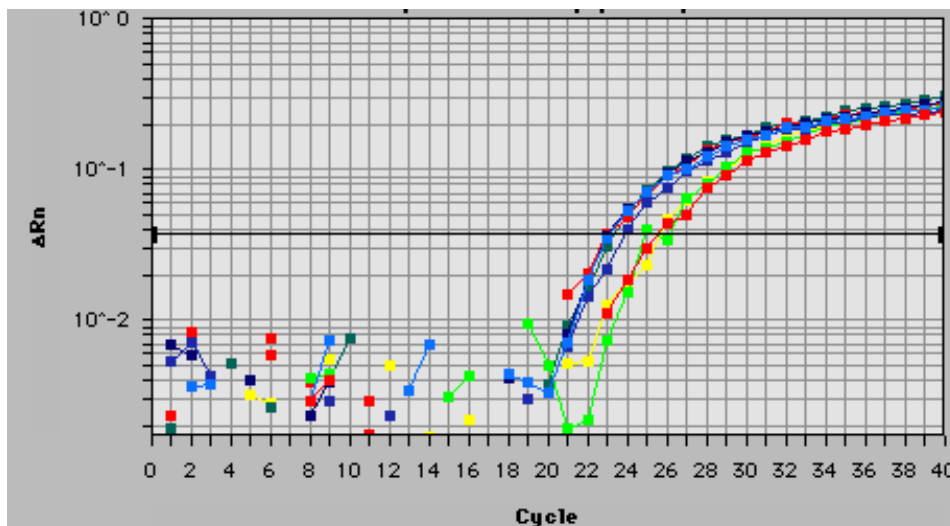


Figure 6: Amplification plot for *pallida* with / without *rostochiensis*. *Rostochiensis* is interfering and causing an additive effect to the *pallida* signal.

The *rostochiensis* primers and probe are specific for *rostochiensis* and are not picking up any *pallida* (Fig 5). The *pallida* primers and/or probe are not as specific and in mixture are picking up *rostochiensis* as *pallida* (Fig 6). High levels of one species does interfere with detection of the other species if at low levels. When both species are at the same level, the detection is on target with expectations.

Primer limiting is being investigated as a method to reduce the interference. Progress at refining the specificity of the duplex primer / probe set is progressing. The next step will be to redesign the primers and probes so that all primers (forward and reverse) are species specific and the probes are also species specific.

Part 2. Sampling strategies

There is considerable variation in sampling methods and field sampling strategies. In this section we have attempted to identify the most commonly used in the field. Using the Mathematical models developed by Shomaker and Been we have developed data to approximate the sampling methods.

These data were entered into the Been and Shomaker model and the range of % probability of detection for each sampling method calculated. Variation of some methods were also calculated and presented in the following series of figures and tables.

We analysed:

- column (various sizes)
- grid (various sizes)
- W pattern
- Zig zag
- Foot sampling method in use in NZ
- Australian large area 50 ha
- central pivot 100 ha
- Accu core methods using W and cluster sampling

It was not the intention or the objective to make selection of best methods but rather to generate a system of comparison between approaches based on the % probability of detecting a given level of PCN infection. This will enable industry to make informed decisions with regard to the most appropriate system for the end use of the survey.

PRELIMINARY CALCULATIONS using existing Dutch data to define the:

- Sample size = 500ml
- Number of cores = 16
- Relative weight of loamy soil = 1.062 g
- Regular core size = $\varnothing 2 \text{ cm} \times 10 \text{ cm} = 31.4 \text{ cc} \times 1.062 = 33.34 \text{ g}$
- Sampled area = 40 ha
- Resulting sampling grid = $100 \times 250 \text{ m}^2$
- Sampling time = immediately post harvest of previous crop
- Lowest density (immediately before planting) giving a visible yield reduction of 35% ($m = 0.15$) = 20 eggs/gram of soil (NZ-assumptions).

The equation for relative yield is Seinhorst's: $y = m + (1-m) \cdot 0.95^{(P/T-1)}$ where m is the minimum yield and T is the tolerance limit.

- Cropping frequency = 1:4
- Post harvest density potato crop $120 / (0.3 \times 0.7^2) = 136 \text{ nem/g}$ (Dutch assumptions)
- Nematodes per cyst = 250
- Post harvest density in cyst/kg = 550
- Average size of infestation focus with central density of 550 cysts/kg soil = 900 m^2
- Focus parameters = The same as in The Netherlands:

Average length gradient is 0.83 and average width gradient 0.64. Both parameters follow a normal bivariate distribution.

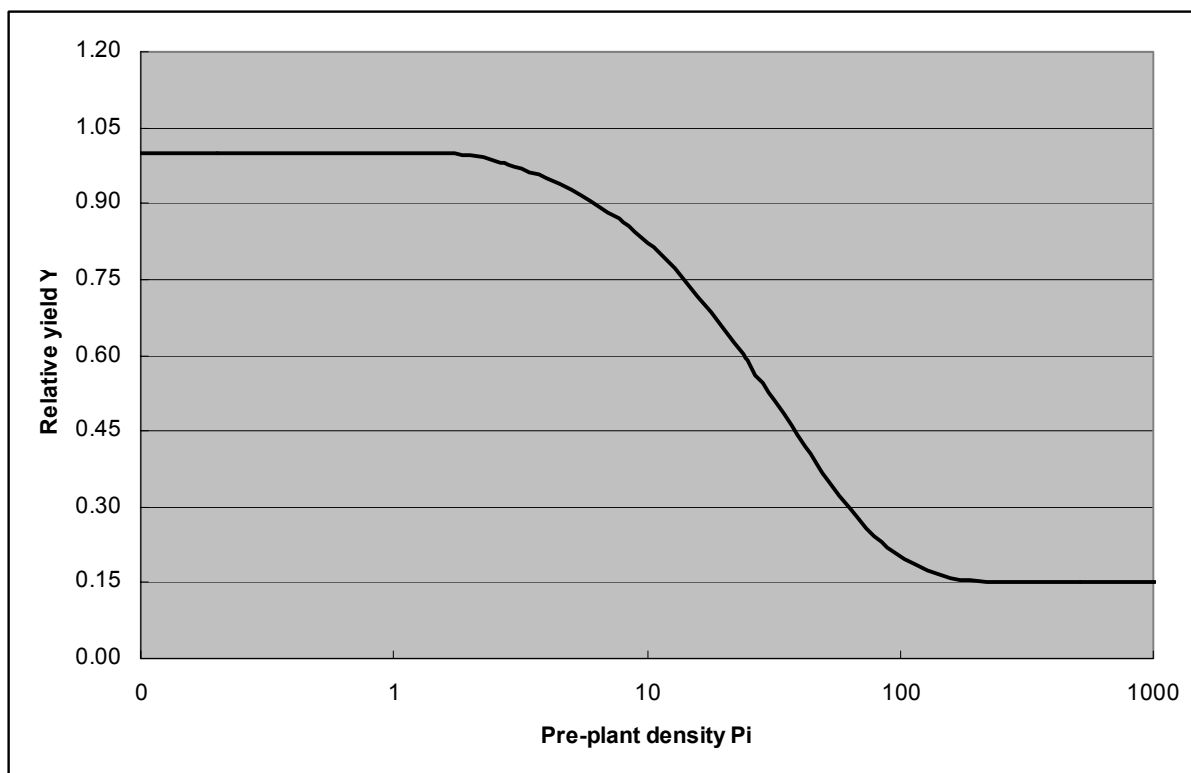


Fig. 1. The relationship between pre-plant density and relative yield according to the Seinhorst equation.

RESULTS

	Core size	Sample size (g)	Sampled area (ha)	Sampling grid (m ²) w x l	Central density focus	% Detection prob.
1	33.34	531	40	100 x 250	550	1.4
2	33.34	531	4	50 x 50	550	13.6
3	33.34	531	1	25 x 25	550	43
4	8.35	531	1	12.5 x 12.5	550	53
5	2.07	531	1	6.25 x 6.25	550	55.8
6	5	1665	1	6 x 5	550	89.3

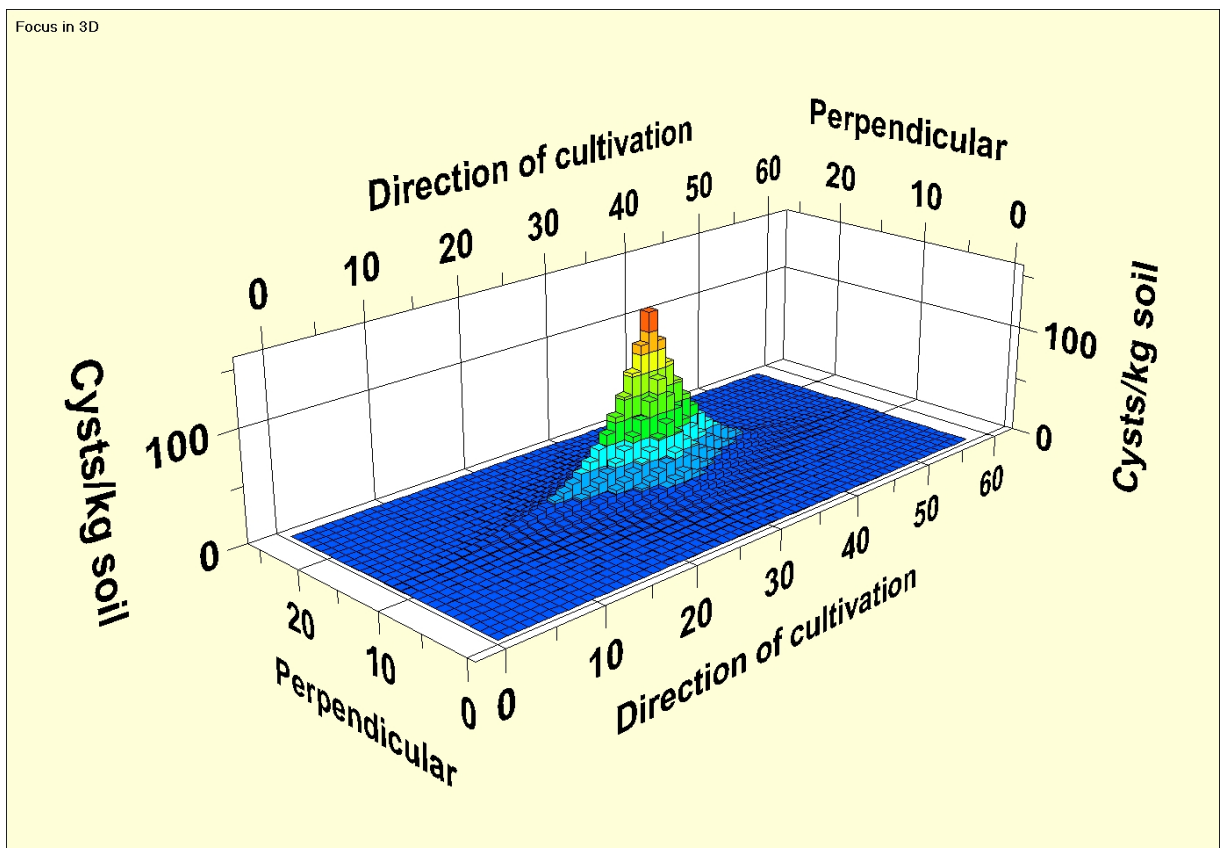
Legend

- First row: Results of above described method
- Second and third row: Decrease of field size with corresponding narrower sampling grid
- Fourth and fifth row: Decrease of sampling grid with corresponding decrease of sample size
- Sixth row: Optimized to 90% detection

NEW ZEALAND

Assumptions based on existing New Zealand data on biology and yield response:

- Minimum yield (m) = 0.15
- Cropping frequency = 1:4
- Sampling purpose = detection
- Sampling time = post harvest of previous potato crop
- Preplant density = 5 e/g soil
- Annual decline for loamy soil = $(38(Pa)+62(Ro))/2 = 50\%$
- Annual decline for organic soil = $(30(Pa)+57(Ro))/2 = 44\%$
- Field size = 10 ha: 400(l)x250(b)
- Nematodes/cyst = 250
- Post harvest density previous crop = 160 cysts/kg of soil (central population density focus)
- Core size = regular: $\varnothing 2 \text{ cm} \times 10 \text{ cm} = 31.4 \text{ cc} \times 1.062 = 33.4 \text{ gram}$
- Number of cores = regular, 16
- Relative weight of loamy soil = 1.062 g
- Focus parameters = The same as in The Netherlands:



Graphic modelling showing shape and density of single PCN infection in field focus with a central population density of 160 cysts/kg. of soil.

Data set calculated for a range of field sampling patterns/ core sizes and methods

Column sampling (* indicates parameter altered)

Scenario 1:

*Core size	33.4
Grid size	7.5 (10 ridges) x 10 m ²
*Sample size/10 ha	44522 g
*Sample size/column	1349 g
Column area:	3000 m ²
Cores per field	1333
Field size	10 ha field (200 m wide 400 m long)
Columns per field `	33.33 columns = 33 subsamples

Scenario 2

Core size	20
Sample size/10 ha	26660 g
Sample size/column	808 g
Otherwise the same as scenario 1	

Scenario 3

Core size	15 g
Sample size/10 ha	19995 g
Sample size/column	606 g
Otherwise the same as scenario 1	

Scenario 4

Core size	10 g
Sample size/10 ha	13330 g
Sample size/column	404 g
Otherwise the same as scenario 1	

Rectangular grid

Scenario 1: New

Core size	16 g
(deviates from EU calculations because of different relative weight of soil samples in New Zealand).	
Sampling grid	6 x 16.7 m ²
Cores/1 ha	100
Foci size	Range from 50-500; see table
Sample size	1500 cc = 1600 g

Scenario 2 New

Core size	16 g
Sampling grid	10 x 10
Otherwise the same as scenario 1.	

Scenario 3 EPPO standard as at 2003

Core size	3.6 g
Sampling grid	7.5 x 7.5 m ²
Cores per ha	178
Sample size/ha	600 cc = 637 g

Historical patterns

Scenario 1: W grid

Core size	16 g
Cores/1ha	100
Sample size	1600 g
Focus size	160 cysts/kg

Scenario 2: Zig-zag grid

Core size	16 g
Cores/1ha	100
Sample size	1600 g
Focus size	160 cysts/kg

Analogue of plant sampling

Scenario Foot: 100% of area sampled

Core size	2122 g
Grid:	7.5 (10 rows) x 10 meters (6 plants) = 75 m ²
Samples/1 ha	133
Sample size/1 ha	282226 g
Focus size	5, 50 and 160 cysts/kg soil
Cyst loss due to visual inspection by experts:	20%
Focus area of focus with a central density of 5 cysts/kg of soil:	67 m ² .

Scenario A: 25% of area sampled (effectively 27%)

Core size	2122 g
Samples per ha	33
Sampling grid	16 x 19 meters
Sample size/ 1 ha	70026 g
Otherwise the same as scenario Foot	

Scenario B: 10% of area sampled (effectively 9.7%)

Core size	2122 g
Samples/1 ha	13
Sampling grid	26 x 29.5 meters
Sample size/1 ha	27568 g
Otherwise the same as scenario Foot	

Scenario C: 2% of area sampled (effectively 2.2%)

Core size	2122 g
Samples/1 ha	3
Sampling grid	57.5 x 58 meters
Sample size/1 ha	6366 g

1. Column sampling

Some background data

Pre-plant density (e/g)	% yield reduction	Post harvest density (c/kg) of prev crop	Infested area prim focus + 3 sec foci in m2	% infested area
1.56	0	50	1480	1.48
1.88	0	60	1510	1.51
2.5	2	80	1570	1.57
3.13	3	100	1620	1.62
3.75	5	120	1660	1.66
4.38	6	140	1700	1.70
5	7	160	1730	1.73
5.63	9	180	1760	1.76
6.25	10	200	1790	1.79

Scenario 1:

Core size	33.4
Column area:	3000 m ²
Sample size/10 ha	45300 g
Sample size/column	1373 g
Grid size	7.5 x 10 m ²

Detection probabilities:

Size* primary focus Post harvest potato crop (n-1)	% Detection probability primary focus	% Detection probability 1 primary focus and 3 secondary "50" foci
50	57.8	96.82
60	63.6	97.26
80	72.3	97.92
100	78.3	98.36
120	82.6	98.69
140	85.8	98.93
160	88.3	99.12
180	90.2	99.26
200	91.7	99.38

* Central population density in cysts/kg of soil

Scenario 2

Core size	20
Column area:	3000 m ²
Sample size/10 ha	26660 g
Sample size/column	808 g
Grid size	7.5 x 10 m.

Detection probabilities:

Population density Pre-plant potato crop n (e/g)	Size* primary focus Post harvest potato crop (n- 1)	% Detection probability primary focus	% Detection probability 1 primary focus and 3 secondary “50” foci
1.6	50	42	95.6
1.8	60	48	96.0
2.5	80	56	96.7
3.1	100	63	97.2
3.8	120	68	97.6
4.4	140	72	97.9
5	160	76	98.2
5.6	180	79	98.4
6.2	200	81	98.6

* Central population density in cysts/kg of soil

Scenario 3

Core size	15 g
Column area:	3000 m ²
Sample size/10 ha	19995 g
Sample size/column	606 g

Detection probabilities:

Population density Pre-plant potato crop n (e/g)	Size* primary focus Post harvest potato crop (n- 1)	% Detection probability primary focus	% Detection probability 1 primary focus and 3 secondary “50” foci
1.6	50	34	72.16
1.8	60	39	74.27
2.5	80	47	77.64
3.1	100	54	80.59
3.8	120	59	82.70
4.4	140	63	84.39
5	160	67	86.08
5.6	180	70	87.34
6.2	200	73	88.61

* Central population density in cysts/kg of soil

Scenario 4

Core size 10 g
 Column area: 3000 m²
 Sample size/10 ha 13330 g
 Sample size/column 404 g

Detection probabilities:

Population density Pre-plant potato crop n (e/g)	Size* primary focus Post harvest potato crop (n-1)	% Detection probability primary focus	% Detection probability 1 primary focus and 3 secondary “50” foci
1.6	50	25	68.3
1.8	60	29	70.05
2.5	80	35	72.58
3.1	100	41	75.11
3.8	120	46	77.26
4.4	140	50	79.03
5	160	54	80.59
5.6	180	57	81.99
6.2	200	60	83.21

* Central population density in cysts/kg of soil

2. Scenario 1 – new

Core size 16 g
 (deviates from EU calculations because of different relative weight of
 soil samples in New Zealand).
 Samples/1 ha 100
 Sampling grid 6 x 16.7 m²
 Foci size See table
 Sample size 1500 cc = 1600 g

Population density in e/g Pre-plant potato crop N	Size* primary focus Post harvest potato crop (n-1)	% Detection probability primary focus	% Detection probability 1 primary focus and 3 secondary “50” foci
1.6	50	28.65	74.08
3.2	100	46.52	80.57
4.8	150	57.99	84.74
5	160	59.80	85.40
7.8	200	65.92	87.62
8	250	71.69	89.72
9.6	300	76.06	91.30
11.2	350	79.48	92.55
12.8	400	82.20	93.53
14.4	450	84.40	94.33
16	500	86.22	94.99

Scenario 2 New

Core size	16 g
Cores/1ha	100
Sampling grid	10 x 10 m ²
Foci size	see table
Sample size	1500 cc = 1600 g

Population density in e/g Pre-plant potato crop n	Size* primary focus Post harvest potato crop (n-1)	% Detection probability primary focus	% Detection probability 1 primary focus and 3 secondary "50" foci
1.6	50	28.19	73.92
3.2	100	45.57	80.23
4.8	150	56.71	84.28
5	160	58.48	84.92
7.8	200	64.46	87.09
8	250	70.14	89.15
9.6	300	74.47	90.73
11.2	350	77.87	91.96
12.8	400	80.60	92.95
14.4	450	82.84	93.77
16	500	84.70	94.44

Scenario 3 EPPO *standard as at 2003*

Core size	3.6 g
Sampling grid	7.5 x 7.5 m ²
Cores per ha	177
Sample size/ha	600 cc = 637 g

Population density in e/g Pre-plant potato crop n	Size* primary focus Post harvest potato crop (n-1)	% Detection probability primary focus	% Detection probability 1 primary focus and 3 secondary "50" foci
1.6	50	12.7	42.02
3.2	100	22.8	48.70
4.8	150	30.6	53.86
5	160	36.8	58.00
7.8	200	41.9	61.42
8	250	46.3	64.31
9.6	300	50.0	66.80
11.2	350	53.3	68.96
12.8	400	56.1	70.86
14.4	450	58.7	72.54
16	500	60.9	74.05

3. HISTORICAL PATTERNS

Scenario 1: *W*

Core size	16 g
Cores/1ha	100
Sample size	1600 g
Focus size	160 cysts/kg

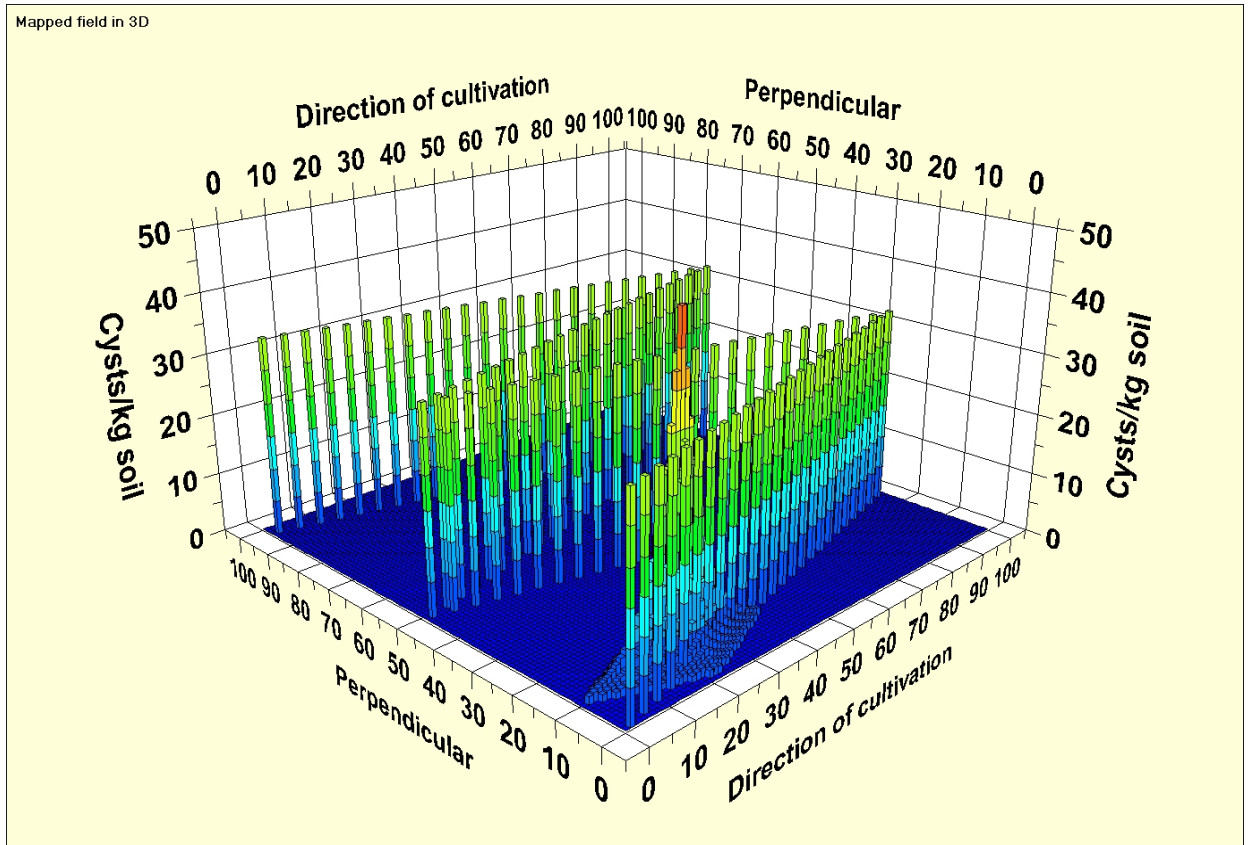


Fig. 3: W-shaped sampling grid with focus imposed (focus shifted over all possible solutions when simulation is carried out)

Scenario 2: Zig-zag

Core size	16 g
Cores/1ha	100
Sample size	1600 g
Focus size	160 cysts/kg

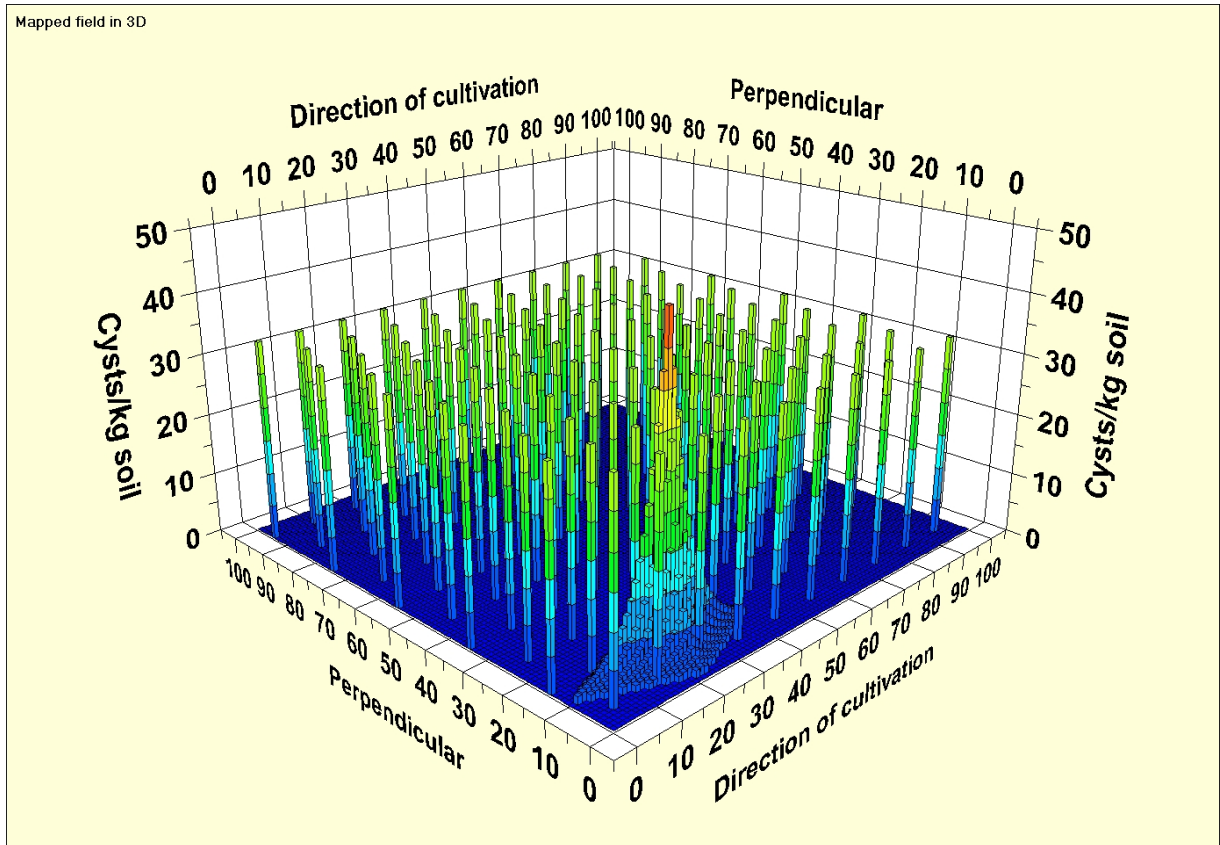


Fig 4. ZigZag sampling grid - compare with Fig 5 of rectangular sampling grid.

Sampling pattern	Size* primary focus Post harvest potato crop (n-1)	Modal* detection probability primary focus	Range of detection probabilities
W	160	0	0-99.5
Zig-zag	160	57.7	36-86
Rectangular	160	57.4	36-86

* The mode is chosen instead of the average because of the skew distribution of detection probabilities in case of the W-pattern. For the zig-zag and the rectangular the mode and the average are the same.

4. PLANT SAMPLING ACCORDING TO THE WOOD & FOOT PAPER

- Original scenario: 100% of area is sampled
- Core size 2122 g
- Grid: 7.5 x 10 meters = 75 m²
- Plant samples/1 ha 133
- Sample size/1 ha 282926.3 g
- Focus size 5, 50 and 160 cysts/kg soil
- Cyst loss due to visual inspection: 20%

Population density Pre-plant potato crop n (e/g)	Size* primary focus Post harvest potato crop (n- 1)	% Detection probability primary focus	% Detection probability 1 primary focus and 3 secondary “50” foci
0.16	5	42.7	100
1.6	50	99.5	100
5	160	100	100

Scenario A1: 25% of area is sampled

- Core size 2122 g
- Samples per ha 33
- Sampling grid 16 x 19 meters
- Sample size/ 1 ha 70026 g
- Focus size 5, 50 and 160 cysts/kg soil
- Cyst loss due to visual inspection: 20%

Population density Pre-plant potato crop n (e/g)	Size* primary focus Post harvest potato crop (n- 1)	% Detection probability primary focus	% Detection probability 1 primary focus and 3 secondary “50” foci
0.16	5	12.23	97.34
1.6	50	68.83	99.06
5	160	88.98	99.67

Scenario B: 10% of area is sampled

Core size 2122 g
 Samples/1 ha 12
 Sampling grid 26 x 32
 Focus size 5; 50 and 160 cysts/kg soil
 Cyst loss due to visual inspection: 20

Population density Pre-plant potato crop n (e/g)	Size* primary focus Post harvest potato crop (n- 1)	% Detection probability primary focus	% Detection probability 1 primary focus and 3 secondary “50” foci
0.16	5	4.9	86.51
1.6	50	35.7	82.94
5	160	55.8	88.26

Scenario C: 2% of area is sampled

Core size 2122 g
 Samples/1 ha 3
 Sampling grid 57.5 x 58
 Focus size 5; 50 and 160 cysts/kg soil
 Cyst loss due to visual inspection: 20

Population density Pre-plant potato crop n (e/g)	Size* primary focus Post harvest potato crop (n- 1)	% Detection probability primary focus	% Detection probability 1 primary focus and 3 secondary “50” foci
0.16	5	1.10	25.79
1.6	50	9.13	31.82
5	160	16.17	37.10

Critical points:

- Expert skill needed to detect nematodes in root systems
- Cysts can only be detected during a distinct period
- Nematode density, needed for advisory, is unknown
- Unknown size
- Unknown species

AUSTRALIA

Assumptions:

- Preplant sampling
- Annual decline non host: 70% (?)
- Rotation: 1:4

Scenario 1

Field	Rectangular, 2 ha
Number of cores	200
Sample size:	2000 g
Subsample	500 g
Sampling grid	10 x 10 m ²
Focus to be detected	A range of central densities, see table.
Core size	10 g

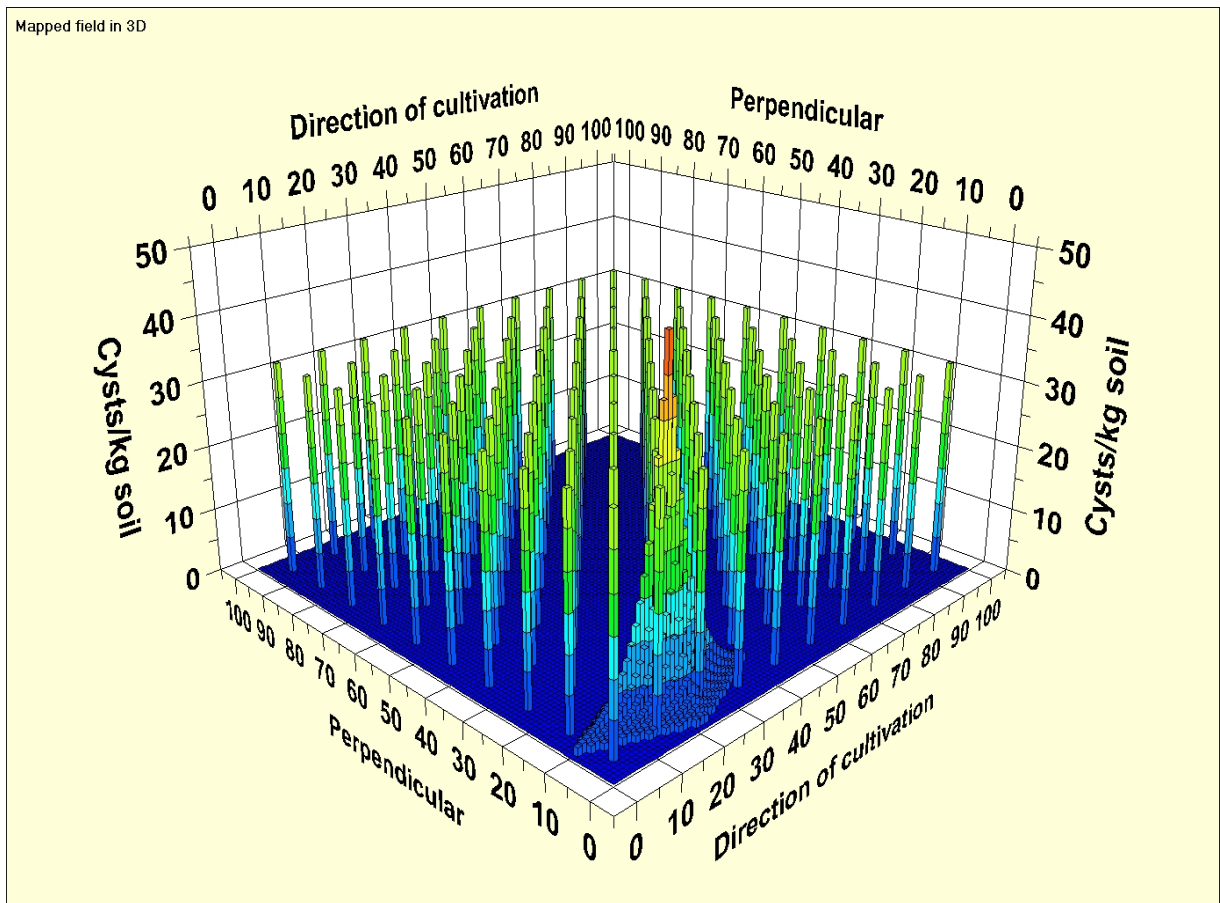


Fig 5. rectangular sampling grid

Result:

Size* focus Post potato	primary harvest crop (n- 1)	% Detection probability primary focus	% Detection probability 1 primary focus and 3 secondary “50” foci
50		19.18	57.33
100		32.83	64.54
150		42.55	69.67
200		49.90	73.55
250		55.66	76.59
300		60.32	79.05
350		64.16	81.08
400		67.29	82.73
450		70.14	84.24
500		72.52	85.49
550		74.59	86.59
600		79.44	87.54
650		78.01	88.39
700		79.44	89.15
750		80.73	89.83
800		81.88	90.43

Scenario 2

Field	Circular 100 ha (central pivot)
Number of cores/2ha	200
Core size	10 g
Sample size/ha	2000 g
Subsample	500 g
Sampling grid	10 x 10 m ²
Focus to be detected	A range of central densities, see table.
Results	As for scenario 1.

Scenario 3 - ACCUcore sampling

Field size 50 ha
 Core size 11g
 Sample 50 g
 Legs 5
 Cluster 3/leg
 Cores/cluster 3
 Cores 45

Size* focus	Number of 1500 cyst foci/50 ha	% Detection probability	% infested area
1500	1	2.3	0.26
1500	2	4.5	0.51
1500	3	6.7	0.77
1500	4	8.9	1.03
1500	5	10.9	1.29
1500	6	13.0	1.54
1500	7	15	1.80
1500	8	17	2.06
1500	9	19	2.32
1500	10	20.7	2.57
1500	99	90.	25.4

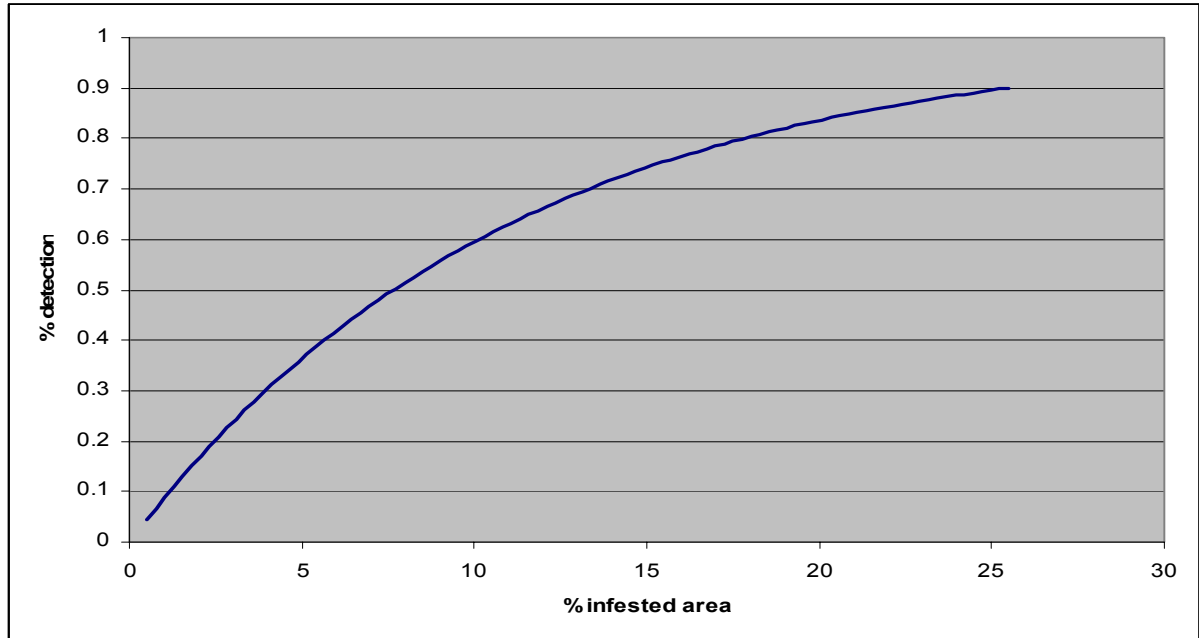


Fig 6. infested area versus detection probability for Australian AccuCore sampling method.

Discussion

Been and Shomaker (2000) have identified a number of assumptions that must be incorporated in to any analysis of reliability.

They are:

- One vital cyst has to be found in a sample
- There are several foci in a field
- PCN follows a negative binomial distribution pattern
- Any cyst in a sample will be recovered by standard extraction methods
- A sampling grid must be rectangular and sampling should cover the whole field
- Detection sensitivity should be able to detect PCN at a level below the crop symptom level
- The number of cores taken has to be high but the size of each sample can be altered to reflect circumstances
- Sample size has a major effect on detection probability
- Probability of detection at least one cyst in the sample should be at least 90%
- Repeated sampling producing negative result could allow for the reduction in sample size for subsequent sampling occasions

Using these assumption the Been and Shomaker analysis identified the preferred system :

Scenario 1: New

Core size	16 g
Sampling grid	6 x 16.7 m ²
Cores/1 ha	100
Foci size	Range from 50-500; see table
Sample size	1500 cc = 1600 g

It is significant that the current PCN survey is based on this concept of fixed-point sampling. The examination of plant roots is used because foliage symptoms are not good indicators of low levels of infestation. The frequency of sampling for PCN and the number of plants examined is therefore based on statistical evidence. While the sampling is statistically sound it also depends on inspector expertise, and climatic and soil conditions. If the primary function of a survey was to collect material rather than examine for the presence or absence of symptoms, it would be possible to increase the emphasis on defined sampling patterns from published epidemiological studies. It would also be possible to objectively set the required level of sampling. Thresholds for detection could then be altered by changing the level of sampling intensity. The distribution of

infestation could then easily be studied with the introduction of GPS-based sampling patterns for a field. This approach would not only provide the coordinates for sampling but would also generate a database on the distribution of diseases and provide evidence for detection, enabling surveys to be delimited and monitored. Such a system would provide evidence of pest-free areas and would also provide a record of paddock history and could be used as a knowledge-based management tool for the farmer to limit the impact of the infestation.

In addition to field sampling, there should also be an increased focus on examining seed and tubers during their preparation for sale. The condition of debris and soil associated with the product is often a better indicator of the health status of the product than a sample of the growing plant that produced it. The major difficulty with this approach is in getting the results of tests back quickly enough to affect the outcome of a line. However, the newly developed PCR tests and quantitative probes, which are both rapid and sensitive, address this issue.

The new DNA-based methods also offer significant potential benefits to the local seed trade, as planting a severely infected seed line can provide inoculum for the development of a disease epidemic the following year, compromising future exports.

Finally, associated with the difficulties of making phytosanitary declarations on the basis of field surveys is the diversity of definitions for freedom from disease. Widely varying definitions make it difficult to define an area of inspection. Strategically, it would be desirable in the medium term to develop the concept of ‘area of production’ as the common unit of quarantine. Aggregations of units of production would constitute an area of freedom or pest-free area. Intensive sampling and diagnosis of diseases would ensure the maintenance of pest-free areas. The reliance on large area of freedom zones is suspect as the chance discovery of disease in the described area of freedom could compromise the entire area. A unit of production infection would be limited to that area and its closest neighbours, and such a concept could be more easily defended on technical grounds if soil examination statistics were developed.

Analysis Results

Population density in e/g Pre-plant potato crop n	Size* primary focus Post harvest potato crop (n-1)	% Detection probability primary focus	% Detection probability 1 primary focus and 3 secondary “50” foci
1.6	50	28.65	74.08
3.2	100	46.52	80.57
4.8	150	57.99	84.74
5	160	59.80	85.40
7.8	200	65.92	87.62
8	250	71.69	89.72
9.6	300	76.06	91.30
11.2	350	79.48	92.55
12.8	400	82.20	93.53
14.4	450	84.40	94.33
16	500	86.22	94.99

Modifying the assumptions included in the analysis indicated that the sample size could be decreased. For instance, longer rotations than 1:3 and known natural decline rate of cysts could both result in a 30% reduction in sample size.

The preferred method identified by the analysis is particularly powerful for phytosanitary measures as it provides a very high level of assurance that PCN is not in the sample. However, before it could be accepted by Australian states and New Zealand for PCN management the cost effectiveness of the proposed method would have to be assessed.

The next step in the evaluation of this modelling system for the identification of optimal sampling regimes would be to sample the infected field sites mentioned earlier (where inoculum levels have been assessed) with several of the identified sampling regimes.

Summary

- Current methods are very labour intensive and require visual searching, detection and enumeration through plant debris before a positive can be confirmed, considerable time and skill is needed to determine the species of the PCN.
- The development of the DNA based diagnostic system for PCN provides an urgently needed improvement in the detection and speciation of PCN.
- With the DNA system we can now determine the species and numbers of nematode eggs present in the sample without having to search the sample.
- This advance will improve the reliability and consistency of the detection process. These attributes are central to acceptance of methods for phytosanitary application as well as providing a reliable basis for PCN management strategies for growers.
- The technique works reliably for *G. rostochiensis* but requires more research to be accurate for *G. pallida* populations and certain mixed populations.
- Sensitivity of detection within the sample is not affected by the DNA methods versus the traditional method but the main advantage is reliability and elimination of operator bias.
- The collection of the soil sample is pivotal to the development of coherent statistically defensible detection and diagnostic systems.
- During this program we analysed all of the common soil sampling strategies.
- Many of the methods are well established in the agricultural sector (eg 'W' across paddock) but do not have any measure of reliability developed for them.
- We engaged the assistance of Professors Been and Shomaker to analyse the reliability of the currently used methods.

References:

Anonymous ACCUCore 2004 New sampling core to collect more representative soil samples. SSP *Spurr soil probes Brighton south SA 5048* 1 pg.

Been .T. H. and Shomaker C.H 2000 . Development and evaluation of sampling methods for fields with infestation foci of potato cyst nematode (*Globodera rostochiensis* and *G. pallida*) *Analytical and theoretical plant pathology*. 99: 647-656.

Bulman, S and Marshall, J.W. 1997 Differentiation of Australasian potato cyst nematode (PCN) populations using the polymerase chain reaction (PCR). *New Zealand Journal of Crop and Horticultural Science*. 25: 123-129.

Marshall, J.W. 1993 Detecting the presence and distribution of *Globodera rostochiensis* and *G. pallida* in mixed populations in New Zealand using DNA probes. *NZ. Journal of Crop and Horticultural Science*. 21: 219-223.

Marshall, J.W. 1997 Potato cyst nematode in New Zealand and Australia. *Chapter 20*. CAB INTERNATIONAL publication entitled: Potato Cyst Nematode. Biology Distribution and Management 353 395.1998.

Turner S. J. 1993. Soil sampling to detect potato cyst-nematodes (*Globodera spp.*). *Annals of applied biology* . 123: 349-357.

Wood F H. , Foot M.A. , Dale P.S. , Barber C.J. 1983. relative efficiency of plant sampling and soil sampling in detecting the presence of low potato cyst nematode infestations. *New Zealand Journal of Experimental Agriculture*. Vol 11: 271-273.

Section 4.

Conclusions

The improvement of monitoring protocols for exotic pathogens is a complex task that requires both the alteration and re-validation of existing protocols or the provision for comprehensive validation of any newly developed protocols. The DNA-based testing procedures developed in this project have proven to be reliable, fast and at least as sensitive as traditional methods. They also both have the advantage of considerable background research by other groups around the world, all of which contributes to strong validation of test performance. Discussions are continuing with the regulatory organisations, MAF in New Zealand and Plant Standards in Victoria, Australia, to determine how best to integrate the new methods into accredited protocols. However, the recent and unfortunate outbreak of PCN in the Koo wee rup region has thrust the new tests into the limelight. The outbreak presented a unique opportunity for this project to implement some of the new protocols under incursion conditions and to begin inter-laboratory validation.

One of the first priorities within the response to the PCN outbreak was to differentiate between *Globodera rostochiensis* and *Globodera pallida*. A PCR assay developed by project staff in New Zealand allowed suspect cysts to be classified to species level within 72 hours of DPI laboratories receiving the samples - this included repeated testing to ensure accuracy and inter-laboratory validation of results between Australia and New Zealand. At the same time, suspect cysts were sent to SARDI for classification on morphological characteristics and to the UK for protein profiling. However, SARDI scientists were unable to definitively classify the cysts and UK scientists were still working on the samples four months after receiving them. The newly developed methods have since been used to assist with six further PCN detections. The PCR has therefore proved indispensable in the rapid identification of PCN species, highlighting the worth of DNA probes in general for fast and accurate response during an incursion situation.

The next step in the transition to DNA-based techniques is large-scale monitoring of crops for area freedom declarations. This study has shown that DNA-based methods are at the very least the equivalent of traditional monitoring techniques. Therefore the only impediments to adoption of the new technology are capacity and cost. Local laboratories must be able to demonstrate the capacity not only to conduct the tests but also the ability to process a large and sudden influx of samples during an incursion. DNA-based methods can be automated, and progress towards that end has been made in this study by the utilisation of real-time PCR and the trialing of robotic equipment. These measures will reduce labour inputs into testing and therefore reduce costs, which are expected to be considerably less than for traditional sample processing and testing.

One of the limitations of PCR-based techniques discovered in this study, however, it is difficult to have 100% confidence in their reliability. For example, *R. solanacearum* primers specific for race 3/biovar 2 also detect closely related organisms like the Blood Disease Bacterium, while probes for *G. pallida* will quantify *G. rostochiensis* under certain conditions. These are minor issues however, and can be solved.

The project has delivered newly developed test protocols that provide industry with:

- 1) Tools to detect both bacterial wilt and PCN in soil and plant tissue with a high degree of sensitivity and specificity
- 2) Improved sampling strategies for the field detection of both pathogens

- 3) The ability to determine the phytosanitary status of a crop using validated monitoring protocols

It is inevitable that DNA-based techniques will one day replace traditional techniques here in Australia and New Zealand. It is important however that we pro-actively seek to make the transition quickly in order to maintain any competitive advantage we have over trading partners, and to reduce the risk that others will use the technology to limit our access to export markets. Embracing the technology will also ensure that our own plant-based industries are protected from exotic diseases.

The tests developed in this study will give regulators with greater confidence in declaring the disease-free status of produce, fields or districts, which will provide obvious benefits to trade and improve market access for Australian and New Zealand potato growers.

Technology Transfer

Industry has been updated on the project's progress through:

- 1) three grower meetings organised by VegCheque where presentations were given by project staff
- 2) one laboratory demonstration at DPI Knoxfield
- 3) Regular discussions with growers and other industry representatives (e.g. Smiths Crisps) while sampling in disease-affected areas
- 4) three short summary articles published in Potato Australia
- 5) Considerable discussion has taken place between VegFed and MAF policy in New Zealand, as well as with the designated organisations contracted to carry out regulatory inspections. Similar discussions occurred in Australia (and are continuing), particularly surrounding the introduction of DNA-based testing to help manage the new PCN outbreak in Koo Wee Rup.

The commercialisation of the techniques developed within this project is being discussed by HAL, DPI and CFR.