Hort Innovation

Final Report

Diagnostic Capability to Detect *Candidatus* Liberibacter solanacearum (CLso)

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Diagnostic Capability to Detect *Candidatus* Liberibacter Solanacearum (CLso) – PT17000

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Content

Summary

Candidatus Liberibacter solanacearum (CLso) causes zebra chip disease in potato and is a serious threat to the Australian potato industry. A recent incursion of the tomato-potato psyllid (TPP) into Western Australia has identified gaps in the diagnostic capacity of Australia and New Zealand to confidently detect CLso in field samples collected as part of surveillance and certification activities. Accurate diagnostics is required to support these requirements.

This project has provided:

- Science-based evidence on the distribution of CLso in field grown potatoes.
- Improved guidelines for sampling of potatoes to be included in the NDP for CLso (NDP 18).
- A validated field deployable tool (Recombinase Polymerase Amplification (RPA)) for the detection of CLso in-field.
- A validated high throughput protocol for testing potato tissue for CLso.
- Evidence for technical proficiency in detecting CLso using the CLso National Diagnostic protocol (NDP) in nine major node laboratories in Australia and New Zealand.

To determine the distribution of CLso in a field grown potato an extensive field trial was established at the PFR Lincoln farm to systematically sample field grown potatoes known to be infected with a well characterised strain of CLso that is associated with the Zebra Chip disease, over a 6-week period. Surprisingly, this type of experiment for CLso has not previously been reported in the literature, rendering this study as one of the first detailed accounts of the natural distribution of CLso in a field grown potato plant.

This field trial determined that:

- Leaf material is not a reliable tissue to sample for the detection of CLso.
- Below ground tissue (stem and tubers) is the most reliable tissue to sample for CLso detection.
- Detailed sub-sampling of individual potato tubers provided evidence of uneven distribution of CLso within the tuber.

Based on these findings our recommendation for sampling potato plants to test for the presence of CLso is to sample below ground tissue (including daughter tubers). Our results do not support pooling of multiple potato plant samples into the one sample, such as the pooling of samples for other bacterial pathogens (*R. solanacearum* and *D. dianthicola*). The inconsistent patterns of CLso distribution observed in this study suggests that multiple samples from the one tuber may be required to accurately test for CLso presence in a tuber. The CLso NDP has been updated to reflect these findings and submitted to the Subcommittee on Plant Health Diagnostics (SPHD) for ratification.

An in-field diagnostic tool using RPA for the detection of CLso was validated and can readily detect CLso at field inoculum levels in potatoes. This capability should be considered for adoption by field officers as it is rapid and easy to use.

Nine laboratories from across Australia and New Zealand participated in the CLso ring test using the NDP (NDP18) for CLso. All laboratories were successfully able to detect CLso using the methods described in the current NDP. The ring test proved to be a great training exercise for the participating laboratories and provides evidence to industry and biosecurity agencies that diagnostic capability to accurately detect CLso exists in multiple labs in Australia.

Keywords

Candidatus Liberibacter solanacearum (CLso), zebra chip, tomato-potato psyllid, field trial, cage trial, pathogen distribution, in-field diagnostics, recombinase polymerase amplification (RPA), diagnostic, ring test, proficiency test, National Diagnostic Protocol (NDP)

Introduction

A recent incursion of the tomato-potato psyllid (TPP) into Western Australia has identified gaps in the diagnostic capacity of Australia & New Zealand to confidently detect *Candidatus* Liberibacter solanacearum (CLso) in surveillance and certification activities in an accurate, cost effective and high throughput manner. CLso causes zebra chip disease in potato and is a serious threat to the Australian potato industry. Following its discovery in New Zealand, zebra chip disease cost potato growers an estimated $NZD 200 million (2006-2012).

CLso is transmitted vertically (by seed tubers) and horizontally (by insect) in potato. Seed transmitted CLso is limited to infected plants that develop potato tubers, while horizontal transmission occurs when TPP feeds on a CLso-infected plant, acquires the bacterium and then transmits the bacterium to other plants in the field or to neighbouring properties. Thus, two mechanisms of CLso transmission must be considered whilst developing a valid sampling strategy to detect CLso present in a potato field. As the bacterium is present in a low proportion of the TPP population and the transmission/acquisition characteristics are complex, analysis of trapped insects does not indicate the extent of infection in the plants. A robust field sampling strategy of potato plants is required to support surveillance and certification programs that tests potential sources of the bacterium in plants and tubers,

A National Diagnostic Protocol (NDP18) has been issued by Subcommittee on Plant Health Diagnostics (SPHD) for CLso (version 1.2, 2012) and a new diagnostic protocol for CLso has recently been approved by the International Plant Protection Convention (IPPC) as DP21. Project 2002 in the Plant Biosecurity Cooperative Research Centre (PBCRC) has developed new approaches for diagnosing bacterial pathogens, including CLso. The Smith laboratory in New Zealand Plant & Food Research (PFR) have developed a quantitative polymerase chain reaction (qPCR) assay deemed to be more specific than the current tests in the IPPC and SPHD protocols, which is currently being validated. In addition to this, the South Australian Research and Development Institute (SARDI) has also developed a qPCR assay. For implementation in Australia, both PFR and SARDI assays need to be performed on European isolates and need to be validated in Australian laboratories.

Ideally, laboratory methods should have high-throughput capabilities and these new protocols need to be compatible with the main industry oriented diagnostic services in Australia which are best placed to service surveillance programs. For application in crop surveillance, sampling strategies need to be developed on potato tubers and other solanaceous crops including capsicum and tomatoes. A critical first step is to determine the maximum sample size (plant and tuber) that can be processed to reliably detect infection by each of the approved and new tests. The size of the composite sample will have a significant impact on development of surveillance sampling strategies. The NDP18 and IPPC DP21 protocols do not provide sampling details to maximise detection, nor guidelines on optimising the sensitivity of assays.

Currently, the only test in use for CLso testing potato tubers in Australia is a “fry test”, this test produces inconsistent results that can be highly variable dependent on tuber variety and interpretation of results is highly subjective. This test has previously been utilised as a research tool and has not been validated as a diagnostic test.

There are existing international standards produced by the European and Mediterranean Plant Protection Organisation (EPPO) for seed potato certification of vascular limited bacterial pathogens such as *Ralstonia solanacearum* (Commission Directive 2006/63/CE of 14 July 2006). Specifically, the tests are designed and validated for detection of latent infections of *R. solanacearum, Clavibacter michiganensis* subspecies *sepedonicus, Pectobacterium* and *Dickeya* species (including *Dickeya dianthicola*) in asymptomatic potato tubers. Given the methods can detect low titre, vascular limited bacteria, this workflow has the potential to also be applicable to testing tubers for CLso. Validation of this method for CLso testing would allow a single molecular test to be added to seed certification tests for international trade. Seed certification testing is already being conducted at several laboratories in Australia for *R. solanacearum*. If successful, a validated protocol for this testing would be submitted to SPHD for national use.

New isothermal assays for CLso have been developed by New Zealand PFR; these assays are fast,

robust and the technology is suitable for in-field surveillance diagnostics. Developing the sampling strategy necessary to fully utilise the capability of CLso qPCR and isothermal diagnostics for potato seed and commercial production is critical to maximise the value of undertaking surveillance for this pathogen.

It is critical that competent technicians are trained and proficient in applying assays outlined in NDPs. Proficiency testing and/or ring tests are an effective means of ensuring protocols are reproducible and that laboratory staff are proficient in using an NDP. To this end current National Association of Testing Authorities (NATA) requirements stipulate that accredited labs actively participate in proficiency testing programs as evidence of lab competency. A ring test is a process in which a set protocol and pre-prepared (blind) samples are provided to several laboratories for testing. The results not only determine the proficiency of participating laboratories but also help determine if the methods in use are reliable and reproducible. This process provides assurance that the results being generated are accurate, specific and sensitive.

Here we report on a series of experiments to determine the distribution of CLso in field grown plants and identify the optimal tissue in a potato plant to recommend for sampling to maximise detection of this pathogen. We also conducted a number of experiments to look at the distribution of CLso within a tuber as this information is critical to formulate sampling procedures for testing batches of tubers. The validation of a potential field deployable diagnostic tool for the detection of CLso is also reported. A ring test was designed and distributed to participating key diagnostic laboratories in Australia and New Zealand to test the proficiency of major node laboratories to use the CLso NDP for the detection of the zebra chip pathogen.

Methodology

Distribution of *Candidatus* Liberibacter solanacearum (CLso) in potato plants and tubers (Appendix 1)

In-field cage trials to determine the distribution and titre of CLso within a potato plant, were set up at the New Zealand PFR farm in Lincoln. For the cage trial, 180 Moonlight (New Zealand; Golden Delight in Australia) tubers were planted in 36 cages (22/11/2017) (Appendix 1, Figure 1). Thirty of these plants across six cages had no TPP released (serving as negative controls). For the remaining 150 plants, contained in 30 cages, 10 TPP that were collected from a CLso-positive colony were released at the base of each plant approximately 7 weeks after tuber planting (9/1/2018). The CLso-positive colony feeds on CLso infected tomato plants and is maintained at NZ PFR – Lincoln, as a source of positive TPP and plant material. The CLso-positive TPP had access to the plants for one week prior to the cages being treated with Avid insecticide (16/1/2018) to kill off the TPP. The cages were further treated with Transform insecticide (31/1/2018) to control the aphid numbers inside the cages. Two plants from each cage were removed from the experiment each week (from 15/1/2018) for 5 weeks and the negative control plants were removed in week 6.

Of the 180 plants planted, 77 plants have been processed (12 replicates for each time point) at PFR, a maximum of 21 tissue samples were taken from each plant. These consisted of tuber, below ground stem, petiole and leaf (consisting of multiple leaflet midribs from the same petiole) samples (Appendix 1, Figure 2). Tuber samples were approximately 1 centimetre $(cm)^3$; below ground stems were cut to 1 cm in length and then quartered; and 1 cm lengths of petiole, leaflet and leaflet midrib tissue were taken.

Extraction of deoxyribonucleic acid (DNA) was performed following the Cetyltrimethylammonium bromide (CTAB) protocol described by Beard et al. (2012). Potato tissue culture plantlets were used as negative extraction controls and tomato plants known to be infected with CLso were used a positive extraction controls. qPCR was performed to detect CLso using the 16S-based assay developed by Li et al. (2009) and on a region of DNA within the potato, cytochrome oxidase (COX) gene, as an internal control to check the quality of the extracted DNA (Weller et al. 2000). The two qPCR reactions were duplexed, and all samples and controls were tested in duplicate. A dilution series of a plasmid with the CLso 16sRNA and potato COX target sequences was used as standards. This enabled approximate copy

numbers of CLso to be determined and for samples to be compared across qPCR plates.

Candidatus Liberibacter solanacearum (CLso) distribution in field infected plants (Appendix 2)

Ten potato plants were rogued from the PFR Lincoln farm. Six of the plants had obvious CLso symptoms and the remaining four had no obvious CLso symptoms. The plants were a range of different varieties/cultivars.

A maximum of 21 tissue samples, consisting of tubers, below ground stem, petioles, and leaf tissue were taken from each plant and DNA extracted as described in the previous section (Appendix 1, Figure 2). Extraction of DNA was performed following the cetyltrimethylammonium bromide (CTAB) protocol described by Beard et al. (2012). Potato tissue culture plantlets were used as negative extraction controls and tomato plants known to be infected with CLso were used a positive extraction controls. Quantitative polymerase chain reaction (qPCR) was performed to detect CLso using the 16S-based assay developed by Li et al. (2009) and on a region of DNA within the potato, cytochrome oxidase (COX) gene, and qPCR was performed as previously described for Appendix 1.

Candidatus Liberibacter solanacearum (CLso) detection in potato tubers (Appendix 3).

To determine if a tuber testing strategy could be used similar to that based on EPPO directive, which relies on concentrating the bacterial component of 200 tubers, a series of tests to determine the distribution and concentration of CLso within a potato tuber were conducted.

In December 2017, 84 Moonlight (New Zealand; Golden Delight in Australia) tubers were obtained from the New Zealand PFR, Lincoln farm. These seed tubers were cultivated the previous season under standard potato growing conditions and their CLso positive/negative status was unknown. Cores approximately 1 cm in diameter, were removed from the stolon end of 84 tubers for CLso screening. Extraction of DNA and qPCR was performed as previously described for Appendix 1.

Three tubers were chosen for a systematic sampling of the tuber to determine if the core taken from the stolon end of the tuber was the most reliable tissue sample (consistent and highest titre) for tuber testing for CLso. Tubers were dissected into a maximum of 63 subsamples (Appendix 3, Figure 1) and extractions and qPCR were performed on each subsample as previously described for Appendix 1.

A further twenty tubers were harvested from ten cages (2 tubers per cage) that had CLso-positive TPP released, and two tubers from a control cage. Core, stolon and rose peel samples were taken from each tuber and DNA extracted and qPCR conducted using a high throughput protocol developed at ArgiBio and utilising the qPCR method that was previously described for Appendix 1.

Validation of the use of in-field isothermal assays for _Candidatus_ Liberibacter solanacearum (CLso) on potato plants (Appendix 4).

For effective management of CLso in the field, early detection is paramount. Currently, molecular detection methods can take several days to be completed as the sample needs to be collected, shipped to an appropriate laboratory, prepared, tested, and finally results reported back to the inspector. RPA is based on isothermal amplification: in contrast to traditional PCR methods, these methods do not cycle through different temperatures to achieve dissociation of the DNA, annealing of primers and extension to achieve amplification. This makes isothermal amplifications simple, fast and robust and amenable to in-field application.

In this study, an RPA was developed at PFR, Lincoln to detect CLso in a selection of cage trial samples. A range of samples from the pool generated for the qPCR analysis was selected based on which plant and which part of the plant they represent as well as the copy number of CLso determined by qPCR (Appendix 4, Table 1). RPA assays were performed using a TwistAmp®exo kit (TwistDX, Cambridge, UK), which allows the detection of amplified product by fluorescence probe, according to the manufacturer's recommendations. Reactions were incubated for 20 minutes in a T8 or T16 isothermal amplification instrument (Axxin, Melbourne, Australia) at 42°C with measurement of fluorescence every 30 seconds. Each assay included a positive and negative control as well as 3 samples in duplicate. The result of a reaction was considered positive if exponential amplification occurred and an assay-

specific threshold was reached.

***Candidatus* Liberibacter solanacearum (CLso) Ring Test (Appendix 5).**

Ring test design, preparation and analysis was conducted by AVR at AgriBio under the guidance of the Australian National Quality Assurance Program (ANQAP) based at AgrBio. For decades ANQAP have been conducting ring tests and proficiency tests and have expertise in this area. Laboratories at both PFR and AVR are internationally recognised in *Liberibacter* diagnostics and were invited to partake in EU proficiency testing in 2016. Sample preparation, distribution and analysis procedures including homogeneity and stability testing will be conducted in consultation with ANQAP staff.

Nine laboratories from eight regions across Australia and New Zealand participated in the CLso ring test were encouraged to perform any in-house protocols for the detection of CLso alongside the National Diagnostic Protocol (NDP18) for CLso (version 1.2, 2012). Ring test results remain anonymous and will be distributed to participating laboratories.

Outputs

Distribution of *Candidatus* Liberibacter solanacearum (CLso) in potato plants and tubers (Appendix 1).

The distribution of CLso within an infected potato plant is inconsistent and the concentration of bacterial cells is generally low. This presents several diagnostic challenges. The aim of this experiment was to systematically sub-sample field grown infected potato plants to determine which tissue type was the most reliable for DNA extraction and CLso detection. This information will inform more reliable sampling procedures to improve detection of CLso in field grown potatoes.

A full report of test results is included in Appendix 1. Key findings include:

- Only one leaf sample (leaflet midrib) from a total of 130 leaf samples tested positive for CLso. This indicates that leaf tissue is not a reliable tissue for CLso detection.
- Below ground stem appears to be a reliable tissue to sample for CLso detection. However, below ground stem tissue does get woody and difficult to cut and homogenize when sampled late in the growing season. Daughter tubers were a reliable tissue source for the detection of CLso in this experiment. No obvious difference in the detection of CLso was observed between the rose and heel/stolon samples from daughter tubers in this experiment (Appendix 1, Figure 3). However, the concentration of CLso cells was, on average, equal to or slightly higher in the rose end of the tuber, when compared to heel/stolon end samples (Appendix 1, Figure 3).
- The CLso bacterium moved systemically from the inoculation point by the CLso-positive TPP on the main stem of the potato plant to the daughter tubers and to additional stems that had emerged from the mother tuber. However, it was difficult to detect CLso in the first 2 weeks following CLso-positive TPP release on to test plants.
- Zebra chip symptoms were visible on inoculated plants in week 4. The concentration of CLso cells was also highest in week 4, and cell copy number decreased in tuber, stem and petiole samples in week 5 (Appendix 1 Figures 3, 4 and 5). On average the concentration of CLso samples was higher in petioles that were sampled midway up the stem when compared to petiole samples collected from the top of a stem (Appendix 1, Figure 6).
- CLso was only detected in the main stem of the inoculated plant in Week 1 (Appendix 1, Figures 4 and 5). After week 1 CLso was detected in both the main stem, 2nd and 3rd stems (below ground) and petioles that were tested. This demonstrates the time required for CLso to move systemically from the point of inoculation on the main stem that the CLso-positive TPP fed on, down into the mother tuber and then migrate to the second and third stems that were tested. This movement is consistent with the movement of solutes in phloem tissue.

***Candidatus* Liberibacter solanacearum (CLso) distribution in field infected plants (Appendix 2).**

The distribution of CLso within a potato plant following field infection was tested and compared to the results from the cage trial. The plants in the cage trial were infested with CLso-positive TPP at a known time point. In order to test if the patterns in CLso distribution are the same for field infected plants, ten potato plants were collected from the PFR Lincoln farm and tested. The timing of infection and the route of infection (TPP or mother tuber) was unknown.

A full report of test results is included in Appendix 2. Key findings include:

- The below-ground stem and tuber were the most reliable tissue to sample for CLso detection.
- CLso was detected in multiple samples from plants not showing symptoms, but a higher proportion of samples taken from the symptomless plants tested negative for CLso. The average CLso copy number was higher in samples from plants with visible symptoms. This was true for all tissue types tested.
- The majority of leaf samples did not contain detectable levels of CLso

- The probability of detecting CLso in a leaf, petiole, stem and tuber sample collected from a symptomatic plant known to be CLso infected is 24%, 83%, 83% and 74%, respectively (Appendix 2: Table 1).
- The probability of detecting CLso in a leaf petiole, stem and tuber sample collected from an asymptomatic plant known to be infected with CLso is 12.5%, 37%, 36% and 70% respectively (Appendix 2: Table 1).
- Overall, the results show a similar pattern in CLso distribution and detection when compared to the Cage results (Appendix 1).

***Candidatus* Liberibacter solanacearum (CLso) detection in potato tubers (Appendix 3).**

Based on the results of this experiment, it is difficult to recommend that pooling of tuber samples is feasible. Given the uneven distribution of CLso within the tuber it is likely that each individual tuber needs to have more than one sample taken from it, all of which test negative. In addition, the tubers tested in this experiment were from a controlled experiment with a known inoculation time and potentially a higher inoculum pressure than would normally be experienced in the field. Further work is required to screen a broader range of potato cultivars, grown in the field, and which have been infected with CLso under natural conditions at various growth stages and preferably under different climatic conditions.

A full report of the experimental results is provided in Appendix 3. Key findings include:

- Sampling a core taken from the stolon end of the tuber proved to be a reliable tissue to test for CLso detection although it was not always the tuber tissue with the highest concentration of CLso (Table 2 Appendix 3).
- A high concentration of CLso was detected in the vascular ring and it may prove to be a reliable tissue for CLso detection.
- The qPCR detection of CLso in tubers without obvious vascular ring symptoms may be more difficult.
- The highest concentration of CLso detected in all of the tests conducted in this experiment was a Ct value of 28.39 from a sample of the vascular ring in tuber 1. The remaining CLso positives were Ct values greater than 30, including many “positive” samples recording a Ct of greater than 35. In a diagnostics context test results with Ct values this high (low pathogen titre equates to a high Ct, which is amplified towards the end of the qPCR cycles), with the assay only running for 40 cycles (Ct value of 40) would be considered inconclusive and re-sampling would be recommended, or the sample would be deemed negative for Ct values greater than 37.

Validation of the use of in-field isothermal assays for *Candidatus* Liberibacter solanacearum (CLso) on potato plants (Appendix 4).

Isothermal amplification methods offer a vast improvement over the common method of visual inspection of crops as symptoms first appear at least three weeks after initial infection, and positive field samples regularly do not show any visual alterations associated with CLso infection.

A full report of the experimental findings is provided in Appendix 4. Key findings include:

- The RPA method used for this study can detect less than 10^3 copies of the target sequence within a sample. In our experience this corresponds to over 80% of all field-collected CLso-infected samples.
- The assay did not produce any false-positive results during its assessment.

Candidatus Liberibacter solanacearum (CLso) Ring Test (Appendix 5).

AVR was able to develop a protocol to produce high quality homogenous samples for CLso plant material and deoxyribonucleic acid (DNA) panels for this ring test (adhering to import permit/quarantine guidelines). Collaborations with New Zealand PFR to receive material for sample preparation and the Australian National Quality Assurance program (ANQAP) for sample preparation, ring test execution and analysis of results was required.

Nine laboratories from eight regions across Australia and New Zealand participated in the CLso ring test were encouraged to perform any in-house protocols for the detection of CLso alongside the NDP (NDP18) for CLso (version 1.2, 2012).

SARDI collaboration

To maintain confidentiality of the participating laboratories, one contact at AVR distributed the ring test, liaised with laboratories, collected all results, processed results and generated the ring test report. The timing of the ring test and distribution of samples was coordinated with SARDI. For this, a list of potential participating laboratories and their primary contact was shared with SARDI. This allowed SARDI to distribute their quantitative polymerase chain reaction (qPCR) protocol, primer and probe reagents at the same time both ring test panels were distributed. Each participant returned all results to the ring test coordinator at AVR, who was able to remove any identifying features and then return the results to the laboratories. This method was implemented to ensure participant anonymity. In total there was 10 sets of results that used the SARDI method of detecting CLso

A full report of the experimental findings is provided in Appendix 5. Key findings include:

- All laboratories were successfully able to detect CLso using the methods described in the current NDP and adhered to deadlines.
- Variations in protocols were seen across participating laboratories for the kits used and template volume used.
- Higher variations in reported results were seen for samples that have lower titres of CLso.
- Individual reports will be returned to laboratories to allow assessment of their performance against other laboratories and address any corrective actions to achieve a better performance if needed.

Outcomes

With the establishment of TPP in Western Australia on-going surveillance and plant certification programs for both TPP and CLso will be required for Australia in the short and medium term. Accurate diagnostics is required to support these requirements. This project has provided:

- Science-based evidence on the distribution of CLso in field grown potatoes.
- Improved guidelines for sampling of potatoes to be included in the NDP for CLso (NDP 18).
- A validated field deployable tool (RPA) for the detection of CLso in-field.
- A validated high throughput protocol for testing potato tissue for CLso.
- Evidence for technical proficiency in detecting CLso using the CLso NDP in nine major node laboratories in Australia and New Zealand.

Significantly this is the first time that such a detailed and controlled experiment of this nature has been conducted. Findings from these experiments have resulted in sampling recommendations for the detection of CLso in potatoes that has been incorporated into the National Diagnostic Protocol (NDP 18) for the detection of CLso. The NDP is currently in SPHDs review and verification process and the project team are awaiting feedback.

Given the current gaps in diagnostic capacity for CLso sampling and testing globally, recommended sampling strategies from this project are likely to be implemented within 2 years of completion of the project in Australia and New Zealand and within 5 years internationally.

The ring test distributed as part of this project facilitated the training of diagnosticians from nine state and regional laboratories in Australia and New Zealand. The results of this ring test has facilitated the improvement of diagnostic standards within Australian and New Zealand laboratories.

To determine the distribution of CLso in a field grown potato an extensive field trail was established at the PFR Lincoln farm to systematically sample field grown potatoes known to be infected with a well characterised strain of CLso that is associated with the Zebra Chip disease, over a period of 6 weeks. Surprisingly, this form of controlled experiment has not previously been reported in the literature, rendering this study as one of the first detailed accounts of the natural distribution of CLso in a field grown potato plant. This field trial determined that:

- Leaf material is not a reliable tissue to sample for the detection of CLso.
- Below ground tissue (stem and tubers) is the most reliable tissue to sample for CLso detection.
- Daughter tubers were also a reliable tissue to sample, particularly from mother plants and tubers expressing symptoms associated with zebra chip disease.
- Detailed sub-sampling of individual potato tubers provided evidence of uneven distribution of CLso within the tuber. The stolon and rose end of the tuber, as well as the vascular ring was the most optimal tissue for sampling. Three tubers were dissected and subsampled extensively in this study. And even with this small sample size, what was of a particularly concern was the hit-miss pattern of CLso detection within the tuber. This inconsistent pattern of detection within the tuber needs to be factored into sampling guidelines for testing protocols.
- Plant tissue, including tubers, derived from asymptomatic plants (i.e. CLso infected, but not showing symptoms) generally generated higher Ct values, indicating a lower concentration of CLso, in the sample tested.

The above results were based on a controlled inoculation procedure of potato plants that had been sprouted in the field. That is, tubers were planted in November and after shoot emergence in early January, 10 CLso-positive TPP insects were placed at the base of the main stem of the potato plant. These CLso-positive psyllids were left to feed on the plant for 1 week. This level of inoculation pressure is likely to be higher than normal with naturally CLso infected potato plants.

To address this potential bias, a further 10 potato plants were sampled from the PFR farm, 6 plants were showing zebra chip symptoms, the remaining 4 plants were asymptomatic. The 10 CLso infected potato plants were the result of natural infection. Similar patterns of CLso distribution were observed in these plants with the below ground stem and tuber samples providing the most consistent results.

The concentration of CLso in CLso-positive plant samples was generally low in all the experiments conducted, with qPCR positive results emerging at relatively high Ct values. The CLso qPCR is run in the lab for 40 cycles (Ct = 40). The lower the Ct value (closer to 1) the higher is the concentration of the target DNA (in this case CLso DNA). The higher the Ct value (closer to 40), the lower is the concentration of the target DNA (in this case CLso DNA). The lowest Ct detected in all the assays conducted across two labs (both labs which were deemed proficient as part of the ring test) was a Ct of 28. The majority of Ct positive results were based on Ct values greater than 30. Of particular concern was a good proportion of these positives were greater than a Ct of 35, which is considered quite a low titre of the pathogen. These collective results do not support the possibility of reliably detecting CLso in pooled samples.

Based on these findings our recommendation for sampling potato plants to test for the presence of CLso is to sample below ground tissue (including daughter tubers). Our results do not support pooling of multiple potato plant samples into the one sample, such as the pooling of samples for other bacterial pathogens (*R. solanacearum and D. dianthicola*).

Uneven distribution of CLso within a tuber remains a concern. Although our sample size is only three tubers, the inconsistent patterns of distribution observed could indicate that multiple samples from the one tuber may be required to ensure CLso freedom.

The CLso NDP has been updated to reflect these findings and submitted to SPHD for ratification.

A high throughput capability for the screening of individual tuber samples for CLso has been established at the AVR labs at AgriBio. In addition to this, molecular protocols outlined in the NDP were able to detect CLso in tuber samples extracted using the adapted extraction method. However, based on current findings, a tuber testing strategy similar to that of the EPPO directive for other low titre vascular limited bacterial pathogens, which relies on concentrating the bacterial component of 200 tubers, is not recommended for seed certification of CLso at this point in time. This is primarily due to the uneven CLso distribution observed within individual tubers. Larger scale research is required to better understand the distribution of this organism within tubers of different cultivars to inform accurate sampling strategies. Additionally, for this tuber testing procedure to be used in a certification scheme, further validation of testing protocols and statistical analysis of the results would be required to assess the level of confidence that CLso can be detected in a subsample of tubers.

Additional findings from this study include evidence that the CLso pathogen can move systemically through the plant within 4 weeks of inoculation by CLso-positive TPP. This finding has implications for certification programs as it provides guidelines for management of seed potato crops and aligns with existing standards for management of insect vectored virus diseases. Seed potato crops need to be “burnt off” within 2-3 weeks post final inspection.

Further work is required to validate the findings presented above. As indicated, although this data was generated from field grown potatoes, the Moonlight potatoes were inoculated with CLso under controlled conditions. Further work is required to asses CLso infection in naturally infected potato plants and should include a variety of cultivars. It is likely that there are potato cultivars that are more tolerant, and more susceptible, than Moonlight to CLso infection. Natural CLso infection of potatoes by CLso positive TPP may well represent a lower inoculum pressure than that used in the experiments in this study. A lower inoculum pressure may result in longer periods of time for the pathogen to move systemically through the plant and build up levels of inoculum within the plant. This factor could have significant implications for the CLso qPCR test to detect the pathogen. The influence of climate and growing conditions also requires further investigation.

The RPA method for the in-field detection of CLso, that was validated in this study, can detect less than 10^3 copies of the target sequence within a sample. In our experience this corresponds to over 80% of all field-collected CLso-infected samples. Additionally, the assay did not produce any false-positive results during its assessment. This capability should be considered for adoption by field officers as it is rapid and easy to use.

Nine laboratories from eight regions across Australia and New Zealand participated in the CLso ring test using the National Diagnostic Protocol (NDP18) for CLso (version 1.2, 2012). All laboratories were successfully able to detect CLso using the methods described in the current NDP and adhered to deadlines. Variations in protocols were seen across participating laboratories for the kits used and template volume used. However, higher variations in reported results were seen for samples that have lower titres of CLso. The ring test proved to be a great training exercise for the participating labs and provides evidence to industry and biosecurity agencies that diagnostic capability to accurately detect CLso exists in multiple labs in Australia.

Monitoring and evaluation

The ring test distributed as part of this project facilitated the training of diagnosticians from nine state and regional laboratories in Australia and New Zealand. The reproducibility of the CLso diagnostic protocols was evaluated when test results from a range of laboratories were compared. The results of this ring test has facilitated the improvement of diagnostic standards within Australian and New Zealand laboratories. Diagnostic labs that produced discordant results for some of the panels have looked at in-house procedures and identified areas for improvement. Participation in the ring test in itself has provided hands-on training to technical staff.

Feedback from participants in the ring test was mostly poisitive.

Comments on the Ring test included:

- “A well-executed ring test.”
- “Thanks for the invite to participate in the ring test. A very worthy exercise for the group.”

Comments from users about the current NDP

- “The current NDP allows flexibility for laboratories to perform extraction methods and which PCRs that have been validated within that laboratory.”
- “For the nested cPCR described in the NDP, on page 25 it states “For nested PCR, the first-stage PCR products are diluted 1:25 (v/v) in water prior to re-amplification using the second-stage PCR primers” but on page 26 it states “For the second stage of the nested PCR for Ca. L. solanacearum pipette 19 µl of reaction mix into each PCR tube then add 1 µl of first stage PCR product as the template.””
- “Page 26, table 3 – for some reason I found this confusing (maybe just me though?), took a while to realise that the middle column for was reaction set up for both nested 1 and housekeeping PCR.”
- “Page 29, table 6 – states 50 cycles, I performed 40 cycles for my testing, the original paper also states 40 cycles.”
- “Section 4.5 on electrophoresis – could potentially be moved to section 4.4.2 or have this section arranged to have the entire conventional PCR procedure together.”

Feedback on the samples provided in the test panels included:

- “sample G gave a Nanoquant reading of 0. We conclude that there was no detectable DNA in this sample”
- -“Non-specific amplification from no template control on internal control primers with four different master mixes: Bioline MyFi master mix, Bioline MangoMix, Sigma PCR master mix and BioLab Phusion master mix. Phusion master mix is the best among the four tested reagents with only a very faint band on no template control.”
- “Sample G was difficult to get a housekeeper response (tested 4 times)”
- “The conventional PCRs and the quantitative PCR used by our lab all worked well and generally agreed with each other”

Recommendations

This project investigated key aspects of CLso infection and distribution in potato plants. Following are some recommendations as a result of these investigations.

It is recommended that:

- Leaf material is not used as sample tissue for CLso testing.
- Below ground tissue (stem and tubers) is the tissue of choice when sampling potato plants for CLso testing.
- Tubers be considered as the preferred tissue for sampling. However, further work is required to better understand the distribution of CLso within a tuber. One-centimetre cores taken from the stolon end of the tuber did produce relatively consistent results in this study. But there was also evidence of the stolon end core testing negative, even though the tuber from which the stolon core was taken from, was CLso positive. Further work is required to determine if each tuber needs to have multiple samples taken, and that each of these subsamples test negative to confirm absence of CLso in that tuber.
- The guidelines for sampling of potatoes be updated on the NDP for CLso (NDP 18) to reflect these findings.
- Sampling protocols, particularly for surveillance purposes, recognise that the concentration of CLso is likely to be lower in potato plants that are not showing symptoms of Zebra Chip.
- Caution be taken in considering pooling of samples taken from different potato plants/tissues into the one test sample. The consistently high Ct values generated by the qPCR for CLso-positive samples clearly indicates that this pathogen can be present at very low concentrations within potato plants. Pooling of multiple plants into the one test sample risks lowering the detection levels beyond the sensitivity of the qPCR assays recommended in the CLso NDP (NDP 18).
- Further research is required to determine the impact of natural inoculation via CLso positive TPP, potato cultivar and variable environmental conditions on CLso infection in potatoes. This data is required to further improve sampling protocols to support accurate detection of CLso in potatoes. The CLso inoculation procedure used in this study is likely to be considered as high inoculum pressure and results from naturally infected tubers may not entirely reflect the findings presented in this report. It is expected that in some instances, naturally infected tubers will have lower titres that will challenge detection levels of recommended qPCR assays. Potato cultivars may be more (or less) susceptible to CLso infection than the Moonlight variety used in this study, and temperature and water stress could also alter results. These factors may also change the time for the pathogen to migrate from the leaf/stem of the plant to the mother tuber, daughter tubers and systemically through the plant.
- That industry considers the adoption of the CLso specific RPA assay for the in-field detection of the zebra chip pathogen.
- That industry and jurisdictions support an on-going proficiency testing program to facilitate the maintenance of a national diagnostic capability for the Australian potato industry.

References

Andersen, M. T., Beever, R. E., Gilman, A. C., Liefting, L. W., Balmori, E., Beck, D. L., Sutherland, P. W., Bryan, G. T., Gardner, R. C., and Forster, R. L. S. 1998. Detection of Phormium yellow leaf phytoplasma in New Zealand flax (Phormium tenax) using nested PCR. Plant Pathology 47:188-196

Babu, B. et al., 2017. A field based detection method for Rose rosette virus using isothermal probe-based Reverse transcription-recombinase polymerase amplification assay. Journal of Virological Methods, 247, pp.81–90.

Beard SS, Pitman AR, Kraberger S, Scott IAW 2012. SYBR Green real-time quantitative PCR for the specific detection and quantification of '*Candidatus* Liberibacter solanacearum' in field samples from New Zealand. Eur J Plant Pathol 136(1): 203-215.

Fukuta, S. et al., 2003. Detection of tomato yellow leaf curl virus by loop-mediated isothermal amplification reaction. Journal of virological methods, 112(1-2), pp.35–40. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12951210 [Accessed March 26, 2018].

Levy J, Ravindran A, Gross D, Tamborindeguy C, Pierson E. 2011. Translocation of '*Candidatus* Liberibacter solanacearum', the Zebra Chip pathogen, in potato and tomato. Phytopathology 101(11): 1285-1291.

Li W, Abad JA, French-Monar RD, Rascoe J, Wen A, Gudmestad NC, Secor GA, Lee IM, Duan Y, Levy L 2009. Multiplex real-time PCR for detection, identification and quantification of 'Candidatus Liberibacter solanacearum' in potato plants with zebra chip. J Microbiol Meth 78(1): 59-65.

Mekuria, T.A., Zhang, S. & Eastwell, K.C., 2014. Rapid and sensitive detection of Little cherry virus 2 using isothermal reverse transcription-recombinase polymerase amplification. Journal of Virological Methods, 205, pp.24–30.

Notomi, T. et al., 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Research, 28(12), p.63e–63.

Piepenburg, O. et al., 2006. DNA Detection Using Recombination Proteins J. Haber, ed. PLoS Biology, 4(7), p.e204.

Rashed A, Workneh F, Paetzold L, Gray J, Rush CM. 2014. Zebra chip disease development in relation to plant age and time of '*Candidatus* Liberibacter solanacearum' infection. Plant Dis 98(1): 24-31.

Ravindran, A., Levy, J., Pierson, E., and Gross, D. C. 2011. Development of primers for improved PCR detection of the potato zebra chip pathogen, 'Candidatus Liberibacter solanacearum'. Plant Disease 95:1542-1546.

Weisberg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. Journal of Bacteriology 173:697–703

Weller SA, Elphinstone JG, Smith NC, Boonham N, Stead DE 2000. Detection of Ralstonia solanacearum strains with a quantitative, multiplex, real-time, fluorogenic PCR (TaqMan) assay. Appl Environ Microbiol 66(7): 2853-2858.

Intellectual property, commercialisation and confidentiality

No commercialisation or intellectual property arose from this body of work.

Acknowledgements

Hort Innovation as the industry partner and funding body

A majority of the work for this project was conducted at New Zealand PFR, Lincoln. Work involved people across the workplace and included involvement from people who maintain the farm site and collect samples/tubers across to laboratory personnel.

The ring test could not have been circulated without PFR supplying CLso-positive plant material and ANQAP staff for their advice and ongoing support throughout the ring test (from sample preparation and distribution to assistance with result collection).

Appendices

Appendix 1: Distribution of Candidatus Liberibacter solanacearum (CLso) in potato plants and tubers

Appendix 2: Candidatus Liberibacter solanacearum distribution in field infected plants

Appendix 3: Candidatus Liberibacter solanacearum (CLso) detection in potato tubers

Appendix 4: Validation of the use of in-field isothermal assays for Candidatus Liberibacter solanacearum (CLso) on potato plants

Appendix 5: Candidatus Liberibacter solanacearum (CLso) Ring Test

Appendix 1: Distribution of *Candidatus* Liberibacter solanacearum (CLso) in potato plants and tubers

Summary

The distribution of *Candidatus* Liberibacter solanacearum (CLso) within an infected potato plant is inconsistent and the concentration of bacterial cells is generally low. This presents several diagnostic challenges. The aim of this experiment was to systematically sub-sample field grown infected potato plants to determine which tissue type was the most reliable for deoxyribonucleic acid (DNA) extraction and CLso detection. This information will inform more reliable sampling procedures to improve detection of CLso in field grown potatoes.

Seed tubers were planted in cages and infested with CLso-positive tomato-potato psyllids (TPP) at flowering. Whole plants were removed and sampled at different time points. DNA was extracted from samples and tested using an internal host control and a CLso quantitative polymerase chain reaction (qPCR) test.

Below ground stem samples were found to be the most reliable for the detection of CLso. Only one leaf sample contained detectable amounts of CLso. Based on this result, using leaf material for the detection of CLso is not recommended.

Introduction

In order to accurately detect potato plants infected with CLso, it is important to understand the distribution of the bacterial cells within the plant. Previous studies have identified leaf and stem material as optimal tissue to test (Levy et al, 2011; Rashed et al, 2014). However, these experiments were performed overseas under different environmental and cultivation conditions, and the effect of these conditions on CLso distribution and titre are not well understood. Several of the previous studies in this area did not use large numbers of field samples and therefore, the robustness of this data remains questionable. Additionally, anecdotal evidence and experience from New Zealand questioned the appropriateness of leaves as the optimal sampling tissue.

To address these issues, a cage trial was established at the New Zealand Plant & Food Research (PFR) farm in Lincoln to provide potato plant and tuber samples for diagnostic testing. The plants were infested with CLso-positive TPP on a known date and the cultivation conditions were recorded. Whole plants were removed for sampling at known time points.

Methods

The trial was designed with six sampling time points and six replicates, resulting in a total of 36 cages (Figure 1). The layout was generated with GenStat using a Latin square design. There was one control cage included in each replicate. These cages did not have TPP released into them and were sampled on week six.

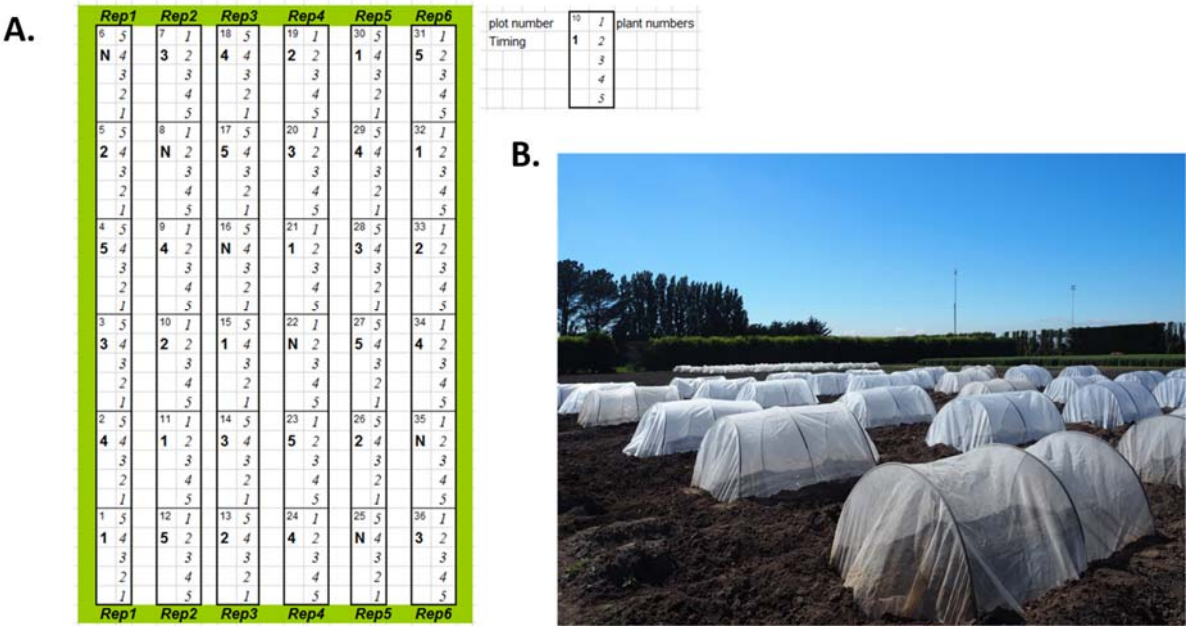

Figure 1: Cage trial design. A. Latin square design of cage trial with six replicates and six sampling time points. N = control cages which did not have TPP released in them. These cages were sampled n week 6. **B.** Cages following set up and planting of Moonlight tubers on 22nd November 2017. Photo is taken from the North West corner of the cages.

Tubers of the potato cultivar "Moonlight" (New Zealand; "Golden Delight" in Australia) were obtained from the PFR Lincoln farm and were left to sprout. Only tubers with strong sprouts were chosen for planting.

Cages were approximately 1 metre (m) x 2 m and consisted of 3 fibreglass poles and insect netting. Five tubers were evenly spaced and planted within each cage. Cages were set up and tubers planted on the 22nd November 2017. Cages were observed for plant emergence and plant height was estimated over the following 4 weeks. Weeds were controlled through glyphosate sprays and hand weeding throughout the trial.

TPP from CLso-positive colonies maintained at PFR Lincoln were used to inoculate the caged potato plants with CLso. The average CLso Ct (Beard et al., 2012) of the CLso-positive TPP colony for the past six months has averaged 16.2, indicating that the inoculum within a TPP individual is high. To inoculate the caged potato plants a tube containing 10 CLso-positive TPP was placed at the base of each plant in a cage on 9th January 2018, and the lid removed. The CLso-positive TPP were allowed to feed for one week before the cages were treated with Avid insecticide on the 16th January 2018 in order to kill the TPP.

The cages were further treated with Transform insecticide on the 31st January 2018 in order to control the aphid numbers inside the cages.

The first plants were removed from the cages six days after TPP release (15th January 2018 – "Week 1"). Plants were removed from cages for the following five weeks, with the control (no TPP) cages being sampled in the last week. Cages were entered from the South end. Plants 3 and 4 were removed for replicates 1, 3, and 5. Plants 2 and 3 were removed for replicates 2, 4, and 6. Whole plants, including tubers, were removed from the plot and immediately bagged. Twelve plants were

sampled each week, except for weeks 5 and 6. By week five many of the plants in the “week 5” cages had died off assumedly due to aphids.

A maximum of 21 sub-samples (Figure 2) were taken from each plant. These consisted of tuber, below ground stem, petiole and leaflet midrib samples. Below ground stems were cut to 1 centimetre (cm) in length and then quartered. Tuber samples consisted of cores which were approximately 1 cm^3 in size. Tuber and stem samples were also finely cut up. One cm lengths of petiole and leaflet midribs were taken. Petiole and leaf samples from the main stem, tuber, and below ground stem samples were homogenised immediately for DNA extraction, while petiole and leaf samples from stems 1 and 2 were freeze-dried and the DNA extracted at a later date.

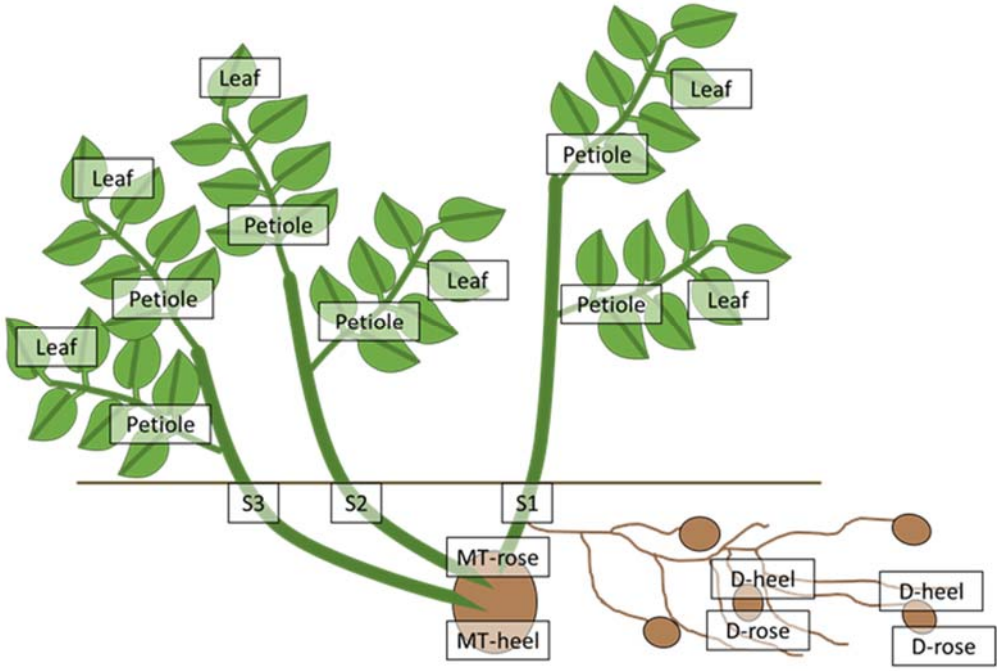

Figure 2: Planned sampling. A maximum of 21 samples were planned for collection from each plant sampled over the cage trial. The samples taken are shown by the white boxes and include; mother tubers, MT (rose and heel), two daughter tubers, D (rose and heel), three stems below ground, S, petioles from the top and middle of each stem and leaf (pooled midrib samples from leaflets of that leaf) from the top and middle of each stem.

Potato plant and tuber samples were homogenised by bead beating then DNA was extracted following the cetyltrimethylammonium bromide (CTAB) protocol described by Beard et al. (2012). Potato tissue culture plantlets were used as negative extraction controls and tomato plants known to be infected with CLso were used a positive extraction controls.

A qPCR specific to the potato cytochrome oxidase (COX) gene was used as an internal control to check the quality of the extracted DNA (Weller et al. 2000). The 16S-based assay developed by Li et al. (2009) was used to detect CLso. The two qPCR reactions were duplexed, and all samples and controls were tested in duplicate.

A dilution series of a plasmid with the CLso 16S and potato COX target sequences was used as standards on each qPCR plate. This enabled approximate copy numbers of CLso present in a sample to be determined and for samples to be compared between qPCR plates by standardising the results.

Results and Discussion

A total of 180 seed potatoes were planted in 36 cages for the cage trial. Only one plant did not emerge (Table 1). The high emergence rate meant that there were extra plants for sampling and this proved useful for weeks 5 and 6 when there was aphid damage on the plants. Plants that were not sampled in weeks 1-6 were left in the ground until March, thus undergoing a full growing cycle. The tubers from these plants were used for the tuber CLso distribution experiments.

Table 1: Summary of plants and sampling used in the cage trial

Cage trial	**Planned**	**Actual**
Tubers planted	180	180
Healthy plants that emerged	180	179
Plants sampled	72	77
Plants left for tuber harvest	107	102
Samples taken and extracted	1512	1180
qPCR (including samples, controls and standards)	4032	3168

The CLso-positive TPP were released onto the potato plants around flowering. Reliable detection of CLso did not occur until week three, however some samples were positive for CLso in weeks one and two. There were no visible symptoms on the plants until week four.

Leaf and petiole samples were not always able to be collected. This became more of a problem later in the season as the plants started to senesce. Many of the leaf samples that were collected did not amplify in the internal host control reaction (Figure 3). Only one leaf sample contained detectable levels of CLso. Based on these results we do not recommend leaf material for the detection of CLso.

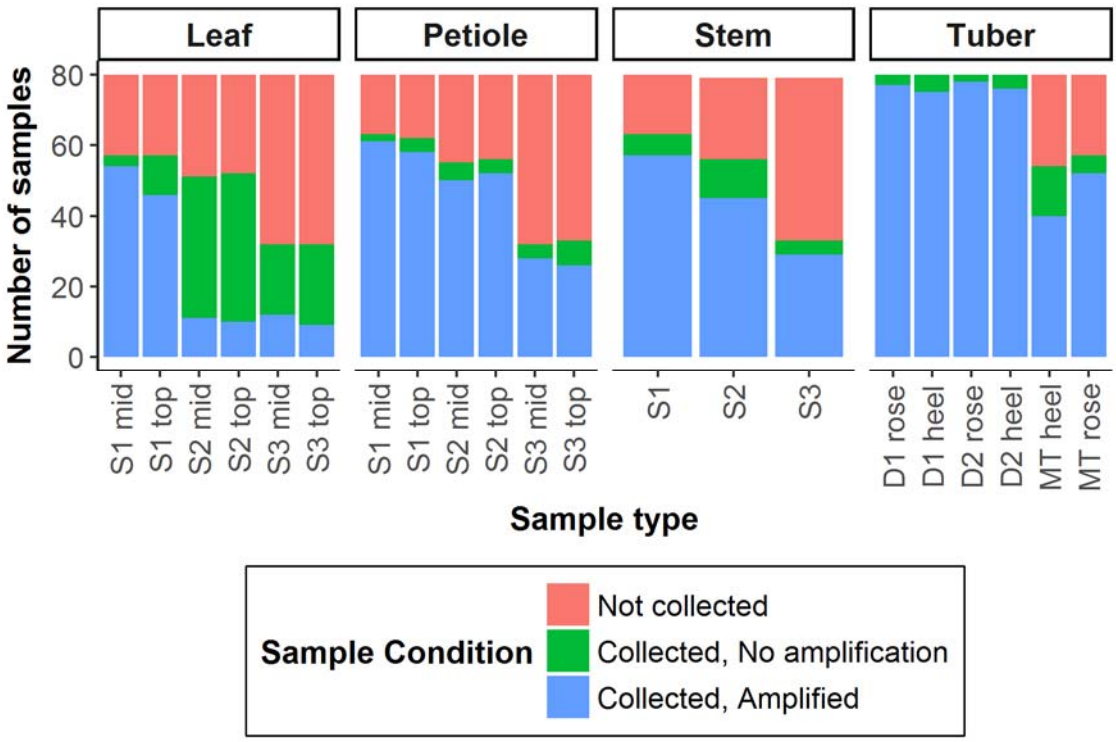

Figure 3: Sample reliability. A maximum of 21 samples were planned for collection from each plant sampled over the cage trial. Samples were not collected (shown in red) if they weren't present on the plant or if the plant was desiccated. The samples that were collected and tested but the internal control reaction did not amplify are shown in green, while the collected samples that did amplify are shown in blue. S1 = stem 1 (the main stem); S2 = stem 2; S3 = stem 3; mid = sample taken from mid-point of stem, top = sample taken from top of stem.

Below ground stem samples were the most reliable tissue for the detection of CLso (Figure 3 and 5), these parts are known to get woody and difficult to homogenise later in the season.

Daughter tubers were another sample type that provided reliable detection of CLso (Figure 3 and 4). CLso was detected in some tuber samples in weeks one and two, and in approximately half the samples in weeks three and four. There was no obvious difference in between rose or heel samples from the daughter tubers. Another advantage of sampling below ground stem or tubers is the higher titre in these samples compared to petiole or leaf.

On average the concentration of CLso samples was higher in petioles that were sampled midway up the stem when compared to petiole samples collected from the top of a stem (Figure 6).

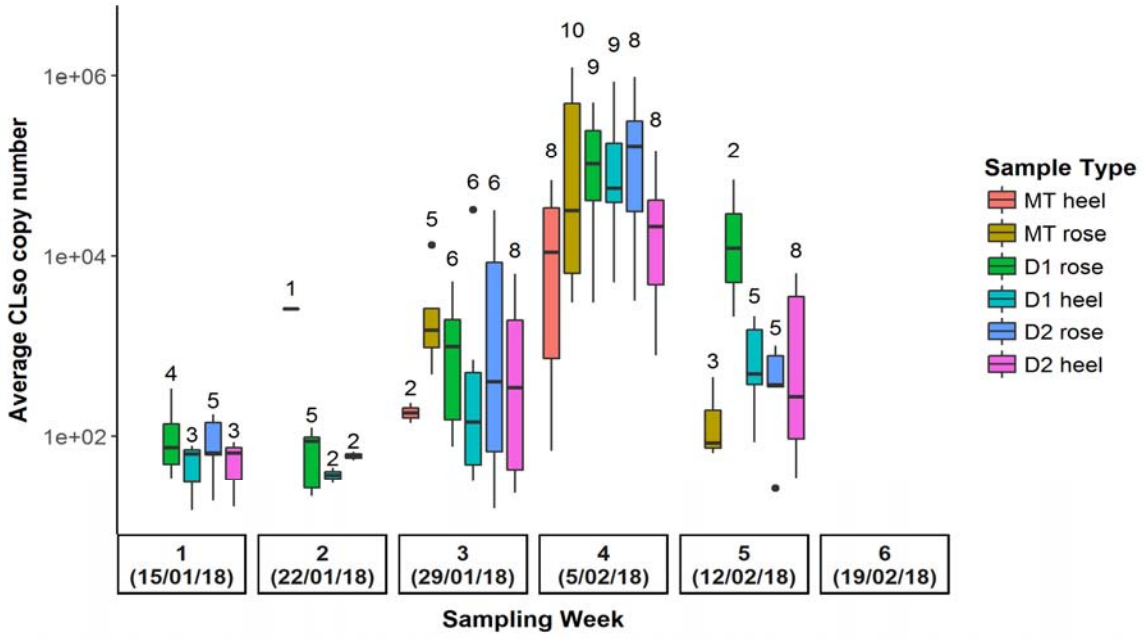

Figure 4: Average copy number of CLso copy number in tuber samples from the cage trial. The number of CLso-positive samples and the average CLso copy number increased from week 1 to week 4. The number of positive samples and the copy number decreased in week 5. The plants sampled in week six were from the control cages which did not have CLso-positive TPP released in them. The number of samples that were collected and had amplification in the host control qPCR test are indicated above the box plots. MT = mother tuber; D = daughter tuber.

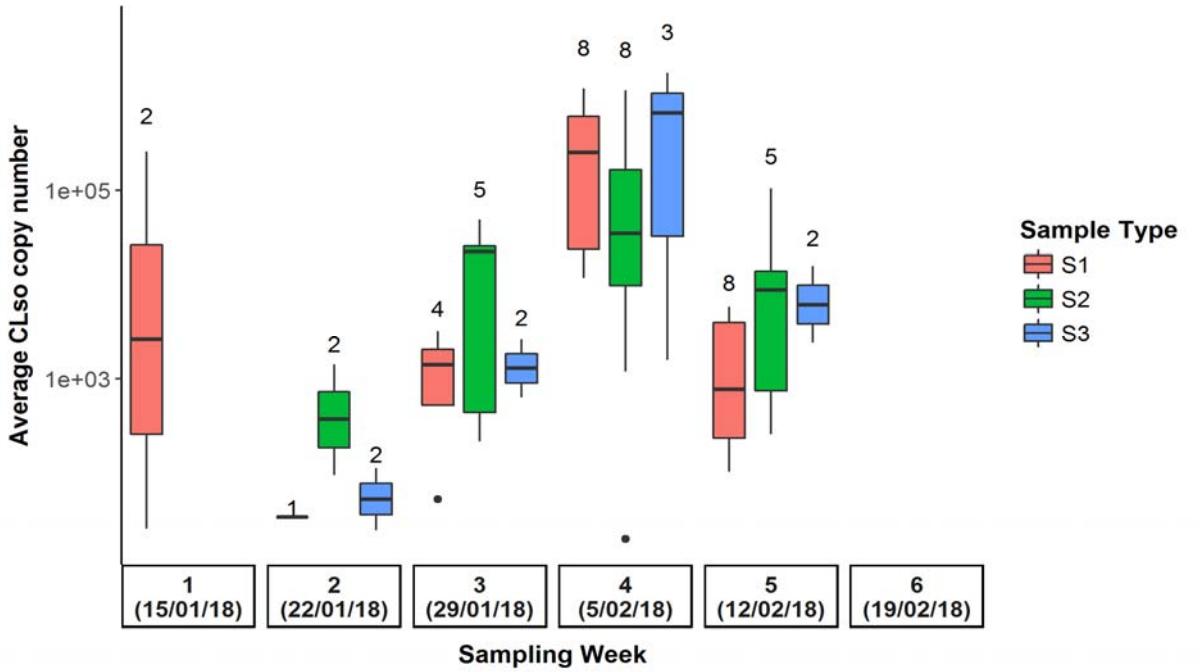

Figure 5: Average copy number of CLso copy number in below ground stem samples from the cage trial. The number of CLso-positive samples and the average CLso copy number increased from week 1 to week 4. The number of positive samples and the copy number decreased in week 5. The plants sampled in week six were from the control cages which did not have TPP released in them. The number of samples that were collected and had amplification in the host control qPCR test are indicated above the box plots. S1 = stem 1 (the main stem); S2 = stem 2; S3 = stem 3.

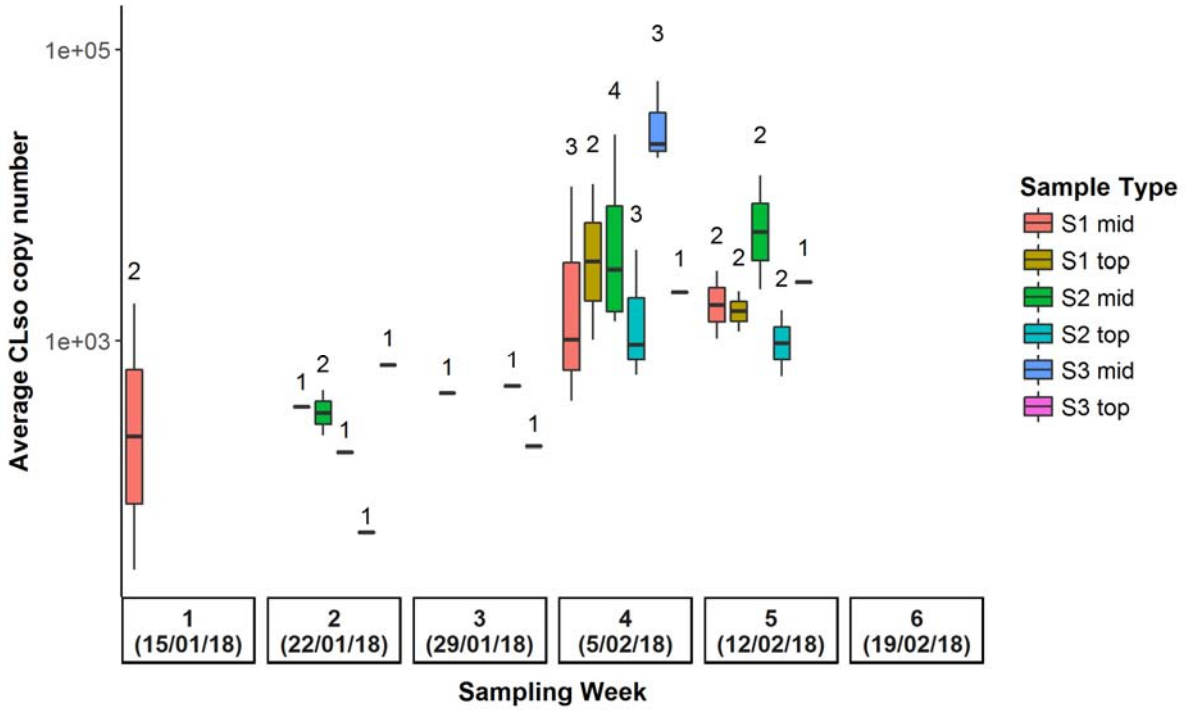

Figure 6: Average copy number of CLso copy number in petiole samples from the cage trial. The number of CLso-positive samples and the average CLso copy number increased from week 1 to week 4. The number of positive samples and the copy number decreased in week 5. The plants sampled in week six were from the control cages which did not have TPP released in them. The number of samples that were collected and had amplification in the host control qPCR test are indicated above the box plots. S1 = stem 1 (the main stem); S2 = stem 2; S3 = stem 3; mid = petiole sample from the midpoint of the stem; top = petiole sample from the top of the stem.

References

Beard SS, Pitman AR, Kraberger S, Scott IAW 2012. SYBR Green real-time quantitative PCR for the specific detection and quantification of '*Candidatus* Liberibacter solanacearum' in field samples from New Zealand. Eur J Plant Pathol 136(1): 203-215.

Levy J, Ravindran A, Gross D, Tamborindeguy C, Pierson E. 2011. Translocation of '*Candidatus* Liberibacter solanacearum', the Zebra Chip pathogen, in potato and tomato. Phytopathology 101(11): 1285-1291.

Li W, Abad JA, French-Monar RD, Rascoe J, Wen A, Gudmestad NC, Secor GA, Lee IM, Duan Y, Levy L 2009. Multiplex real-time PCR for detection, identification and quantification of 'Candidatus Liberibacter solanacearum' in potato plants with zebra chip. J Microbiol Meth 78(1): 59-65.

Rashed A, Workneh F, Paetzold L, Gray J, Rush CM. 2014. Zebra chip disease development in relation to plant age and time of '*Candidatus* Liberibacter solanacearum' infection. Plant Dis 98(1): 24-31.

Weller SA, Elphinstone JG, Smith NC, Boonham N, Stead DE 2000. Detection of Ralstonia solanacearum strains with a quantitative, multiplex, real-time, fluorogenic PCR (TaqMan) assay. Appl Environ Microbiol 66(7): 2853-2858.

Appendix 2: *Candidatus* Liberibacter solanacearum distribution in field infected plants

Introduction

The distribution of *Candidatus* Liberibacter solanacearum (CLso) within a potato plant following field infection was tested and compared to the results from the cage trial (Appendix 1). The plants in the cage trial were infested with CLso-positive tomato-potato psyllid (TPP) at a known time point. In order to observe if the patterns in CLso distribution are the same for field infected plants, ten potato plants were collected from the PFR Lincoln farm and tested. The timing of infection of these potato plants and the route of infection (TPP or mother tuber) was not known.

Methods

Ten potato plants were rogued from the PFR Lincoln farm. Six of the plants had obvious CLso symptoms and the remaining four plants had no obvious CLso symptoms. The plants consisted of a range of different varieties/cultivars.

A maximum of 21 tissue samples were taken from each plant. These consisted of tuber, below ground stem, petiole and leaf (consisting of multiple leaflet midribs from the same petiole) samples. Tuber samples were approximately 1 centimetre $(cm)^3$ cores. Below ground stems were cut to 1 cm in length and then quartered, and 1 cm lengths of petiole and leaflet midrib tissue were also taken. All samples were macerated and freeze-dried, and the deoxyribonucleic acid (DNA) was extracted at a later date.

Extraction of DNA was performed following the cetyltrimethylammonium bromide (CTAB) protocol described by Beard et al. (2012). Potato tissue culture plantlets were used as negative extraction controls and tomato plants known to be infected with CLso were used as positive extraction controls. Quantitative polymerase chain reaction (qPCR) was performed to detect CLso using the 16S-based assay developed by Li et al. (2009) and on a region of DNA within the potato, cytochrome oxidase (COX) gene, as an internal control to check the quality of the extracted DNA (Weller et al. 2000). The two qPCR reactions were duplexed, and all samples and controls were tested in duplicate. A dilution series of a plasmid with the CLso 16S and potato COX target sequences was used as standards. This enabled approximate copy numbers of CLso to be determined and for samples to be compared across qPCR plates.

Results and Discussion

The qPCR tests detected CLso in some samples from each of the ten field infected potato plants, despite not all of the plants having obvious CLso symptoms. However, less samples tested positive in symptomless plants when compared to symptomatic plants. The average CLso copy number was higher in sample from plants with visible symptoms. This was true for all tissue types tested.

Sample reliability was analysed (Figure 1; Table 1). Several mother tubers were not recovered from the plants, as would be expected close to harvest. A large proportion of leaf and tuber samples did not amplify in the internal control reaction suggesting these regions are less reliable samples for CLso detection.

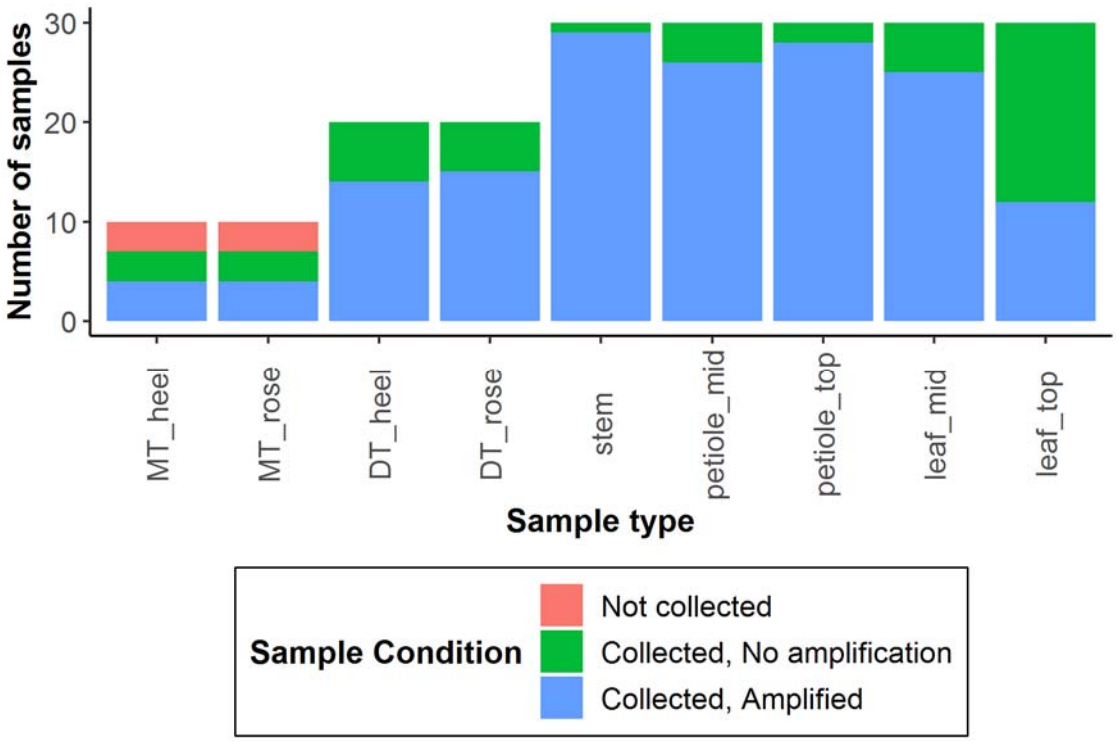

Figure 1: Analysis of sample reliability from field potato plants. A maximum of 21 samples were planned for each of the ten plants sampled. Samples were not collected (red) if they were not present on the plant. Samples that were collected but the internal control reaction did not amplify are shown in green, while the collected samples that did amplify are shown in blue. MT = mother tuber, DT = daughter tuber, mid = petiole or leaf sample from a mid-point on the stem, top = petiole or leaf sample from the top of the stem.

Table 1: Summary of CLso qPCR results

Symptomatic plants (6)

	Leaf	Petiole	Stem	Tuber
CLso-positive	5	29	15	20
CLso-negative	16	6	3	7
No amplification	15	1	0	7
Absent	0	0	0	2
Total	36	36	18	36

Asymptomatic plants (4)

	Leaf	Petiole	Stem	Tuber
CLso-positive	2	7	4	7
CLso-negative	14	12	7	3
No amplification	8	5	1	10
Absent	0	0	0	4
Total	24	24	12	24

All plants (10)

	Leaf	Petiole	Stem	Tuber
CLso-positive	7	36	19	27
CLso-negative	30	18	10	10
No amplification	23	6	1	17
Absent	0	0	0	6
Total	60	60	30	60

The below ground stem sample was the most reliable tissue type for CLso detection (Table 1, Figure 2). CLso was detected in 15/18 stems from plants with visible symptoms and 4/12 stems from plants without visible symptoms. The internal control reaction did not amplify for one of the stem samples.

Petiole tissue also gave good results for CLso detection (Table 1, Figure 2); 29/36 samples from plants with visible symptoms had detectable CLso and 7/24 samples from the plants without visible symptoms tested positive for CLso. There was a higher failure rate of the internal control reaction with these samples compared to the stem samples.

The majority of leaf samples did not contain detectable levels of CLso (Table 1, Figure 2). Only 7/60 samples were positive for CLso. Many of the samples did not amplify in the internal control reaction (23/60). These results are in agreement with the cage trial results (Appendix 1) and do not support the use of leaf material for CLso detection.

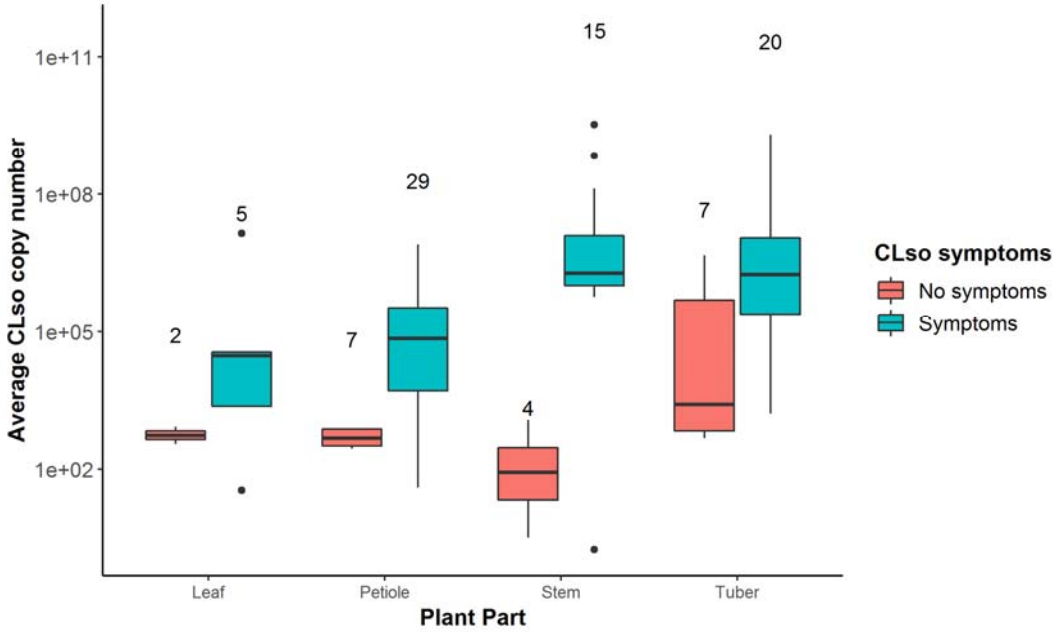

Figure 2: Average copy number of *Candidatus* Liberibacter solanacearum in field potato plant samples. The number of samples that were collected and had amplification in the internal control reaction are indicated above the box plots.

The Ct values for the CLso qPCR (Li et al., 2009) ranged from Ct 21-25 for CLso infected stems from symptomatic plants to Ct values greater than 35 for leaves, petioles and stems on non-symptomatic

plants. It is important to note that these Ct values greater than 35 are on the limits of detection and although they were considered CLso positive in this study, in many situations these Ct values would be considered inconclusive and result in re-sampling.

To summarise, the probability of detecting CLso in a leaf, petiole, stem, and tuber sample collected from a symptomatic plant known to be CLso infected is 24%, 83%, 83% and 74%, respectively (Table 1). The probability of detecting CLso in a leaf, petiole, stem and tuber sample collected from an asymptomatic plant known to be infected with CLso is 12.5%, 37%, 36% and 70% respectively (Table 1).

Conclusions

CLso-infected potato plants from the PFR Lincoln farm were dissected and tested for CLso distribution to test if the cage trial results were reflected. Testing these plants showed similar issues with sample availability and DNA extraction as the cage trial. DNA was not consistently extracted from leaf, petiole and tuber samples.

Overall these results show a similar pattern in CLso distribution and detection ability when compared to the cage trial results. The below ground stem and tubers is the most reliable tissue to sample. Leaf material is not very reliable as it may not be available for sampling especially later in the season and frequently does not have detectable levels of CLso despite the plant being infected. The titre of CLso was highest in plants with visible CLso symptoms, and less samples from plants showing no symptoms tested positive compared to symptomatic plants.

The reliability of the qPCR assay to detect CLso in plants known to be infected with this pathogen was only 74% in tubers harvested from symptomatic plants and 70% in tubers harvested from asymptomatic plants. Although the sample size for this experiment was 10, the implications of this result is significant, particularly when using this assay to certify plant material. Sampling procedures will need to be adjusted to accommodate a 70% sensitivity score the recommended CLso qPCR assay.

References

Beard SS, Pitman AR, Kraberger S, Scott IAW 2012. SYBR Green real-time quantitative PCR for the specific detection and quantification of 'Candidatus Liberibacter solanacearum' in field samples from New Zealand. Eur J Plant Pathol 136(1): 203-215.

Li W, Abad JA, French-Monar RD, Rascoe J, Wen A, Gudmestad NC, Secor GA, Lee IM, Duan Y, Levy L 2009. Multiplex real-time PCR for detection, identification and quantification of 'Candidatus Liberibacter solanacearum' in potato plants with zebra chip. Journal of Microbiological Methods 78(1): 59-65.

Weller SA, Elphinstone JG, Smith NC, Boonham N, Stead DE 2000. Detection of Ralstonia solanacearum strains with a quantitative, multiplex, real-time, fluorogenic PCR (TaqMan) assay. Appl Environ Microbiol 66(7): 2853-2858.

Appendix 3: *Candidatus* Liberibacter solanacearum (CLso) detection in potato tubers

Introduction

Currently, the best available method for detecting *Candidatus* Liberibacter solanacearum in potato tubers is the "fry" test. However, there are limitations to this test which include the fact that this assay is very subjective, it is not clear whether all infected potato cultivars will discolour, the assay has not been validated under Australian conditions and the assay is not intended for use in certification programs.

The stolon end of the tuber which attaches to the stem of the potato plant, has been recommended as the tissue to sample for symptomatic and non-symptomatic tubers using PCR (ICPP 2013 Draft Annex to ISPM 27 – Candidatus Liberibacter solanacearum (2013-001)). There is also a desire to apply high throughput tuber screening methods such as those employed by the European Plant Pathology Organisation (EPPO) for detection of *Dickeya* and *Ralstonia* species, which are able to screen 200 cores as one sample. However, the distribution of CLso and uniformity of infection throughout the tuber is unknown. It remains unclear which tissue is the most reliable tissue to sample from the tuber for testing.

The following experiments investigated the distribution of CLso within the potato tuber and tested the reproducibility of detection of CLso in a tuber using qPCR.

Methods

Experiment 1: Initial tuber testing

A total of 84 Moonlight (New Zealand; Golden Delight in Australia) tubers were obtained from the New Zealand Plant & Food Research (PFR) Lincoln farm. These seed tubers were cultivated the previous season under standard potato growing conditions and their CLso positive/negative status was unknown

Cores approximately 1 centimetre (cm) in diameter, were removed from the stolon end of 84 tubers for CLso screening. Extraction of deoxyribonucleic acid (DNA) was performed following the cetyltrimethylammonium bromide (CTAB) protocol described by Beard et al. (2012). The single-plex quantitative polymerase chain reaction (qPCR) to detect CLso using the 16S RNA-based assay developed by Beard et al. (2012) was also used to screen all cores. Two qPCR replicates were performed for all 84 core samples.

Additional screening using the qPCR protocol by Li et al. (2009) targetting the 16S RNA CLso region and a region of DNA within the potato, cytochrome oxidase (COX) gene (Weller et al. 2000), was also performed on 24/80 of the tuber cores. The COX screening serves as an internal control to check the quality of the extracted DNA (Weller et al. 2000). The two qPCR reactions were duplexed and two replicates were performed.

Experiment 2: Systematic sampling of three individual potato tubers

Three tubers were chosen for further testing of the whole tuber to determine if the core taken from the stolon end of the tuber was the most reliable sample (consistent and highest titre) for tuber testing of CLso. Tubers were dissected as described in Figure 1, with a maximum of 63 samples per tuber collected for testing. Quantitatve PCR was performed on all remaining samples in duplex as previously mentioned.

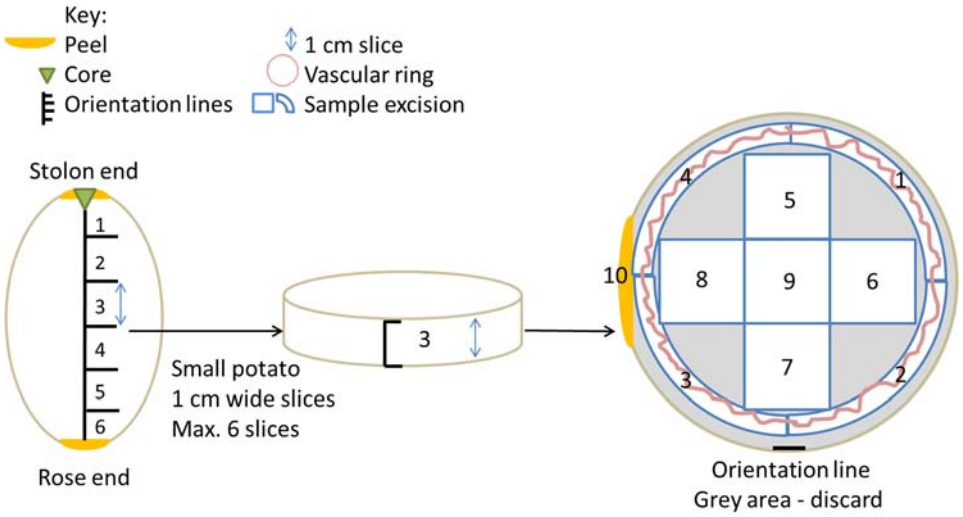

Figure 1: Schematic describing the sampling strategy for tubers. Samples 1-4 include the vascular ring, samples 5-8 include the outer medulla, sample 9 includes the pith and sample 10 is an external peel of the tuber slice

Experiment 3: Secondary tuber testing

For the secondary round of tuber testing, tubers from the cage trial (Appendix 1) were used as they were from plants known to be infected with CLso. Tubers were selected from plants left in cages until harvest on 28/03/2018. The harvested tubers were stored for several months before testing. Twenty tubers from ten cages that had CLso-positive TPP released, and two tubers from a control cage were dissected as described in Figure 1. Stolon and rose peel samples were taken using a potato peeler. Core samples, approximately 1 cm^3 in size were taken from the stolon end of the tuber (once peel was removed). Slices of the tuber were then cut and sampled, all samples were freeze-dried and shipped to AgriBio (Melbourne, Australia) for DNA extraction and qPCR testing.

A high throughput DNA extraction method was developed for the freeze-dried tuber samples in 96 well plate format. Initial screening of the core and peels (from the stolon and rose ends) was performed. A single 3 millimetre (mm) stainless steel ball bearing was added to each tube containing the tuber sample and pulverised using the GenoGrinder. CTAB was added to all samples and the MagJET Plant Genomic DNA kit (Thermo Fisher Scientific) was used to extract nucleic acids on the KingFisher Flex robot (Thermo Fisher Scientific). The duplex qPCR was performed for the detection of CLso and internal control using Li et al. (2009) and Weller et al. (2000) primers and probes

Results and discussion

Initial screening in Experiment 1 detected CLso in 21/84 (25%) core samples, using the Beard et al (2012) method and/or the Li et al (2009) method. Given that the CLso infection status of the 84 selected was previously unknown, it is unclear how effective sampling a core from the stolon end of the tuber is. All samples were positive for the COX internal control showing that the extraction method was successful indicating that extraction of DNA that can be amplified by Taq-based polymerases is reproducible.

Three tubers known to be infected with CLso were selected for further sampling of the entire tuber for the detection of CLso (Figure 1). CLso was detected throughout the entire first and second tubers, excluding the stolon peel (Table 1a and 1b). For both tubers, the highest levels of CLso was detected in the vascular ring sections, followed by the core, outer medulla section and section 10 peels. The levels of CLso appear to be higher in the rose end of the vascular ring for the first tuber (Table 1a), however for the second tuber (Table 1b) CLso concentration appears to be slightly higher throughout the middle of the tubers vascular ring.

Of the 44 individual qPCR assays conducted on tuber 1 (Table 1a) the lowest Ct (Li et al., 2009) value of 28.29 was obtained from the vascular ring sampled towards the rose end of the tuber. The average Ct value for the vascular ring, outer medulla, pith and slice peel samples was 33.15, 35.42, 35.85 and 36.80 respectively, with the core for tuber 1 resulting in a Ct of 34.80

Of the 53 individual qPCRs conducted on tuber 2 (Table 1b), the lowest Ct (Li et al., 2009) value of 30.89 was obtained in the vascular ring towards the middle of the tuber. The average Ct value for the vascular ring, outer medulla, pith and slice peel tissue was 33.07, 34.63, 34.46 and 36.48 respectively. The Ct value of the core for tuber 2 was 34.09.

The third tuber was selected for further testing as the CLso qPCR on the original core screening gave a negative result using the Li et al assay (2009). Further testing on samples collected from this tuber showed that the tuber was in fact infected with CLso and that the pathogen was detected mostly at the rose end of the tuber in the vascular ring, outer medulla and pith tissue (Table 1c). For this tuber, CLso was not detected in either the core or rose end peel but was detected in the stolon peel at a similar level to the pith samples (mid-range CLso detection). Of the 63 individual qPCR samples conducted on Tuber 3 only 32 were positive. The lowest Ct measured was 31.84 and found in the vascular ring towards the rose end of the tuber (Table 1c). The average Ct value of the vascular ring, outer medulla, pith and slice peel tissue was 36.42, 36.41, 35.12 and 38.64 respectively.

Table 1. Mapping of qPCR Ct values (Li et al., 2009) for each tuber. Red colouring shows the highest amount of CLso detected, yellow to orange show the mid-range levels of CLso and green shows the lowest amount of CLso detected. Tuber sections with uncoloured cells with are labelled as negative indicating no detection of CLso.

Table 1a. CLso detection mapping for tuber 1 (171206-53)

	Tuber 1 (171206- 53)													
	Vascular ring					Outer medulla					Pith	Peel			
Section	1	2	3	4	Average	5	6	7	8	Average	9	10	Stolon	Rose	Core
Slice 1	32.26	34.36	34.28	34.22	33.78	35.85	35.85	34.98	34.82	35.38	36.13	35.70	Negative		34.80
Slice 2	32.49	34.37	37.92	34.24	34.75	35.16	35.58	36.86	36.56	36.04	35.46	36.05			
Slice 3	32.84	33.33	35.54	33.97	33.92	34.59	34.51	34.93	35.39	34.86	34.64	37.65			
Slice 4	32.24	33.17	32.71	29.25	31.84	34.13	34.86	34.82	37.85	35.41	37.17	37.81			
Slice 5	31.31	28.29			29.80									32.36	
	Average				33.15	Average				35.42	35.85	36.80			
	Lowest Ct				28.29	Lowest Ct				34.13					
	Highest Ct				37.92	Highest Ct				37.85					

Table 1b. CLso detection mapping for tuber 2 (171211-24)

	Tuber 2 (171211- 24)														
	Vascular ring					Outer medulla					Pith	Peel			
Section	1	2	3	4	Average	5	6	7	8	Average	9	10	Stolon	Rose	Core
Slice 1	34.45	34.75	31.67	30.98	32.96	34.20	35.76	35.03	33.67	34.67	33.70	39.42	Negative		34.09
Slice 2	32.41	33.80	34.32	32.17	33.18	33.07	33.99	35.34	37.94	35.09	34.01	35.35			
Slice 3	32.35	33.31	30.89	31.68	32.06	33.80	34.10	34.77	33.39	34.01	36.12	36.91			
Slice 4	32.69	33.27	34.28	32.31	33.14	34.74	34.47	35.05	33.68	34.49	34.56	36.70			
Slice 5	34.14	33.52	35.66	32.84	34.04	34.56	34.38	34.22	36.40	34.89	33.91	34.04		34.07	
	Average				33.07	Average				34.63	34.46	36.48			
	Lowest Ct				30.89	Lowest Ct				33.07					
	Highest Ct				35.66	Highest Ct				37.94					

Table 1c. CLso detection mapping for tuber 3 (171211-22)

	Tuber 3 (171211- 22)														
	Vascular ring					Outer medulla					Pith	Peel			
Section	1	2	3	4	Average	5	6	7	8	Average	9	10	Stolon	Rose	Core
Slice 1	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	37.97	Negative	33.34		Negative
Slice 2	Negative	Negative	Negative	Negative	Negative	Negative	37.72	Negative	Negative	37.72	Negative	Negative			
Slice 3	37.15	36.03	Negative	38.96	36.59	35.16	Negative	Negative	Negative	35.16	34.33	39.69			
Slice 4	34.82	37.04	39.49	38.68	37.51	36.88	Negative	Negative	Negative	36.88	33.35	37.86			
Slice 5	32.97	37.19	Negative	31.84	35.08	32.82	38.80	34.57	38.95	36.29	34.81	38.38		Negative	
	Average				36.42	Average				36.41	35.12	38.64			
	Lowest Ct				31.84	Lowest Ct				32.82					
	Highest Ct				39.49	Highest Ct				38.95					

Various factors can affect transmission and distribution of CLso in tubers. Therefore, selecting tubers from the cage trial conducted at the PFR farm at Lincoln allowed a more in-depth, controlled experiment to assess the distribution of CLso in the 'Moonlight' ('Golden Delight') variety. Twenty tubers, all symptomatic for CLso with a darkened vascular ring, were selected for further testing. Core, stolon and rose peel tissues were sampled from each tuber and tested using the CLso qPCR by Li et al assay (2009) (Table 2). In this case the core sample did appear to be the most consistent sample to detect CLso (100% of cores), followed by the stolon peels (50%) and rose peels (20%).

Of the 20 positive core samples, the lowest Ct obtained was 30.98 and the highest Ct was 37.65. For the 10/20 positive stolon peel samples that tested positive, the lowest Ct was 33.66 and the highest Ct was 39.66. For the rose peel samples 4/20 tested positive, the lowest Ct was 37.34 and the highest was 39.99 (which is 0.01 Ct from being undetected completely by the qPCR). The two negative control tubers, which originated from a negative control plot and appeared symptomless, were negative for CLso in the core, stolon and rose end peels.

Table 2. Screening of 20 tubers that were a result of the cage trial (Appendix 1). All samples tested in duplicate, results marked with an asterisk (*) denote results where only one of the duplicates tested positive for CLso.

	Tuber sample		
Tuber	**Core**	**Stolon peel**	**Rose peel**
1	34.74	Negative	37.34
2	33.35	Negative	Negative
3	34.71	Negative	Negative
4	31.03	Negative	Negative
5	32.99	Negative	Negative
6	32.82	37.54	Negative
7	32.14	36.05	Negative
8	31.97	34.90	36.44
9	34.12	35.18	*39.99
10	31.65	*39.66	Negative
11	34.43	37.29	38.78
12	35.72	38.73	Negative
13	36.41	33.66	Negative
14	33.94	38.73	Negative
15	34.53	Negative	Negative
16	32.59	33.96	Negative
17	32.53	Negative	Negative
18	35.02	Negative	Negative
19	37.46	Negative	Negative
20	36.01	Negative	Negative
Lowest Ct	30.98	33.66	37.34
Highest Ct	37.64	39.66	39.99
Number of positive samples	20	10	4
Percentage of positive samples	100%	50%	20%

Summary

In this experiment, sampling the core did prove to be a reliable tissue to test for CLso detection although it was not always the tuber tissue with the highest concentration of CLso. A high concentration of CLso was detected in the vascular ring and it may also prove to be a reliable tissue for CLso detection.

However, this finding needs to be considered carefully as the CLso positive tubers that were subsampled and tested in this study were derived from mother plants that were infected two months after planting with 10 CLso positive tubers that were left to feed on the mother plant for a week (Appendix 1). This is likely to be higher inoculum pressure than would normally be experienced in the field. The CLso infected plants were then left to grow for a further two months before senescence. It is feasible to consider that plants grown under normal field conditions may be infected later in the season, and that other potato cultivars may be more tolerant to CLso infection than Moonlight/Golden Delight.

Further work is required to understand CLso transmission and distribution for symptomatic and non-symptomatic tubers. The results generated in this experiment indicate that the qPCR detection of CLso in tubers without obvious vascular ring symptoms is still poorly understood. The Ct values from the CLso positive tuber that was harvested from a symptomless mother plant (tuber 3) were higher than the Ct values obtained from the tubers from the symptomatic plants. Although not unsurprising it does demonstrate that CLso can be present in a very low concentration in the tuber.

If the core is determined to be the most reliable sample point for CLso in tubers, further work is also needed to assess the likelihood of detecting one CLso positive core in pooled samples, with the ultimate goal of pooling of one infected tuber amongst 199 negative cores. Application of this testing will allow high throughput screening of CLso with other vascular pathogens such as *Dickeya* and *Ralstonia* species. Given the results obtained in this study, this outcome appears to be less feasible. However, the highest concentration of CLso detected in all of the tests conducted in this experiment was a Ct value of 28.39 from a sample of the vascular ring in tuber 1. The remaining CLso positives were Ct values greater than 30, including many “positive” samples recording a Ct of greater than 35. In a diagnostics context Ct values in this higher range, would be considered inconclusive and re-sampling/testing would be recommended, or the sample would be deemed negative for Ct values greater than 37.

Based on the results of this experiment, it is difficult to recommend that pooling of tuber samples is feasible. Given the uneven distribution of CLso within the tuber it is likely that each individual tuber needs to have more than one sample taken from it, all of which need to test negative in order for the tuber to be deemed negative for CLso. Further work is required to screen a broader range of potato cultivars, grown in the field, and which have been infected with CLso under natural conditions and preferably under different climatic conditions.

References

Beard SS, Pitman AR, Kraberger S, Scott IAW 2012. SYBR Green real-time quantitative PCR for the specific detection and quantification of 'Candidatus Liberibacter solanacearum' in field samples from New Zealand. Eur J Plant Pathol 136(1): 203-215.

Li W, Abad JA, French-Monar RD, Rascoe J, Wen A, Gudmestad NC, Secor GA, Lee IM, Duan Y, Levy L 2009. Multiplex real-time PCR for detection, identification and quantification of 'Candidatus Liberibacter solanacearum' in potato plants with zebra chip. Journal of Microbiological Methods 78(1): 59-65.

Weller SA, Elphinstone JG, Smith NC, Boonham N, Stead DE 2000. Detection of Ralstonia solanacearum strains with a quantitative, multiplex, real-time, fluorogenic PCR (TaqMan) assay. Appl Environ Microbiol 66(7): 2853-2858.

Appendix 4: Validation of the use of in-field isothermal assays for *Candidatus* Liberibacter solanacearum (CLso) on potato plants

Summary

Isothermal amplification methods offer a vast improvement over the common method of visual inspection of crops as symptoms first appear at least three weeks after initial infection, and positive field samples regularly do not show any visual alterations associated with CLso infection. The Recombinase polymerase amplification (RPA) method used for this study can detect less than 10^3 copies of the target sequence within a sample. In our experience this corresponds to over 80% of all field-collected CLso-infected samples. Additionally, the assay did not produce any false-positive results during its assessment.

Introduction

For effective management of CLso in the field, early detection is paramount. Currently, molecular detection methods can take several days to be completed as the sample needs to be collected, shipped to an appropriate laboratory, prepared, tested, and finally results reported back to the inspector. Over the past 15 years, a range of field-deployable technologies for molecular detection of plant pathogens have been introduced (e.g. Fukuta et al. 2003; Mekuria et al. 2014; Babu et al. 2017), including Loop-mediated isothermal amplification (LAMP) (Notomi et al. 2000) and RPA (Piepenburg et al. 2006). Both LAMP and RPA are based on isothermal amplification: in contrast to traditional PCR methods, these methods do not cycle through different temperatures to achieve dissociation of the DNA, annealing of primers and extension to achieve amplification. Instead, specific enzymes are used to undertake the melt, anneal and amplification phases. This makes isothermal amplifications simple, fast and robust.

For RPA, a recombinase enzyme is used bind primers to DNA. A single strand binding protein prevents the displacement of the primers, and a strand-displacing polymerase then elongates from the primer. The RPA is targeted at a unique sequence only present in CLso and is therefore highly specific for this organism.

The LAMP assay protocol has a number of critical criteria that must be met. It is not suitable for organisms with a low GC genome content such as CLso due to the specific secondary structures of its primers. Additionally, it has no multiplexing capacity, making DNA extraction controls and internal amplification controls more complicated. And its complex design, consisting of up to 10 individual primers, presents a challenge during development, and optimisation of the LAMP assay. The RPA assay does not have these disadvantages. RPA can amplify target sequence form low GC genomes, has multiplexing capacity through the use of different probes, and due to a significant lower number of components, it is more robust and can easily be optimised.

Methods

In this study, an RPA was developed at Plant & Food Research, Lincoln to detect CLso in a selection of cage trial samples. A range of samples from the pool generated for the qPCR analysis was selected based on which plant and which part of the plant they represent as well as the copy number of CLso determined by qPCR. Those samples are listed in Table 1.

RPA assays were performed using a TwistAmp®exo kit (TwistDX, Cambridge, UK), which allows the detection of amplified product by fluorescence probe, according to the manufacturer's recommendations. Reactions were incubated for 20 minute in a T8 or T16 isothermal amplification instrument (Axxin, Melbourne, Australia) at 42°C with measurement of fluorescence every 30

seconds. Each assay included a positive and negative control as well as 3 samples in duplicate. The result of a reaction was considered positive if exponential amplification occurred and an assay-specific threshold was reached.

Results and Discussion

As can be seen in table 1, the RPA was able to correctly identify all samples with a copy number higher than 1000. This is in agreement with the previously documented detection range of this CLso detection method (Kalamorz et al., unpublished data). A copy number of 1000 or higher is encountered in more than 80% of all field-collected samples of infected plants (Kalamorz et al., unpublished data). Although a rate of 20% false-negatives may seem high, this problem is in practice easily circumvented by testing multiple samples from each plant and multiple plants at each site.

Table 1: Results of RPA

No.	Cage	Plant	Sample ID	Sample type	Copy number	Ct*	RPA result
1	3	4	3A12	Tuber D2 heel	0.00		Negative
2	7	2	3C02	Stem (S2)	0.00		Negative
3	7	3	3D08	Tuber M rose	0.00		Negative
4	28	4	3I01	Stem (S1)	0.00		Negative
5	28	4	3I17	Petiole (S2 top)	0.00		Negative
6	2	4	4A13	Petiole (S1 top)	0.00		Negative
7	24	2	4G09	Tuber D1 rose	0.00		Negative
8	34	2	4K17	Petiole (S2 top)	0.00		Negative
9	31	2	5K01	Stem (S1)	0.00		Negative
10	13	2	5O01	Stem (S1)	0.00		Negative
11	31	2	5K13	Petiole (S1 top)	175	37.1	Negative
12	14	4	3E02	Stem (S2)	386	35.3	Negative
13	27	3	5J11	Tuber D2 rose	907	35.5	Negative
14	36	3	3L08	Tuber M rose	979	34.0	Negative
15	18	4	4E17	Petiole (S2 top)	988	38.4	Positive
16	2	4	4A14	Petiole (S2 mid)	1145	38.1	Positive
17	36	2	3K08	Tuber M rose	1693	33.3	Positive
18	5	1	5N01	Stem (S1)	1828	33.7	Positive
19	2	3	4B12	Tuber D2 heel	1897	34.5	Positive
20	3	4	3A09	Tuber D1 rose	2063	32.7	Positive
21	14	3	3F03	Stem (S3)	2768	32.4	Positive
22	27	4	5I01	Stem (S1)	2859	33.7	Positive
23	27	3	5J12	Tuber D2 heel	3615	33.8	Positive
24	34	3	4L07	Tuber M heel	4904	34.2	Positive
25	5	2	5M01	Stem (S1)	5678	32.7	Positive
26	3	3	3B12	Tuber D2 heel	6184	31.1	Positive
27	27	4	5I12	Tuber D2 heel	6942	32.9	Positive
28	12	3	5D02	Stem (S2)	8830	32.1	Positive
29	12	3	5D03	Stem (S3)	9723	31.9	Positive
30	34	2	4K07	Tuber M heel	75001	30.4	Positive

No. – Number of sample. Cage, plant – gives the position of the plant in the field trial set-up. Sample ID – unique identifier of each sample. Sample type – description of sample. Copy number – copy number as determined using qPCR diagnostic. RPA result – negative = no CLso detected, positive = CLso detected. *Ct values have not been adjusted for plate-to-plate variation, and copy numbers should be used.

In summary, the RPA provides a fast, robust and reliable method which is easily deployable in the field, for example in the back of a car or a temporary testing station. In contrast to sampling and processing in a laboratory, which usually takes between 2 and 5 days depending on shipping times and methods, RPA sampling and results can be generated within an hour of arriving on site.

References

Babu, B. et al., 2017. A field based detection method for Rose rosette virus using isothermal probe-based Reverse transcription-recombinase polymerase amplification assay. Journal of Virological Methods, 247, pp.81–90. Available at: http://www.ncbi.nlm.nih.gov/pubmed/28583856 [Accessed March 26, 2018].

Fukuta, S. et al., 2003. Detection of tomato yellow leaf curl virus by loop-mediated isothermal amplification reaction. Journal of virological methods, 112(1-2), pp.35–40. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12951210 [Accessed March 26, 2018].

Mekuria, T.A., Zhang, S. & Eastwell, K.C., 2014. Rapid and sensitive detection of Little cherry virus 2 using isothermal reverse transcription-recombinase polymerase amplification. Journal of Virological Methods, 205, pp.24–30. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24797461 [Accessed March 26, 2018].

Notomi, T. et al., 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Research, 28(12), p.63e–63. Available at: https://academic.oup.com/nar/article-lookup/doi/10.1093/nar/28.12.e63 [Accessed March 27, 2018].

Piepenburg, O. et al., 2006. DNA Detection Using Recombination Proteins J. Haber, ed. PLoS Biology, 4(7), p.e204. Available at: http://dx.plos.org/10.1371/journal.pbio.0040204 [Accessed March 27, 2018].

Appendix 5: Candidatus Liberibacter solanacearum (CLso) Ring Test

Summary

Nine laboratories from eight regions across Australia and New Zealand participated in the CLso ring test were encouraged to perform any in-house protocols for the detection of CLso alongside the National Diagnostic Protocol (NDP18) for CLso (version 1.2, 2012).

All laboratories were successfully able to detect CLso using the methods described in the current NDP and adhered to deadlines. Variations in protocols were seen across participating laboratories for the kits used and template volume used. Higher variations in reported results were seen for samples that have lower titres.

Individual reports were returned to laboratories to allow assessment of their performance against other laboratories and address any corrective actions to achieve a better performance if needed.

AVR was able to develop a protocol to produce high quality homogenous samples for CLso plant material and deoxyribonucleic acid (DNA) panels for this ring test (adhering to import permit/quarantine guidelines). Collaborations with New Zealand Plant & Food Research (PFR) to receive material for sample preparation and the Australian National Quality Assurance program (ANQAP) for sample preparation, ring test execution and analysis of results was required.

SARDI collaboration

To maintain confidentiality of the participating laboratories, one contact at AVR distributed the ring test, liaised with laboratories, collected all results, processed results and generated the ring test report. The timing of the ring test and distribution of samples was coordinated with SARDI. For this, a list of potential participating laboratories and their primary contact was shared with SARDI. This allowed SARDI to distribute their quantitative polymerase chain reaction (qPCR) protocol, primer and probe reagents at the same time both ring test panels were distributed. Each participant returned all results to the ring test coordinator at AVR, who was able to remove any identifying features and then return the results to the laboratories. This method was implemented to ensure participant anonymity. In total there was 10 sets of results that used the SARDI method of detecting CLso

Ring test design and laboratory instructions

This ring test was developed under the guidance of the ANQAP, based at AgriBio. One of the highest priorities of a quality assurance program is to ensure participating laboratory confidentiality.

CLso is exotic to Australia. Therefore, the original material used in the ring test was generated at Plant and Food Research in Lincoln and freeze dried before transport- a strict requirement of the import permit. Extensive preparation was required for the preparation and additional transfer to each laboratory of these samples under quarantine restrictions.

Samples for the ring test were generated with a high homogenous quality for both freeze-dried plant tissue and for nucleic acids.

The current National Diagnostic Protocol (NDP) is currently under review. Therefore, laboratories were encouraged to perform their own standard operating procedures (SOPs) and any additional testing methods, in addition to the methods described in the NDP. This enabled novel methods to be

assessed on both panels. Various methods were performed, predominantly those described in the NDP, but also included magnetic bead extraction methods and various conventional and qPCR detection methods.

Only protocols described in the NDP were included in the overall ring test report.

Challenging samples, with high to low levels of CLso were intentionally included in this ring test. This was able to give laboratories a chance at assessing their detection limits for the pathogen that is known to be present at varying titres.

A document containing instructions was circulated to all participants and can be seen in Appendix 5a.

Results

All laboratories were successfully able to detect CLso using the methods described in the current NDP and adhered to the prescribed deadlines. Results were classed as concordant, concordant with minor variation and discordant.

A copy of the ring test report can be seen in Appendix 5b.

Variations in protocols were seen across participating laboratories for the kits used and template volume used. Higher variations in reported results were seen for samples that had lower titres. For Li et al 2009 qPCR and the conventional nested polymerase chain reaction (cPCR) method variation in input template volume appeared to cause variation.

A range in extraction kits and methods, cPCR and qPCR kits that were used by participating laboratories are stated in Table 1. Additional methods used by participating laboratories for the detection of CLso are mentioned in Table 2.

Table 1: Extraction, cPCR and qPCR kits used by participants of the ring test.

Extraction kits	Qiagen DNeasy plant kit Promega Maxwell RSC FFS DNA Kit In house method Bioline, Isolate II Plant DNA Kit
cPCR kits	Promega GoTaq G2 green mastermix Invitrogen™ - Platinum™ Taq DNA Polymerase Promega GoTaq® Green Master Mix BioLine MyFi master mix Bioline MyTaq HS Mix (cat.#BIO-25045). Qiagen HotStarTaq Plus 2 x MyFi
qPCR kits	Qiagen Rotor-gene multiplex PCR Applied Biosystems TaqMan Fast Advanced Master Mix Promega GoTaq® probe qPCR Master Mix RealMasterMix Probe (5 Prime) Qiagen QuantiTect Probe Kit Bioline Immolase Invitrogen Platinum Taq Invitrogen Platinum Quantitative PCR Supermix-UDG AgPath-ID™ One-Step RT-PCR Reagents Bioline qPCR master mix

Table 2: Additional PCR protocols performed by participating laboratories

Additional methods performed for CLso	LsoTx primers Ravindran et al. 2011 cPCR in house qPCR in house SARDI in house Plant and Food Research Lincoln in house
Additional housekeepers/internal controls	Gd1 and Berg54 16S rRNA Andersen et al. 1998 Fd1/rp2 16S rRNA Weisberg et al. 1991 qPCR COX in house COX plant material Weller et al. 2000

Monitoring and Evaluation of the Ring test:

Ring test feedback from laboratories

- "A well-executed ring test."
- "Thanks for the invite to participate in the ring test. A very worthy exercise for the group."

Comments from users about the current NDP

- "The current NDP allows flexibility for laboratories to perform extraction methods and which PCRs that have been validated within that laboratory."
- "For the nested cPCR described in the NDP, on page 25 it states "For nested PCR, the first-stage PCR products are diluted 1:25 (v/v) in water prior to re-amplification using the second-stage PCR primers" but on page 26 it states "For the second stage of the nested PCR for *Ca.* L. solanacearum pipette 19 µl of reaction mix into each PCR tube then add 1 µl of first stage PCR product as the template.""
- "Page 26, table 3 – for some reason I found this confusing (maybe just me though?), took a while to realise that the middle column for was reaction set up for both nested 1 and housekeeping PCR."
- "Page 29, table 6 – states 50 cycles, I performed 40 cycles for my testing, the original paper also states 40 cycles."
- "Section 4.5 on electrophoresis – could potentially be moved to section 4.4.2 or have this section arranged to have the entire conventional PCR procedure together."

Feedback on the samples

- "sample G gave a Nanoquant reading of 0. We conclude that there was no detectable DNA in this sample"
- "Non-specific amplification from no template control on internal control primers with four different master mixes: Bioline MyFi master mix, Bioline MangoMix, Sigma PCR master mix and BioLab Phusion master mix. Phusion master mix is the best among the four tested reagents with only a very faint band on no template control."
- "Sample G was difficult to get a housekeeper response (tested 4 times)"
- "The conventional PCRs and the quantitative PCR used by our lab all worked well and generally agreed with each other"

References

Ravindran, A., Levy, J., Pierson, E., and Gross, D. C. 2011. Development of primers for improved PCR detection of the potato zebra chip pathogen, 'Candidatus Liberibacter solanacearum'. Plant Disease 95:1542-1546.

Andersen, M. T., Beever, R. E., Gilman, A. C., Liefting, L. W., Balmori, E., Beck, D. L., Sutherland, P. W., Bryan, G. T., Gardner, R. C., and Forster, R. L. S. 1998. Detection of Phormium yellow leaf phytoplasma in New Zealand flax (Phormium tenax) using nested PCR. Plant Pathology 47:188-196

Weisberg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. Journal of Bacteriology 173:697–703

Weller, S. A., Elphinstone, J. G., Smith, N. C., Boonham, N., & Stead, D. E. (2000). Detection of Ralstonia solanacearum strains with a quantitative, multiplex, real-time, fluorogenic PCR (TaqMan) assay. Applied Environmental Microbiology 66:2853–2858.

Appendix 5a: *Candidatus* Liberibacter solanacearum (CLso) Ring Test Participants Information

Candidatus Liberibacter solanacearum (CLso) Ring Test Participants Information

Important notes for recieveing samples

- Complete section "5 Sign-off" the Biological Movement Record (attached and emailed) and return to AgriBio Compliance agribio.compliance@ecodev.vic.gov.au and Jacqui Morris jacqui.morris@ecodev.vic.gov.au
- Check that all samples are intact (no signs of damaged vials/tubes)
- Please, notify Jacqui Morris immediately if the package or the material received is damaged or material is missing.

Contents of Test Package

#	**Sample / Item ID** *(unique identifier)*	**Taxon** *(common and scientific name)*	**Sample type** *(tissue or culture type, fresh/lyophilised, etc.)*
1	Panel 1, Sample A, Biosecurity material	Solanum lycoperisicum infected with Candidatus Liberibacter solanacearum	<0.01 grams of freeze-dried
2	Panel 1, Sample B, Biosecurity material	Solanum lycoperisicum infected with Candidatus Liberibacter solanacearum	<0.01 grams of freeze-dried
3	Panel 1, Sample C, Biosecurity material	Solanum lycoperisicum infected with Candidatus Liberibacter solanacearum	<0.01 grams of freeze-dried
4	Panel 1, Sample D, Biosecurity material	Solanum lycoperisicum infected with Candidatus Liberibacter solanacearum	<0.01 grams of freeze-dried
5	Panel 1, Sample E, Biosecurity material	Solanum lycoperisicum infected with Candidatus Liberibacter solanacearum	<0.01 grams of freeze-dried
6	Panel 2, Sample F, Released	Nucleic acid extracted from Solanum lycoperisicum infected with Candidatus Liberibacter solanacearum	50 microlitres of nucleic acid
7	Panel 2, Sample G, Released	Nucleic acid extracted from Solanum lycoperisicum infected with Candidatus Liberibacter solanacearum	50 microlitres of nucleic acid
8	Panel 2, Sample H, Released	Nucleic acid extracted from Solanum lycoperisicum infected with Candidatus Liberibacter solanacearum	50 microlitres of nucleic acid
9	Panel 2, Sample I, Released	Nucleic acid extracted from Solanum lycoperisicum infected with Candidatus Liberibacter solanacearum	50 microlitres of nucleic acid
10	Panel 2, Sample J, Released	Nucleic acid extracted from Solanum lycoperisicum infected with Candidatus Liberibacter solanacearum	50 microlitres of nucleic acid
11	Panel 2, Sample K, Released	Nucleic acid extracted from Solanum lycoperisicum infected with Candidatus Liberibacter solanacearum	50 microlitres of nucleic acid

Participant number:

Questions/Contact

Jacqui Morris at Agriculture Victoria Research, DEDJTR
P: +61 3 9032 7145
M: +61 400 165 411
E: jacqui.morris@ecodev.vic.gov.au

Ring test information

As part of the Horticulture Innovation Australia funded project PT17000 "Diagnostic capability to detect *Candidatus* Liberibacter solanacearum (CLso)" a milestone of this project is to conduct a ring test for CLso diagnostic testing across Australia and New Zealand. The aim of this project is to ensure that different laboratories can adhere to specified diagnostic protocols and correctly interpret results. More so, the ring test provides each participating laboratory with an opportunity to use the CLso National Diagnostic Protocol (NDP) to detect CLso in infected plant tissue and CLso positive nucleic acid extracts.

The ring test includes two panels:

Panel 1: Freeze dried leaf material (5 samples, A to E) (Quarantine material, must comply under the permit 0002254334)

Panel 2: Nucleic acid (6 samples, F to K)

Panel 1 (freeze dried leaf material) is subject to quarantine, under section 2 of Permit 0002254334 attached. Once the nucleic acids are extracted and the waste material is disposed of appropriately, these samples will not be subject to quarantine and are considered released.

Panel 1 will follow NDP 18, version 1.2, for nucleic acid extraction (with additional recommendations provided below for the freeze-dried material) and molecular identification procedures.

Panel 2 (nucleic acid) will not be under quarantine and will follow molecular identification procedures only.

<u>No haplotyping or sequencing will be required as part of the ring test.</u>

As NDP 18, version 1.2 is currently under review, your laboratory is encouraged to perform any additional molecular detection methods for CLso. Please include information on the method with your Results Reporting Form.

Expected timelines

Samples are expected to be sent out on the 30th of July 2018.
Please return results no later than the 24th of August 2018.
These dates may vary depending on sample transfer timing.

Confidentiality

To protect the identity of participating laboratories, each laboratory will be allocated a confidential Participant number on this document and via email.

The identity of all participating laboratories will be known only to Jacqui Morris and all results returned only to Jacqui. This includes any additional molecular identification procedures. Once any laboratory identification is removed from reported results, Jacqui will return the additional results to the requesting test institute.

Panel 1 – Sample opening and processing information

To open the vials for Panel 1 please proceed with caution following the below guide.

- Using flat forceps push the outer aluminium ring down at the base of the arrow (Figure 1A-B)
- Push the aluminium ring upwards slightly to reveal the grey rubber bung (Figure 1C)
- Securely clamp the raised aluminium inner circle and carefully peel the lid off in the direction of the arrow (Figure 1D-E).
- Remove the aliminium seal off the sample vial (Figure 1F) and discard foil in a sharps quarantine container.

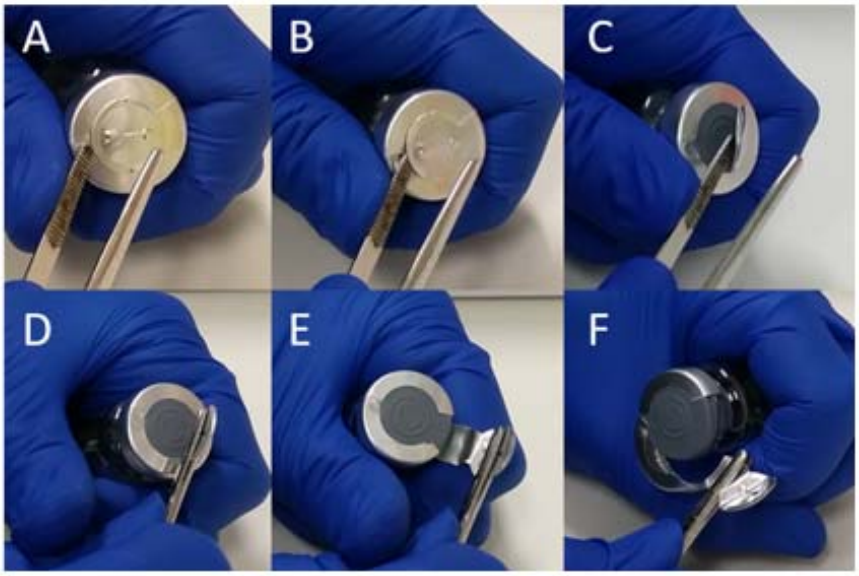

Figure 1. Opening the sealed vials

Panel 1 – Processing the vials of plant material

- With the grey rubber bung remaining sealed, tap the vial on the bench to bring down any loose material, another option is to "flick" the contents down.
- To reduce the chance of cross contamination between samples, complete this step one vial at a time. There will be plant material that remains stuck to the bung and the first introduction of air into the vial may create a small vortex of the loose material within the vial.
- Open the rubber bung very slowly.
- Add 600 µL of extraction buffer to the vial and seal the vial again with the bung. Proceed to the next vial.
- When extraction buffer has been added to all samples check they are all sealed well and mix the contents by gently inverting several times.
- Incubate the suspension at room temperature for 30-60 minutes, gently inverting at least twice during this time.
- Tap the vials on the bench or flick to bring down liquid that may be stuck in the bung.
- Carefully open the vial again (there will be liquid within the grooves of the bung) and transfer 500 µL of the plant material suspension from the vial to a clean 1.5 mL eppendorf tube.
- No grinding is required for these samples. Continue with the extraction procedure at step 2 as describe in the NDP 18, version 1.2- page 22.

Reporting Results

Please use the attached Results Reporting Forms to report all results.

Fill in one form per method (for example if you perform a 16S Housekeeping Conventional PCR, a Nested Conventional PCR and a Quantitative PCR, please fill in two Conventional PCR forms and one Quantitative PCR form).

To ensure your results remain confidential, return all results to Jacqui Morris jacqui.morris@ecodev.vic.gov.au only by the 24th of August 2018.

Final Results Report

Electronic results reports on this ring test are scheduled to be sent in September, 2018.

Appendix 5b: HIA project PT17000 "Diagnostic capability to detect Candidatus Liberibacter solanacearum (CLso)" Ring Test Report

HIA project PT17000 "Diagnostic capability to detect *Candidatus* Liberibacter solanacearum (CLso)" Ring Test Report

Aim

The aim of this project is to ensure that different laboratories can adhere to specified diagnostic protocols and correctly interpret results. More so, the ring test provides each participating laboratory with an opportunity to use the CLso National Diagnostic Protocol (NDP) to detect CLso in infected plant tissue and CLso positive nucleic acid extracts.

Samples

The Ring Test was made-up of two different panels; Panel 1, 5 samples of freeze dried leaf material (named A to E) (Quarantine material, which must comply under the permit 0002254334 in Australia) and Panel 2, 6 samples of nucleic acid (named F to K). The samples were prepared under the guidance of the Australian National Quality Assurance Program (ANQAP) which is a NATA Accredited Proficiency Testing program. Their methods of producing homogenous and stable samples were adopted to produce high quality samples that meet the requirements of ISO/IEC 17043:2010 Conformity assessment – General requirements for proficiency testing.

Panel 1 Sample Identification List
Sample A – Healthy eggplant
Sample B – Weak positive
Sample C – Strong positive
Sample D – Healthy eggplant
Sample E – Weak positive

Panel 2 Sample Identification List
Sample F – Healthy eggplant
Sample G – Positive*
Sample H – Positive
Sample I – Positive
Sample J – Positive
Sample K – Healthy eggplant

*Sample G was a very dilulted low positive sample. It was included in the panel to test the Laboratory's Limit of Detection of their test.

Testing

For Panel 1, laboratories were instructed to follow NDP 18, version 1.2, for nucleic acid extraction (with additional recommendations provided for the freeze-dried material) and for molecular identification.

For Panel 2, laboratories were instructed to follow their standar operating procedures (SOPs) for molecular identification.

Laboratories were instructed that no haplotyping or sequencing was required for this project.
In addition, laboraties were encouraged to perform any additional molecular identification procedures.

The following report includes the nested conventional polymerase chain reaction (PCR) method by Liefting *et al.* 2009b and the quantitative PCR by Li *et al.* 2009 only.

Classification

The results are presented as reported by each individual laboratory however rounding to two significant figures was applied. The classification is only dependent on the test results reported by the laboratories and does not consider preliminary testing results. Each laboratory was classified using the median and Acceptable Variation Range (AVR), where applicable. Consideration was given to those laboratories who reported Ct results with minor variation when considering the AVR.

In relation to the qPCR, "Concordant results" were those that fell within two Cts of the median, "Concordant results with minor variation" were those that fell within three Cts of the median, and any results that were three or more Cts above/below the median were deemed as "Discordant".

In relation to the cPCR, "Concordant results" were those that correctly identified the sample as either Positive or Negative (or equivalent) when comparing to the median and those that were deemed as "Discordant" were those that did not correctly identify the sample.

Participants with results that are identified as "Discordant" are encouraged to review their methods to ensure appropriate sensitivity, repeatability and reproducibility is maintained.

Collated Test Results

Table 1. Panel 1 Nested cPCR collated laboratory results.

Laboratory Number	Sample A	Sample B	Sample C	Sample D	Sample E	Classification
0	Negative	Negative	Positive	Negative	Negative	Samples A, C & D - Concordant Samples B & E - Discordant
0	Negative	Positive	Positive	Negative	Negative	Samples A-D - Concordant Sample E - Discordant
0	Negative	Positive	Positive	Negative	Positive	Concordant
0	Negative	Positive	Positive	Negative	Positive	Concordant
0	Negative	Positive	Positive	Negative	Positive	Concordant
0	Negative	Positive	Positive	Negative	Positive	Concordant
Median	**Negative**	**Positive**	**Positive**	**Negative**	**Positive**	

Table 2. Panel 1 Li et al 2009 qPCR Collated Laboratory Results.

Laboratory Number	Sample A	Int.	Sample B	Int.	Sample C	Int.	Sample D	Int.	Sample E	Int.	Classification
0	Undeter.	Neg	34.79	Pos	26.04	Pos	Undeter.	Neg	36.45	Pos	Samples A & D - Concordant Samples B, C & E - Discordant
0	-	Neg	25.68	Pos	19.77	Pos	-	Neg	26.56	Pos	Samples A-D - Concordant Sample E - Concordant result with minor variation
0	Undeter.	Neg	27.73	Pos	18.86	Pos	Undeter.	Neg	28.11	Pos	Concordant
0	NA	Neg	26.73	Pos	19.51	Pos	NA	Neg	29.15	Pos	Concordant
0	Undeter.	Neg	27.14	Pos	19.74	Pos	Undeter.	Neg	29.01	Pos	Concordant
0	-	Neg	30.90	Pos	24.14	Pos	-	Neg	31.36	Pos	Samples A, D & E - Concordant Sample B - Concordant result with minor variation Sample C - Discordant
0	0.00	Neg	27.00	Pos	19.00	Pos	33.00	Suspect Pos	26.00	Pos	Samples A-C - Concordant Sample D - Discordant Sample E - Concordant result with minor variation
0	Undeter.	Neg	34.72	Pos	26.35	Pos	Undeter.	Neg	36.26	Pos	Samples A & D - Concordant Samples B, C & E - Discordant
0	Undeter.	Neg	34.78	Pos	26.49	Pos	Undeter.	Neg	35.52	Pos	Samples A & D - Concordant Samples B, C & E - Discordant
Median Interpretation	**Negative**		**Positive**		**Positive**		**Negative**		**Positive**		
Median Ct	**N/A**		**27.73**		**19.77**		**N/A**		**29.15**		
Acceptable Variation Range	**N/A**		**25.00-30.00**		**17.00-22.00**		**N/A**		**27.00-32.00**		

Table 3. Panel 2 Nested cPCR Collated Laboratory Results.

Laboratory Number	Sample F	Sample G*	Sample H	Sample I	Sample J	Sample K	Classification
0	Negative	Negative	Positive	Positive	Positive	Negative	Concordant
0	Negative	Negative	Positive	Positive	Positive	Negative	Concordant
0	Negative	Negative	Positive	Negative	Positive	Negative	Samples F, H, J & K - Concordant Sample I - Discordant
0	Negative	Positive	Positive	Positive	Positive	Negative	Concordant
0	Negative	Negative	Positive	Negative	Positive	Negative	Samples F, H, J & K - Concordant Sample I - Discordant
0	Negative	Negative	Positive	Positive	Positive	Negative	Concordant
0	Negative	Unknown	Positive	Positive	Positive	Negative	Concordant
Median	**Negative**	**Negative**	**Positive**	**Positive**	**Positive**	**Negative**	

*Sample G: This sample was a very dilulted low positive sample. It was included in the panel to test the Laboratory's Limit of Detection of their test.

Table 4. Panel 2 Li et al 2009 qPCR Collated Laboratory Results.

Laboratory Number	Sample F	Int.	Sample G	Int.	Sample H	Int.	Sample I	Int.	Sample J	Int.	Sample K	Int.	Classification
0	Undeter.	Neg	38.66	Suspect Pos	25.29	Pos	37.81	Pos	32.03	Pos	Undeter.	Neg	Samples F, G & K - Concordant Samples H & J - Concordant result with minor variation Sample I- Discordant
0	0	Neg	34.00	Suspect Pos	19.00	Pos	33.00	Suspect Pos	26.00	Pos	0.00	Neg	Samples F, I & K - Concordant Samples G, H & J - Concordant result with minor variation
0	Undeter.	Neg	33.61	Pos	19.16	Pos	30.32	Pos	26.29	Pos	Undeter.	Neg	Samples F & K - Concordant Samples H, I & J - Concordant result with minor variation Sample G - Discordant
0	no amp	Neg	38.54	Unknown	22.23	Pos	32.80	Pos	29.33	Pos	no amp	Neg	Concordant
0	NA	Neg	37.70	Pos	37.70	Pos	37.44	Pos	29.24	Pos	NA	Neg	Samples F, G, J & K - Concordant Samples H & I - Discordant
0	-	Neg	37.57	Indeter.	20.53	Pos	31.75	Pos	28.17	Pos	-	Neg	Concordant
0	Undeter.	Neg	38.25	Pos	25.11	Pos	34.82	Pos	30.68	Pos	Undeter.	Neg	Samples F, G, I, J & K - Concordant Sample H - Concordant result with minor variation
0	-	Neg	34.52	Late Pos	20.03	Pos	32.51	Late Pos	28.20	Pos	-	Neg	Samples F, H, I, J & K - Concordant Sample G - Concordant result with minor variation
0	Undeter.	Neg	Undeter.	**Negative, further testing needed (see comment)	25.21	Pos	36.96	Pos	32.38	Pos	Undeter.	Neg	Samples F & K - Concordant Samples H & J - Concordant result with minor variation Samples G & I- Discordant
Median Interpretation	**Negative**		**Positive**		**Positive**		**Positive**		**Positive**		**Negative**		[redacted]
Median Ct	**N/A**		**37.64**		**22.23**		**33.00**		**29.24**		**N/A**		[redacted]
Acceptable Variation Range	**N/A**		**35.00-39.00**		**20.00-25.00**		**31.00-35.00**		**27.00-32.00**		**N/A**		[redacted]

Laboratory #

Your results for this test have been classified as
Concordant.
Concordant result with minor variation.
Discordant.

If you have any queries, please do not hesitate to contact me.

Yours sincerely,

Jacqui Morris

Ring Test Coordinator

References:
Li, W., Abad, J.a., French-Monar, R.D., Rascoe, J., Wen, A. and Gudmestad, etal. (2009) Multiplex real-time PCR for detection, identification and quantification of "Candidatus Liberibacter solanacearum" in potato plants with zebra chip. J Microbiol Methods 78: 59–65.

Liefting, L.W., Weir, B.S., Pennycook, S.R., and Clover, G.R.G. (2009b) "Candidatus Liberibacter solanacearum", associated with plants in the family Solanaceae. Int J Syst Evol Microbiol 59: 2274–2276.

This is the End of the Report