Hort Innovation

Final Report

Improving diagnostics and biosecurity for graft-transmissible diseases in citrus

Project leader: Nerida Donovan

Project authors: Nerida Donovan, Grant Chambers, Anna Englezou, Paul Holford

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Level 7
141 Walker Street
North Sydney NSW 2060

Telephone: (02) 8295 2300

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Public summary

The Australian and global citrus industries are under threat from graft-transmissible diseases that can cause reduced yields, fruit quality or tree death. There are no cures, and management strategies rely on prevention. The disease-causing agents may be present in plants without symptoms or symptoms may be delayed, but these plants are a source of future infections. Early detection of exotic diseases soon after they breach our borders is critical because delays in diagnostic results can impact the success of eradication. It is also important to detect diseases in propagation material before its use to ensure the planting of healthy orchards, the basis for a sustainable industry. This project strengthened Australia's ability to combat graft-transmissible citrus diseases through improved knowledge of citrus pathogens and how to diagnose them.

Detecting sap- or graft-transmissible pathogens can be difficult because field symptoms may be confused with other disorders and the pathogen may be present below detectable levels or unevenly distributed within the plant. It is important that diagnostic tests are specific to the target organism, sensitive, and efficient. A previous Hort Innovation project (CT14009) enhanced Australia's capability to detect and manage major pathogen threats, such as huanglongbing, and built an experienced citrus biosecurity team. However, gaps in knowledge remained and diagnostic technologies continued to evolve, therefore the investment continued via this second Hort Innovation project to ensure industry and government are armed with appropriate tools and knowledge to protect Australian citrus from diseases that threaten industry sustainability.

Biosecurity, communication, and capability are priorities for the Australian citrus industry (Citrus Strategic Investment Plans 2017-2022 and 2022-2026). Project CT17007 supported a citrus biosecurity team that worked on research and diagnostic issues deemed important by industry, but team members were available to work on suspect and confirmed incursion responses. Improved detection methods were evaluated or developed for 42 pathogens (20 exotic and 22 endemic). National diagnostic capability for significant biosecurity threats was expanded to other state diagnostic laboratories in Queensland, Western Australia, and the Northern Territory. The adoption of detection tools recommended by the project improved the accuracy and/or speed of diagnostic results. The testing of survey samples from around Australia for the exotic diseases, huanglongbing and citrus variegated chlorosis, supported our early warning system and provided evidence of absence data. The samples were also tested for endemic diseases which expanded our pathogen collection through the addition of new accessions, enabled the robustness of our test methods to be checked and increased our understanding of the incidence of endemic viruses and viroids. Industry engagement enhanced awareness of citrus biosecurity issues. The project also linked with other projects on citrus biosecurity threats to increase project impact. The work of the NSW DPI citrus pathology team, and their collaborators in universities, industry, and government, has enhanced the national capability to manage biosecurity threats to Australian citrus.

Keywords

Citrus; biosecurity; exotic; endemic; detection; graft-transmissible; budwood; rootstock seed

Acronyms

ACD	Australian citrus dieback
ACIAR	Australian Centre for International Agricultural Research
CaAV-1	citrus associated ampelovirus 1
CaAV-2	citrus associated ampelovirus 2
CiaRV	citrus associated rhabdovirus
CBCVd	citrus bark cracking viroid (also called citrus viroid IV)
CBLVd	citrus bent leaf viroid (also called citrus viroid I)
CBLVd-LSS	citrus bent leaf viroid – low sequence similarity
CCDaV	citrus chlorotic dwarf associated virus
CCGaV	citrus concave gum associated virus
CCSV	citrus chlorotic spot virus
CDVd	citrus dwarfing viroid (also called citrus viroid III)
CEVd	citrus exocortis viroid
CiCSV	citrus chlorotic spot virus
CiLV-C	citrus leprosis virus cytomplasmic
CiLV-C2	citrus leprosis virus-C2
CiLV-C2H	hibiscus strain of citrus leprosis virus C2
CiLV-N	citrus leprosis virus nuclear
CiNSV	citrus necrotic spot virus
CiVA	citrus virus A
CiVB	citrus virus B
CiVC	citrus virus C
CiYMaV	citrus yellow mottle-associated virus
CiYSV	citrus yellow spot virus
CLBV	citrus leaf blotch virus
CLBV-2	citrus leaf blotch virus 2
CLRV	citrus leaf rugose virus
CPsV	citrus psorosis virus
CSDaV	citrus sudden death associated virus
CTLV	citrus tatterleaf virus
CTV	citrus tristeza virus
Ct	cycle threshold
CVC	citrus variegated chlorosis
CVd-IIb	citrus viroid IIb (also called cachexia)

CVd-V	citrus viroid V
CVd-VI	citrus viroid VI
CVd-VII	citrus viroid VII
CVEV	citrus vein enation virus
CVV	citrus variegation virus
CYMV	citrus yellow mosaic virus
CYVaV	citrus yellow vein-associated virus
CYVCV	citrus yellow vein clearing virus
DAFF	Department of Agriculture, Fisheries and Forestry
DNA	deoxyribonucleic acid
DTBIA	direct tissue blot immunoassay
EMAI	Elizabeth Macarthur Agricultural Institute
HGSV-2	hibiscus green spot virus 2
HLB	huanglongbing
HSVd	hop stunt viroid or citrus viroid II
HTS	high-throughput sequencing
ICRSV	Indian citrus ringspot virus
IOCV	International Organisation of Citrus Virologists
LAMP	loop mediated isothermal amplification
NAQS	Northern Australia Quarantine Strategy
NGS	next generation sequencing
NSW DPI	New South Wales Department of Primary Industries
NT DITT	Northern Territory Department of Industry, Tourism and Trade
OFV	orchid fleck virus
PCR	polymerase chain reaction
PDR	People's Democratic Republic
PHA	Plant Health Australia
PII	Primary Industries Institute
QAAFI	Queensland Alliance for Agriculture and Food Innovation
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
RPA	recombinase polymerase amplification
RT-LAMP	reverse transcription loop mediated isothermal amplification
RT-PCR	reverse transcription polymerase chain reaction
RT-qPCR	reverse transcription quantitative polymerase chain reaction
SDV	satsuma dwarf virus
UQ	University of Queensland
WA DPIRD	Western Australian Department of Primary Industries and Regional Development

Introduction

Diseases can destroy an industry, and the Australian citrus industry is no exception. Graft-transmissible diseases are of most concern because they can kill trees and there is no cure. The disease-causing agents (pathogens) are spread by infected plant material or on infected cutting tools during pruning and hedging. A small number of these pathogens are also spread by aphids or other insect vectors. Management strategies rely on prevention through quarantine and the use of high health status propagation material.

Major graft-transmissible citrus diseases, such as huanglongbing (HLB) and citrus variegated chlorosis (CVC), are not known to occur in Australia. However, within our country, there are graft-transmissible viruses and viroids that can cause stunting, yield loss and even death in some scion and rootstock combinations, yet other varieties may be symptomless carriers. Examples of endemic graft-transmissibles include citrus exocortis viroid (CEVd), cachexia (citrus viroid IIb - CVd-IIb) and citrus tristeza virus (CTV).

Citrus tristeza virus is the most economically damaging citrus virus globally, but little is known about the economic impact of the diversity of CTV variants found in Australia. Increasing our knowledge of CTV variants will enhance diagnostic tool development and allow isolates to be identified that may be used to inoculate trees to protect them from devastating orange or lime stem pitting diseases, caused by severe variants of CTV.

Citrus exocortis viroid infection can cause yield loss of nearly 50% on citrange and 69% on trifoliata rootstocks during the first 8 years (Bevington and Bacon 1977). There is also a limited understanding of some of the citrus viroid strains found in Australia, their distribution, and the long-term impact of co-infection when more than one viroid is present in a tree. Increasing our knowledge of Australian citrus viroids will allow a more accurate assessment of viroid risk, enhance diagnostic tool development, and inform recommendations to growers who are using dwarfing viroids in high density plantings.

For sap- and graft-transmissible agents, even those for which we have a recommended diagnostic test, newly published assays will need to be evaluated to see if they are more sensitive, specific, or efficient, and additional controls should be sourced to include different strains and to replenish stocks. There are also biosecurity threats for which we are unprepared. For most graft-transmissible diseases, symptoms may not be seen in nursery trees, and the signs will only appear a few years later in the orchard. By that time, the disease is likely to have spread to surrounding trees. Nothing can be done to save infected trees.

Australian quarantine, managed by the Federal Department of Agriculture, Fisheries and Forestry (DAFF), significantly reduces the risk of entry of graft-transmissible diseases into Australia. Graft-transmissible diseases are managed in Australian citrus by:

- surveillance programs for early detection to increase the chance of eradication—mostly undertaken by the DAFF Northern Australia Quarantine Strategy (NAQS) who conduct surveillance and testing in high-risk areas of Australia and offshore;
- the DAFF post-entry quarantine system in which newly imported citrus varieties are tested for exotic and endemic plant pathogens before release;
- the National Citrus Repository Program, in which foundation trees of commercial citrus varieties are maintained in insect-proof repositories and tested for citrus pathogens—these trees supply small quantities of high health status, true to type budwood to Auscitrus or private variety owners for rapid nursery multiplication; and
- the Auscitrus propagation scheme, managed by an industry non-profit organisation (Australian Citrus Propagation Association) that supplies high health status, true to type budwood and rootstock seed to nurseries for tree production.

Independent testing of the repository and Auscitrus supply trees is provided by the NSW Department of Primary Industries (NSW DPI) citrus pathology team based at Elizabeth Macarthur Agricultural Institute (EMAI). This work is part of the NSW DPI Citrus Pathology Program which aims to protect the health status of the Australian citrus industry by expanding our knowledge and capability on disease threats and maintaining the resources to respond to new threats.

To date, 60 sap- or graft-transmissible viruses, viroids, bacteria and bacterial-like agents have been described that can infect citrus. Of these, 38 have not been reported in Australia. However, there are strains of each of these agents and variants of each strain; therefore, even if a graft-transmissible agent is known to occur in Australia, we must be cautious of exotic strains with potentially devastating impacts on infected plants. Single infections of some agents may not be significant, but their impact may be detrimental when co-infected with other agents. Some strains of viruses and viroids can also be managed to benefit a production system, such as cross protective viral variants and commercial dwarfing viroids.

Detection and management of graft-transmissible diseases can be difficult because the pathogens may be present in plants without symptoms or symptoms may be delayed, but these plants are a source of future infections. Therefore, it is important that these diseases can be detected soon after they enter Australia or in propagation material before its use. Early detection of new diseases soon after they breach our borders is critical because delays in diagnostic results can reduce the chance of eradicating the agent from Australia. Accurate testing of budwood and rootstock seed supply trees is also crucial to ensure the propagation of healthy plants and the planting of healthy orchards.

There are other issues with detecting graft-transmissible agents in citrus plants; notably field symptoms may be confused with other disorders, and the pathogen may be present below detectable levels or unevenly distributed within the tree; therefore, not all samples will contain the pathogen. It is critical that diagnostic tests are specific to the target organism, sensitive (i.e., will detect even at low levels such as in the early stages of infection), and efficient in terms of time and cost. The gaps in knowledge of graft-transmissible diseases affecting citrus in Australia impact our ability to detect and manage these diseases and assess their realised or potential impact on the industry. Project (CT17007) and the previous project (CT14009) enhanced Australia's capability to detect and manage major pathogen threats and built an experienced citrus biosecurity team.

The objective of this project was to improve our ability to detect and define graft-transmissible threats to Australian citrus. More specific objectives include:

- improved detection of graft-transmissible citrus pathogens;
- improved understanding of the range and risk of endemic and new graft-transmissible citrus pathogens;
- collaborating with projects CT17001 and MT17006; and
- responding to industry requests for diagnostic support.

Methodology

Diagnostics

Citrus pathogen collection

Graft-transmissible pathogens are not culturable. The NSW DPI citrus pathogen collection is maintained at EMAI, stored in infected citrus trees in controlled-environment greenhouses (endemic pathogens only) or as nucleic acid extracts (endemic or exotic pathogens). All accessions in the collection are catalogued in a database and their test history is recorded.

During the project, new accessions were added to the collection. New and existing accessions were used to evaluate published or develop new diagnostic tools and were shared with other Australian diagnostic laboratories to increase national capability and biosecurity preparedness.

Extraction methods

The most time consuming and therefore costly part of the molecular diagnostic process is extracting nucleic acid (DNA or RNA) from plant or insect samples. There is also potential for chronic injuries to develop in laboratory technicians who routinely perform large numbers of extractions, and more efficient methods are needed to handle the large numbers of samples submitted during an incursion. When testing samples for both DNA- and RNA-based targets, two extractions are also needed, adding to the cost. For example, when testing for citrus viruses and viroids, RNA is extracted from plant tissue, and when testing for the casual agents of HLB and CVC, DNA is extracted from plant tissue. More efficient RNA and DNA extraction methods were evaluated in this project. The most suitable tissue type for the detection of viruses and viroids in citrus plant material was also confirmed.

Diagnostic methods

New diagnostic methods for citrus viruses and viroids were evaluated in the previous citrus diagnostic project CT14009. Some methods were recommended and adopted resulting in significant improvements in efficiency and in our ability to detect the pathogens. However, diagnostic gaps remain given not all pathogen threats had been considered and there were still challenges with detecting some pathogens and with distinguishing between different strains. Detection technologies also continuously evolve, from near-field tests to sequencing.

During the project, published diagnostic assays were evaluated, or new assays were developed, to ensure Australian diagnostic laboratories have the capability to detect exotic or endemic graft-transmissible citrus pathogens and identify strain differences. Near-field detection tests were also developed for two key endemic viroids, CEVd and cachexia (CVd-

IIb, cachexia variant of hop stunt viroid HSVd). Multi-pathogen assays were evaluated or developed to improve efficiency of testing (time and cost).

Huanglongbing (HLB)

Huanglongbing is currently deemed to be the biggest threat to the Australian citrus industry. The real-time PCR method described by Li et al. (2006) is routinely used internationally, but it is widely recognised that this method can produce inconclusive results, particularly if the level of pathogen in the sample is low - cycle threshold (Ct) value between 32 and 40. There is also an issue distinguishing between '*Candidatus* Liberibacter asiaticus' ('*Ca*. L. asiaticus') and '*Candidatus* Liberibacter africanus' ('*Ca*. L. africanus') using this assay.

During the project, newly published detection methods were evaluated for the putative causal agents of HLB; '*Ca*. L. asiaticus', '*Ca*. L. africanus' and '*Candidatus* Liberibacter americanus' ('*Ca*. L. americanus) in leaf or psyllid tissue. This information was used to write Section 9 'Diagnostic Procedures to Support Surveillance' for the National Diagnostic Protocol for '*Ca*. L. asiaticus' (NDP 25 V1) and to revise the entire protocol to create Version 2.

Diagnostic testing

Surveillance samples were submitted to EMAI for pathogen testing from industry and government surveillance programs including the First Detector Network (later called CitrusWatch), the Northern Australia Quarantine Strategy (NAQS) and the National Plant Health Surveillance Program. Samples were tested for two high priority bacterial pathogens ('*Ca*. L. asiaticus' and *Xylella fastidiosa* subsp. *pauca*) identified in the National Citrus Biosecurity Surveillance Strategy, and endemic and exotic viruses and viroids.

Challenging diagnostic cases were also investigated to not only provide an answer to the submitter, but to build knowledge and enhance diagnostic capability.

Research

Citrus viroid VII (CVd-VII) and other newly detected viroids

A new viroid, tentatively named CVd-VII, was discovered by the EMAI citrus pathology team through their work with Auscitrus, and the detection was confirmed using tools validated in project CT14009. During this project, research was undertaken to improve our ability to determine the impact of this viroid, such as its distribution, host range, transmission, and the impact of co-infection with other viroids, including citrus viroids V (CVd-V) and VI (CVd-VI). Further molecular characterisation informed the development of improved detection tools.

Citrus tristeza virus (CTV)

Several variants of CTV have been identified, and these have been grouped into at least seven major strains (genetically distinct groups). It is not well understood how genotypes and phenotypes are connected. The variants within a strain are genetically similar, but they can cause very different phenotypes, that is they can induce severe, moderate, or mild symptoms or none. Most field trees contain more than one variant of CTV, belonging to more than one strain (a CTV sample from an individual tree is called an isolate). Field symptoms induced by CTV include stem pitting or quick decline, although in smaller indicator plants other symptoms can be seen including seedling yellows, stunting and vein clearing. Symptom expression and severity depends on the scion variety, the rootstock, and the variant (or mixture of variants) of CTV present.

The plants containing CTV in the EMAI living pathogen collection were mostly produced through single aphid transmission to reduce the number of viral variants present in each plant. However, each plant may still house more than one variant of this virus. During this project, molecular assays and sequencing were used to identify which strain groups the variants present in the pathogen collection plants belonged to. Strains were also characterized in field samples sourced from around Australia.

Citrus pathology support

During this project, the EMAI citrus pathology team provided diagnostic support and scientific advice on citrus pathogen threats to industry and government, including the provision of support for emergency responses. Team members participated in extension and scientific conference activities and advisory committees.

Collaboration with other projects

The project team communicated with project leaders and team members working on 'CT17001: Improving biosecurity preparedness of the Australian citrus industry'. Surveillance samples submitted by the First Detector Network (later called CitrusWatch) were tested for graft-transmissible citrus pathogen threats.

The citrus diagnostic project team collaborated with the EMAI project team working on 'MT17006: Improving preparedness of the Australian horticultural sector to the threat posed by *Xylella fastidiosa*'. The citrus team evaluated five new diagnostic tools for *X*. *fastidiosa* subsp. *pauca* (the causal agent of CVC) on behalf of the diagnostic team working on MT17006.

Intensifying a citrus orchard can improve efficiency and productivity and is one management tool used in HLB affected regions overseas. Citrus dwarfing viroid strain IIIb (CDVd-IIIb or CVd-IIIb) induces mild dwarfing in inoculated trees and can be used as a management tool for high density plantings in Australia. Greater experience with dwarfing viroids in the Australian environment will prove useful if HLB is introduced and unable to be eradicated. Two viroid field trials were established at NSW DPI's Dareton Primary Industries Institute (PII) in a collaborative effort with the team working on Hort Innovation funded project 'AS18000 National tree crop intensification in horticulture (citrus)'. Trial 1 will evaluate the impact on citrus field trees of co-infection of the commercial dwarfing viroid and newly discovered or detected viroids under Australian conditions. The second trial will evaluate the impact of co-infection of the commercial dwarfing viroid with pathogenic viroids and determine if the inoculation order (i.e. which viroid enters the tree first) has any impact. During this project, the EMAI team tested trial trees using reverse transcription quantitative polymerase chain reaction (RT-qPCR) prior to finalizing the experimental design, assisted with the field inoculation and tested trial 1 trees 12 months after inoculation to confirm viroid presence.

Capability building

The capability of experienced technicians in the EMAI team was enhanced through exploring new research directions, thereby improving the ability of NSW DPI to respond to industry needs. The capability of other Australian diagnostic laboratories to detect graft-transmissible citrus pathogens was enhanced by adoption of diagnostic tools recommended by this project, exchange of protocols and resources between diagnostic laboratories and collaboration with the diagnostic hubs at the University of Queensland, Queensland Alliance for Agriculture and Food Innovation (UQ QAAFI), and the Western Australian Department of Primary Industries and Regional Development (WA DPIRD).

Results and discussion

Diagnostics

Citrus pathogen collection

During the project, the pathogen status of new and existing accessions in the NSW DPI citrus pathogen collection was confirmed. Appendix 1 outlines the accessions included in the collection. Appendix 2 outlines new accessions obtained during the project term.

Protocols and pathogen extracts or fresh samples were provided to the other Australian diagnostic laboratories. Citrus exocortis viroid RNA extracts and plant tissue containing CEVd were sent to the WA DPIRD diagnostic laboratory in south Perth for validation of a new RT-LAMP detection method. The south Perth laboratory already had considerable experience with testing for Liberibacter species, due to testing samples for '*Candidatus* Liberibacter solanacearum' after the incursion of the tomato potato psyllid in WA in 2017.

DNA extracts and plant material containing '*Ca*. L. asiaticus' (imported under permit from the Lao People's Democratic Republic and stored in ethanol) were sent to Biosecurity Queensland in Brisbane, in exchange for DNA of '*Ca*. L. africanus'. '*Ca*. L. asiaticus' extracts were also sent to the Northern Territory Department of Industry, Tourism and Trade (NT DITT) diagnostic laboratory in Darwin. Citrus survey samples collected in Darwin were split and tested for '*Ca*. L asiaticus' at both the Darwin and EMAI laboratories serving as proficiency testing.

The UQ QAAFI diagnostic laboratory is experienced in testing plant material for viruses and viroids. The UQ QAAFI and EMAI diagnostic laboratories increased their experience with testing for the hibiscus strain of citrus leprosis virus C2 (CiLV-C2H) and other leprosis viruses. Note that leprosis viruses are not systemic or graft-transmissible but are transmitted by sap and mite vectors. Some species pose a significant biosecurity threat to Australian citrus hence their inclusion in this project.

When positive control material is difficult to obtain or in short supply, synthetic controls can be used. A synthetic oligonucleotide containing primer binding and associated amplicon sequences of both '*Ca*. L. americanus' and a plant malate dehydrogenase gene control were designed and evaluated for use in qPCR testing for '*Ca*. L. americanus'.

Extraction methods

The traditional extraction method used in the citrus pathology laboratory was to put a sample in a tube, freeze using liquid nitrogen and then homogenize by grinding with a micro-pestle. On average, 24 samples were extracted in each

batch.

During the project, sample extraction was streamlined by freeze drying a sample then using either the FastPrep™ machine or the Tissue Lyser II to disrupt and homogenise the plant tissue in one simple and reliable step. Once the sample is homogenized an extract of it can then be made. Further efficiencies have been gained using a KingFisher™ instrument which performs automated nucleic acid extraction using magnetic technology; processing 96 samples in approximately one hour. The Kingfisher™ method will prove useful in an incursion where high throughput of samples is necessary for PCR testing and has been adopted by the Auscitrus program to test rootstock and budwood seed source trees. Trials were also run to determine that samples could be bulked without compromising the integrity of results; therefore, the Auscitrus samples are now submitted in batches of 180.

It was determined through trials that by excluding the DNase treatment step during the extraction process, an extract was produced that can be used to test for both RNA- and DNA-based organisms, eliminating the need for two separate extractions.

Plants inoculated with CVd-VII were used to evaluate a new, rapid extraction method where slithers of petiole were placed into a tube and frozen for a minimum of 30 minutes. Tris buffer was then added to each tube and this mixture was used directly in a PCR reaction. Results obtained using the rapid extraction method were comparable to a commercial extraction kit, except at low titres. However, the rapid method is considerably cheaper to perform and would be useful in situations where resources are limited but viroid titre is not limiting.

Replicated tissue sampling experiments, conducted using CVd-VII-infected lemon and etrog plants, determined that green bark was the best citrus tissue to sample when testing for viroids, compared with leaf midrib, lamina or grey bark from older branches or leaf midrib and lamina material from younger branches. Approximately ten times more viroid was present in the green bark of etrog than lemon plants, and there was up to a 100-fold difference in viroid titre in the different tissues sampled within each plant. This knowledge will be useful for optimising sampling strategies to reliably detect viroids.

Diagnostic methods

Appendices 3, 4, 5, and 6 outline the detection methods evaluated or developed during this project and whether they are recommended for use in Australian diagnostic laboratories.

Some published methods failed to detect Australian strains of the target pathogen; therefore, new methods were developed, for example for CTV. Whole genome sequences were obtained of selected CTV variants in the EMAI collection to assist with identifying target regions for primers with the ability to detect a wider range of CTV variants. A new 'degenerate' probe was subsequently designed to use with the Osman et al. (2015) assay and validation of this assay is in progress. Other examples include assays developed for citrus psorosis virus (CPsV) and citrus tatterleaf virus (CTLV) which have subsequently been adopted.

Huanglongbing (HLB)

The National Diagnostic Protocol for '*Ca*. L. asiaticus' (NDP 25) was reviewed and Section 9 'Diagnostic Procedures to Support Surveillance' was written. Version 2 of the protocol was submitted to Plant Health Australia (PHA) and is under review.

The project team collaborated with scientists from the United States, Brazil and South Africa on an improved molecular detection assay for '*Ca*. L. asiaticus'; the assay was developed by scientists at the University of California Davis and evaluated in the collaborating laboratories. This assay has been published (Appendix 16) and will have an impact in regulatory, diagnostic and research programs globally.

Internationally, there has been considerable research investment and discussion to identify the best method and tissue type to test for the causal agent of HLB and the search continues for an effective means of early detection before symptoms can be seen on infected plants. Early detection will increase the chance of eradication in an incursion and reduce spread by identifying infected trees that have yet to express symptoms but are an inoculum source for further infections.

'*Candidatus* Liberibacter asiaticus' was found to be more evenly distributed in the roots of infected plants compared to the canopy (Louzada et al. 2016). Park et al. (2018) reported a new method to detect '*Ca*. L asiaticus' in plant roots. The study found the root test to be more sensitive than the leaf test, and that '*Ca*. L. asiaticus' could be detected in root tissue before symptom expression. This method was evaluated through collaborative work with Western Sydney University and the Bhutanese Ministry of Agriculture. The Park et al. (2018) assay and the widely accepted Li et al. (2006) assay both harness real-time PCR technology and were compared using root and leaf tissue from field trial trees sampled from

different altitudes in Bhutan. Results to date suggest little difference. However, there were some issues with false positives possibly due to contamination from humic acids and organisms inhabiting the root rhizosphere. These issues were not alleviated using a 'clean up' kit to improve the quality of extracted DNA.

The Asian citrus psyllid (ACP) is associated with transmission of '*Ca*. L. asiaticus'. PCR assays were evaluated for psyllid identification (Boykin et al. 2012) and to ensure that '*Ca*. L. asiaticus' can be detected in infected psyllids.

Multiplex assays

Multiplex assays for simultaneous detection of more than one target are outlined in Appendix 6.

A multiplex probe-based, RT-qPCR assay was evaluated for simultaneous detection of CEVd, HSVd and citrus bark cracking viroid (CBCVd) (Osman et al. 2017). This assay has been used successfully on samples from a wide range of geographic locations, including samples collected from Australia and overseas. The assay has now been adopted by Auscitrus to test the health status of budwood supply trees for CEVd, HSVd and CBCVd.

A multiplex RT-qPCR assay was evaluated to simultaneously detect CPsV, CTLV and citrus leaf blotch virus (CLBV). The assay is based on Osman et al. (2015) but uses probes and primers designed by our group to include detection of Australian isolates of CPsV and CTLV. This assay is now routinely used within our program, including testing of new Australian varieties prior to inclusion in the National Citrus Repository Program, existing foundation trees in the repositories and the Auscitrus rootstock seed and budwood supply trees.

A method was evaluated for the simultaneous detection of X. *fastidiosa* subsp. *pauca* and '*Ca*. L. asiaticus'. The RT-qPCR assay has been working well on DNA extracts from plants, insects, and pure cultures of Xylella.

Near-field technologies

Loop mediated isothermal amplification (LAMP) is a diagnostic technology that can be used in a mobile laboratory, otherwise known as near-field detection. It is difficult to design primers for viroid detection given the number of primers (6) needed to fit on the small viroid genome. Six RT-LAMP detection methods for viroids have been published but none of these target CEVd. A RT-LAMP method to detect CEVd was developed, validated by WA DPIRD, and submitted for publication.

A published recombinase polymerase amplification (RPA) assay to detect HSVd (Kappagantu et al. 2017), and a commercially available kit test did not detect all Australian isolates. A new RT-LAMP assay has been designed and will undergo further evaluation prior to publication.

Simple extraction techniques, ideal for use in a mobile laboratory or potentially in the field, were successfully trialled using fresh leaves from the EMAI living pathogen collection.

Sequencing technologies

EDNA2 is a web-based plant pathogen detection tool based at Oklahoma State University. Our colleagues at the University of California, Riverside allowed us to test some of our high-throughput sequencing (HTS) data using eProbes that they designed as part of EDNA2 to detect CTV, CEVd and '*Ca*. L. asiaticus'.

Diagnostic testing

Surveillance samples submitted to EMAI for pathogen testing from industry and government surveillance programs were tested for two high priority bacterial pathogens ('*Ca*. L. asiaticus' and *X. fastidiosa*) identified in the National Citrus Biosecurity Surveillance Strategy, and endemic and exotic viruses and viroids. The results are summarised in Appendix 7. Most viroids and viruses were either not detected or were found at low incidence. CTV is the only graft-transmissible virus found in Australia that is also transmitted by insects; therefore, the incidence was expected to be higher than other pathogens. CTV was detected in 64% of citrus samples tested—this was potentially an underestimate given the limitations of CTV detection using molecular assays. Our most reliable method for CTV presence or absence remains the serological technique of direct tissue blot immunoassay (DTBIA), used annually to check the foundation trees in the National Citrus Repository.

Citrus viroid VII has only been detected in trees on one Sunraysia property. There have been no viroid detections in samples from budwood source trees, national surveys, and general diagnostics (>1200). This implies limited distribution of the viroid in Australia and limited impact on the industry currently.

Research

Citrus viroid VII (CVd-VII) and other newly detected viroids

A new diagnostic assay using RT-qPCR technology was developed to detect the newly discovered CVd-VII (Appendix 15). Citrus viroid VII can be detected in the leaves, bark, and fruit (peduncle, central column, outer seed coat and embryo) of infected trees. The viroid is readily transmitted by grafting or mechanical inoculation. Viroids are not known to be seed transmitted in citrus (Durán-Vila and Semancik 2003). Preliminary trials show no evidence of seed transmission of CVd-VII and a larger trial is in progress.

Host range trials were established to investigate whether CVd-VII can replicate in a range of citrus and non-citrus plants including tomato, cucumber, luffa, zucchini, grape, avocado, bean, and chrysanthemum. Results suggest that CVd-VII can replicate in other citrus varieties including sweet orange, lime, grapefruit, and mandarin in addition to the two lemon cultivars in which it was originally discovered but the viroid has not been found to replicate in non-citrus plants.

The impact of CVd-VII infection in single and mixed infections with other viroids (CEVd, citrus bent leaf viroid low sequence similarity CBLVd-LSS, cachexia and non-cachexia variants of HSVd, CVd-V, and CVd-VI) was evaluated in pot trials with etrog plants, a biological indicator for citrus viroids. Growth was reduced in plants infected with CVd-VII, although the impact does not appear to be greater in mixed infections (i.e. no apparent synergistic effect). Symptoms observed on etrog plants inoculated with CVd-VII include leaf bending and curling, stem growth bending, leaf midvein necrosis and fine bumps on the stem.

In March 2022, CVd-VII was ratified by the International Committee on Taxonomy of Viruses as a distinct viroid species, *Apscaviroid cvd-VII*.

Citrus tristeza virus (CTV)

Some plants in the citrus pathogen collection tested positive for CTV using a direct tissue blot immunoassay (Garnsey et al. 1993) and PCR assays (Gillings et al. 1993; Herrera-Isidron et al. 2009) but not using CTV strain-differentiating assays (Roy et al. 2010; Cook et al. 2016). These results either indicate the Australian collection contains additional strains not previously reported, or the strain-differentiating tests did not pick up the Australian variants given their genetic information was not available to the test developers.

To determine which scenario applies, total RNA from several isolates was used for HTS using Illumina technology. Associate Professor Roberto Barrero at the Queensland University of Technology leads a Hort Innovation funded project 'MT18005 Improving plant industry access to new genetics through faster and more accurate diagnostics using Next Generation Sequencing'. In collaboration with this project, 20 RNA extracts obtained from citrus plants infected with viruses or viroids, and from healthy control plants, were sent for small RNA sequencing. Samples were sent to two service providers to generate comparative data: the Australian Genome Research Facility in Melbourne and the Ramaciotti Centre for Genomics in Sydney. Additional isolates were sequenced at other times during the project.

The data sets generated were analysed with help from researchers at Stellenbosch University and Citrus Research International in South Africa who have developed a bioinformatic pipeline for CTV genotype detection. This information was combined with the results from the strain characterisation PCR assays to enhance our understanding of the genetic diversity of CTV isolates in Australian citrus. However, more work is needed before the biological impact of these isolates (pathogenicity) can be understood given the link between genotype and phenotype is not clear and is further complicated by mixed infections inducing different responses in different hosts.

Other pathogens

Citrus virus A (CiVA) is a newly discovered virus, and symptom expression on a range of indicator plants is not known. A trial was established to determine if there is any symptom expression other than in Symons sweet orange. Symptoms have been observed on inoculated Dweet Tangor, Duncan grapefruit and Leng early navel plants, but not on Rusk citrange. A manuscript which includes this information, and the genome characterization of an Australian isolate, has been accepted for publication.

Citrus pathology services

Team members processed diagnostic samples for the NSW DPI Plant Health Diagnostic Service and managed enquiries from around Australia, including from the Biosecurity Hotline.

The project leader participated in meetings and undertook work to support the Auscitrus Executive Committee, the Citrus Pest and Disease Prevention Committee (CPDPC), the HLB Taskforce which has now been absorbed by the CPDPC, the National Citrus Surveillance Steering Committee which has become the CitrusWatch Steering Group and the Project Reference Group for project MT18005.

Technical advice was provided to industry and government as requested, including editing of resources and participation

in workshops. For example, the Citrus Triage Workshop held in Mildura in 2019 which provided training to representatives from industry and government in field triage, sample submission and surveillance. Nerida assisted the workshop organisers by presenting on citrus disease symptoms, answering enquiries from participants during the field exercise via the MyPestGuide app, and triaging all samples collected to provide feedback on sample collection, diagnose samples where possible and identify samples for laboratory testing. A selection of samples from the workshop were tested at EMAI for targeted exotic and endemic graft-transmissible pathogens of citrus.

Collaboration with other projects

The project team evaluated diagnostic tools for *X*. *fastidiosa* subsp. *pauca* (the causal agent of CVC) and gave that information to the diagnostic team working on MT17006.

As mentioned above, surveillance samples from the Hort Innovation funded citrus biosecurity projects were tested for the exotic pathogens, '*Ca*. L. asiaticus' and *X. fastidiosa* subsp*. pauca* (Appendix 7) as part of Australia's early warning system.

Two field trials evaluating the long-term impact of viroid co-infection were established at Dareton PII in collaboration with the team working on the Hort Innovation funded project 'AS18000 National tree crop intensification in horticulture (citrus)'. The trials were established during the project term. The EMAI team tested trial trees using real-time PCR prior to finalizing the experimental designs, assisted with the field inoculation and tested a subset of trees for viroids 12 months after inoculation. No viroids were detected. These trees will be regularly tested to contribute to our understanding of the time it can take for viroids to replicate to detectable levels in field trees.

Capability building

Grant Chambers is a member of the EMAI project team and is enrolled in a PhD program at the University of Queensland under the supervision of Associate Professor Andrew Geering (UQ, QAAFI), Dr Nerida Donovan (Citrus Pathologist and project leader, NSW DPI), Professor Georgios Vidalakis (University of California, Riverside) and Professor Paul Holford (Western Sydney University). Grant is studying viroids in Australian citriculture, with a particular focus on the newly discovered CVd-VII.

Two project team members, Nerida and Grant, presented at the Joint Conference of the International Organisation of Citrus Virologists (IOCV) and the International Research Conference on Huanglongbing held in Riverside, California in March 2019, and participated in the post-conference California Citriculture Tour.

The project leader, Nerida Donovan, travelled to Lao PDR in February 2020 in her role as key mentor with the Crawford Fund Lao PDR program. Nerida identified and advised on diseases and disorders of citrus and increased the capability of local staff and Australian volunteers working in southern Lao PDR. In turn, Nerida gained additional experience with field symptoms of exotic citrus disease threats and their vectors, including HLB and citrus canker. Nerida also collected leaf and psyllid samples which tested positive for '*Ca*. L. asiaticus', contributing to the stocks of positive control material. Samples also contained exotic strains of citrus viruses and viroids, including citrus bent leaf viroid which is not known to occur in Australia – the Australian variant is CBLVd-LSS. The trip was funded by the Crawford Fund and sample testing was funded by this project.

Outputs

Table 1. Output summary

Output	Description	Detail
Pathogen collection	Pathogens maintained in living plants or as nucleic acid extracts—existing and new accessions tested during the project	Validated pathogen material (>270 accessions) held in the NSW DPI citrus pathogen collection at EMAI for use as positive controls in diagnostics and research.
Extraction method	Freeze dry, machine homogenize, Kingfisher automatic extraction	Improved efficiency of nucleic acid extraction from citrus plant tissue (from 24 to 96 samples / batch).
Diagnostic assays	Improved diagnostic assays evaluated	More efficient, sensitive, and specific tests for the detection of graft-transmissible citrus pathogens in Australian citrus germplasm for use by diagnosticians, researchers, and regulators.
Diagnostic assays	Improved diagnostic assays developed	More efficient, sensitive, and specific tests (including near-field tests) for the detection of graft-transmissible citrus pathogens in Australian citrus germplasm for use by diagnosticians, researchers, and regulators.
Data – exotic pathogens	Data providing evidence of absence in citrus samples collected nationally for key exotic threats	Data contained in Appendix 7 can be used by industry or government when defining risk, exporting budwood, tracing during an incursion response or in trade negotiations.
Data – established pathogens	Survey data on the incidence of established viruses and viroids in citrus samples collected nationally	Data contained in Appendix 7 can be used by industry or government when defining risk, exporting budwood or in trade negotiations.
Extension publication	The team of scientists on the frontline of disease prevention. Australian Citrus News, Spring 2021 p 31	Industry magazine article about the work of the NSW DPI Citrus Pathology team. Appendix 8 and available online at https://citrusaustralia.com.au/wp-content/uploads/Citrus-News-Spring-2021-FINAL-web.pdf
Industry conference	Donovan N, Chambers G, Englezou A, 2019. Protecting Australian citrus germplasm through improved diagnostic tools. Citrus Technical Forum, Adelaide 6-7 March 2019	Poster viewed by industry stakeholders and scientists at the industry forum attended by industry representatives and stakeholders from across Australia. Appendix 9.
Industry conference	Donovan N, Herrmann T, Chambers G, Englezou A, Forbes W, Dando A (2022) Protecting our industry from incurable diseases. Poster. Citrus Technical Forum, Sunshine Coast, Australia 8-9 March 2022	Poster viewed by industry stakeholders and scientists at the industry forum attended by industry representatives and stakeholders from across Australia. Appendix 10.

Table 1 continued

Output	Description	Detail
Scientific conference	Chambers G, Englezou A, Webster J, Bogema D, Donovan N, 2019. Using Next generation sequencing (NGS) to characterize Australia's living pathogen collection. Joint Conference of the International Organization of Citrus Virologists and the International Research Conference on Huanglongbing, Riverside California, United States, 10-15 March 2019	Conference presentation to inform and gain feedback from the international scientific community (209 delegates from 23 countries). Abstract at Appendix 11 and available online at https://escholarship.org/uc/item/1zp421bn
Scientific conference	Chambers GA, Geering A, Holford P, Vidalakis G, Donovan N. 2021. Determining the potential economic impact of the newly discovered citrus viroid VII. Proceedings of the 23rd Biennial Australasian Plant Pathology Conference, 23-26th November 2021 p 73 online	Conference presentation to inform and gain feedback. Abstract at Appendix 12 and available online at https://appsconference.com.au/wp-content/uploads/2021/11/2021-APPS-Conference-Handbook-1.pdf
National Diagnostic Protocol	Subcommittee on Plant Health Diagnostics (2022) National Diagnostic Protocol for '*Candidatus* Liberibacter asiaticus', the putative causal agent of huanglongbing (HLB) – NDP 25 V2. Authors Donovan NJ, Englezou A, Chambers G, Holford P. Submitted for review	Protocol to facilitate accurate identification of '*Ca*. L. asiaticus' in a suspect plant sample or specimen of a potential vector. Once endorsed, it will be available online at https://www.plantbiosecuritydiagnostics.net.au/national-diagnostic-protocol-list/
Journal paper	Chambers GA, Bogema DR, Englezou A, Donovan NJ (2020) First Report of Citrus viroid V and Citrus viroid VI in Australia infecting Citrus. Plant Disease DOI 10.1094/PDIS-12-19-2662-PDN	First report of CVd-V and VI in Australia. These viroids have been added to the test schedule for Auscitrus budwood supply trees, including foundation trees in the National Citrus Repository, and surveillance samples will continue to be tested to determine the distribution and incidence of these viroids in Australia. Appendix 13 and available online at https://apsjournals.apsnet.org/doi/10.1094/PDIS-12-19-2662-PDN
Journal paper	Donovan NJ, Chambers GA, Englezou A, Phanthavong S, Daly A, Wildman O, Holford P, Burgess LW (2020) First report of citrus exocortis viroid, citrus bent leaf viroid, hop stunt viroid and citrus dwarfing viroid in Lao PDR. Australasian Plant Pathology 49: 661-663 DOI 10.1007/s13313-020-00740-6	First report for four viroids in Lao PDR to inform their regulation and management of citrus viroids. The work provided the team with positive controls facilitating validation of detection tools. Appendix 14 and available online at https://link.springer.com/article/10.1007/s13313-020-00740-6
Journal paper	Donovan NJ, Englezou A, Chambers GA, Phanthavong S, Daly A, Saleh F, Holford P, Burgess LW (2021) First report of citrus tristeza virus in Lao PDR. Australasian Plant Pathology DOI:10.1007/s13313-021-00818-9	First report for CTV in Lao PDR to inform their regulation and management of the virus. The work provided the team with positive controls facilitating validation of detection tools. Appendix 15 and available online at https://link.springer.com/article/10.1007/s13313-021-00818-9

Table 1 continued

Output	Description	Detail
Journal paper	Chambers GA, Geering ADW, Holford P, Vidalakis G, Donovan NJ. 2022. Development of a one-step RT-qPCR for the newly described citrus viroid VII. Journal of Virological Methods DOI: 10.1016/j.viromet.2021.114330	Improved detection method for use in diagnostic and research laboratories, particularly for phytosanitary schemes regulating citrus propagation material, and surveys to establish the distribution of CVd-VII globally. Appendix 16 and available online at https://www.sciencedirect.com/journal/journal-of-virological-methods/vol/299/suppl/C
Journal paper	Osman F, Dang T, Bodaghi S, Haq R, Lavagi-Craddock I, Polek M, Rawstern A, Rodriguez E, Wulff N, Pietersen G, Englezou A, Donovan N, Vidalakis G. An updated multiplex qPCR detection assay for the detection of three *Candidatus* Liberibacter species associated with citrus Huanglongbing. Phytofrontiers DOI: 10.1094/PHYTOFR-04-22-0046-Fl	Improved detection method for '*Ca*. L. asiaticus' for use in diagnostic and research laboratories. Appendix 17 and available online at https://apsjournals.apsnet.org/doi/10.1094/PHYTOFR-04-22-0046-FI
Journal paper	Donovan NJ, Englezou A, Phanthavong S, Chambers GA, Dao HT, Phitsanoukane P, Daly A, Cowan S, Holford P, Beattie GAC, Vilavong S, Burgess LW. Investigating the impact of huanglongbing in citrus in southern Lao PDR. Journal of Citrus Pathology 9 DOI:10.5070/C49155026	Survey results and discussion on HLB in Lao PDR to inform their regulation and management of the disease. The work provided the team with positive controls facilitating validation of detection tools. Appendix 18 and available online at https://escholarship.org/uc/item/5tv0s54r
Journal paper	Donovan NJ, Chambers GA, Englezou A, Forbes W, Dando A, Holford P. First report of citrus virus A in Australia. Journal of Citrus Pathology (in press)	First report of CiVA in Australia. This virus has been added to the test schedule for the Auscitrus budwood supply trees, including foundation trees in the National Citrus Repository. Surveillance samples will continue to be tested to determine its distribution and incidence in Australia.
Journal paper	Chambers GA, Geering ADW, Holford P, Kehoe MA, Vidalakis G, Donovan NJ A reverse transcription loop-mediated isothermal amplification assay for the detection of citrus exocortis viroid in Australian citrus. Australasian Plant Pathology (submitted)	An additional diagnostic tool, with near-field application, for the pathogenic CEVd to inform management decisions, complementing but not replacing laboratory testing.

Outcomes

Table 2. Outcome summary

Outcome	Alignment to fund outcome, strategy and KPI	Description	Evidence
Efficiency	Outcome 1: Protect the production base through robust biosecurity systems, ensuring access to superior scion varieties and proactively monitoring potential crop protection regulatory threats.	Improved efficiency of testing citrus plant tissue for graft-transmissible diseases using automated extraction processes and multiplex assays, enhances diagnostic capability by reducing the cost of testing without compromising the quality of results.	Faster and more effective nucleic acid extraction process, from 24 to 96 samples per batch or 180 when two samples are bulked into one. Testing for more than one pathogen in one assay e.g. CPsV, CTLV and CLBV e.g. CEVd, HSVd, CBCVd
Capability	Outcome 1: Protect the production base through robust biosecurity systems, ensuring access to superior scion varieties and proactively monitoring potential crop protection regulatory threats.	Enhanced ability to test for graft-transmissible citrus pathogen threats	Protocols and positive control material shared with other diagnostic laboratories (4). Improved diagnostic tools published and available for use in diagnostics and research. National Diagnostic Protocol for the causal agent of HLB reviewed.
Knowledge	Outcome 1: Protect the production base through proactively monitoring potential crop protection regulatory threats and building foundational knowledge to develop effective, socially acceptable, and environmentally sound crop protection solutions.	Enhanced knowledge of graft-transmissible citrus pathogen threats	Survey data to determine distribution of endemic viruses and viroids, informing risk. CVd-VII transmission, host range, impact on growth in single and mixed infections. CTV strain characterisation to inform cross protection strategies.
Awareness	Outcome 3: Communication, extension, and capability – raise awareness to encourage best practice and aid early detection of new threats.	Enhanced awareness of graft-transmissible citrus pathogens	Industry forum presentations (2) Scientific conference presentations (2) Extension publications (1) Scientific publications (8) National Diagnostic Protocol (1)
Reduced risk	Outcome 1: Protect the production base through robust biosecurity systems to minimize the impact of pathogen threats.	Reduced risk posed by graft-transmissible citrus pathogens	Greater awareness, surveillance, and diagnostic capability to facilitate early detection of threats. Foundational knowledge to determine risk and inform management decisions.

Monitoring and evaluation

Table 3. Key Evaluation Questions

Key Evaluation Question	Project performance	Continuous improvement opportunities
Effectiveness		
Are sensitive and efficient diagnostic tools available?	Yes, for 20 exotic and 22 endemic pathogen threats	Develop capability for pathogen threats for which we have limited or no capability. Improve existing methods and enhance specificity where generic assays are being used.
Do we have diagnostic capability in more than 1 laboratory?	Yes, capability enhanced for significant threats in 4 laboratories.	Expand capability to additional pathogens and laboratories.
Do we have greater knowledge of new graft-transmissible citrus pathogens and their risk to industry?	Yes, greater knowledge of transmission, host range, distribution, and impact of CVd-VII. Greater understanding of the distribution of endemic viruses and viroids to inform assessment of risk. Greater understanding of the genetic diversity of CTV.	Long-term impact of single and mixed infections of viroids unknown. Relationship between genotype and phenotype of Australian CTV variants and strains is largely unknown.
Has awareness of graft-transmissible diseases increased?	Yes, through presentations at industry forums and extension publications (Appendices 8, 9, 10, 19).	Greater awareness needed to increase adoption of high health status propagation material and enhance surveillance efforts to support the early warning system for exotic pathogen threats.
Relevance		
Do significant graft-transmissible citrus pathogen threats exist which we cannot test for?	Yes, as per Appendix 1.	Develop the capability to test for pathogens for which we have limited or no capability.
Are extension materials available for significant graft-transmissible citrus pathogen threats?	Yes, fact sheets, NSW DPI Citrus Plant Protection Guide (regularly updated), articles in Australian Citrus News (Appendices 8, 19).	Improve or develop new resources, in collaboration with other citrus biosecurity projects to prevent duplication.
Process appropriateness		
Are diagnostic tools available for use?	Yes, diagnostic tools are published, appendices 14 (CBCVd), 16 (CVd-VII), 17 (*Ca*. L. asiaticus) or publication is in progress (CEVd).	Publish new diagnostic tools as they are developed and validated.
Is the industry aware of significant pathogen threats?	Yes, through presentations at industry forums and extension publications (Appendices 8, 9, 10, 19).	Raise awareness at industry forums in partnership with other biosecurity projects and industry bodies.
Efficiency		
Has the efficiency of existing and new diagnostic tools been compared?	Yes, as part of the validation process for newly published or developed diagnostic tools.	Validate new tools against existing tools prior to recommending them for adoption.

Recommendations

Diagnostic tools recommended for adoption

- Improved diagnostic tools are recommended for use in diagnostic and research (Appendix 1).

Future research to improve diagnostic capability

- Identify methods to successfully detect those pathogens which we are not confident in our ability to detect (Appendix 1).
- Identify methods to effectively differentiate between viral strains (e.g. CTV, HSVd).
- Multiplex more tests to further improve efficiency.
- Continue to evaluate the diagnostic potential of new sequencing technologies.
- Develop additional field detection assays.
- Continue to trial newly published diagnostic methods for endemic and exotic pathogens of citrus to improve specificity, sensitivity (early detection) and efficiency (time and cost) compared to current methods.

Managing biosecurity threats to Australian citrus

The following strategies are recommended to enhance Australia's national capability for detecting and managing biosecurity threats (endemic and exotic).

- Continue to improve diagnostic tools for graft-transmissible citrus pathogens and extend this diagnostic capability to other laboratories.
- Publish newly developed diagnostic tools in peer-reviewed publications to make information accessible to other Australian diagnostic providers.
- Determine the long-term impact of single and mixed infections of citrus viroids in field trials to inform the use of dwarfing viroids in high density plantings.
- Make the use of health-tested propagation material from Auscitrus mandatory to reduce the impact of graft-transmissible diseases (by reducing the incidence of endemic graft-transmissible diseases or the spread of a new graft-transmissible diseases). Mandatory nursery registration would also facilitate tracing and surveillance in an incursion and increase participation and engagement with awareness activities.
- Continue to build awareness in industry and government of the biosecurity threat posed by graft-transmissible diseases to improve the likelihood of early detection and increase support for programs designed to reduce disease impact (e.g. use of pathogen tested propagation material from Auscitrus).
- Continue to link with the citrus biosecurity project and CitrusWatch program, other citrus biosecurity projects and surveillance programs, to add value to activities and reduce duplication.

Refereed scientific publications

Journal articles

Chambers GA, Bogema DR, Englezou A, Donovan NJ. 2020. First Report of Citrus viroid V and Citrus viroid VI in Australia infecting Citrus. Plant Disease doi:10.1094/PDIS-12-19-2662-PDN

Donovan NJ, Chambers GA, Englezou A, Phanthavong S, Daly A, Wildman O, Holford P, Burgess LW. 2020. First report of citrus exocortis viroid, citrus bent leaf viroid, hop stunt viroid and citrus dwarfing viroid in Lao PDR. Australasian Plant Pathology 49: 661-663 doi:10.1007/s13313-020-00740-6

Donovan NJ, Englezou A, Chambers GA, Phanthavong S, Daly A, Saleh F, Holford P, Burgess LW. 2021. First report of citrus tristeza virus in Lao PDR. Australasian Plant Pathology doi:10.1007/s13313-021-00818-9

Chambers GA, Geering ADW, Holford P, Vidalakis G, Donovan NJ. 2022. Development of a one-step RT-qPCR for the newly described citrus viroid VII. Journal of Virological Methods doi:10.1016/j.viromet.2021.114330

Osman F, Dang T, Bodaghi S, Haq R, Lavagi-Craddock I, Polek M, Rawstern A, Rodriguez E, Wulff N, Pietersen G, Englezou A, Donovan N, Vidalakis G. 2022. An updated multiplex qPCR detection assay for the detection of three *Candidatus*

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Chambers GA, Geering ADW, Holford P, Kehoe MA, Vidalakis G, Donovan NJ. A reverse transcription loop-mediated isothermal amplification assay for the detection of citrus exocortis viroid in Australian citrus. Australasian Plant Pathology submitted

Appendix 19 lists refereed scientific publications that were not direct project outputs but were produced by the program on the topic of graft-transmissible citrus pathogens and therefore contribute to the knowledge base and awareness of citrus biosecurity threats.

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Intellectual property

There is shared IP with Hort Innovation associated with project reports, extension articles and other publications.

Accessions in the citrus pathogen collection remain the IP of NSW DPI.

Technology (i.e. test methods) published by others and validated via the project will not be subject to IP provisions under the Hort Innovation Research Agreement. Test protocols developed within project CT17007 will be made available to end users by publications and direct contact with the project team.

There are no commercialisation or confidentiality issues to report.

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Appendices

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Appendix 7: Incidence of graft-transmissible pathogens detected in Australian citrus surveillance samples from October 2018 to August 2022

Appendix 8: Extension article – The team of scientists on the frontline of disease prevention. Australian Citrus News, Spring 2021 p 31

Appendix 9: Industry forum – poster – Donovan N, Chambers G, Englezou A, 2019. Protecting Australian citrus germplasm through improved diagnostic tools. Citrus Technical Forum, Adelaide 6-7 March 2019

Appendix 10: Industry forum – poster – Donovan N, Herrmann T, Chambers G, Englezou A, Forbes W, Dando A (2022) Protecting our industry from incurable diseases. Poster. Citrus Technical Forum, Sunshine Coast, Australia 8-9 March 2022

Appendix 11: Scientific conference – abstract – Chambers G, Englezou A, Webster J, Bogema D, Donovan N, 2019. Using Next generation sequencing (NGS) to characterize Australia's living pathogen collection. Joint Conference of the International Organization of Citrus Virologists and the International Research Conference on Huanglongbing, Riverside California, United States, 10-15 March 2019

Appendix 12: Scientific conference – abstract – Chambers GA, Geering A, Holford P, Vidalakis G, Donovan N. 2021. Determining the potential economic impact of the newly discovered citrus viroid VII. Proceedings of the 23rd Biennial Australasian Plant Pathology Conference, 23-26th November 2021 p 73 online

Appendix 13: Scientific journal paper – Chambers GA, Bogema DR, Englezou A, Donovan NJ (2020) First Report of Citrus viroid V and Citrus viroid VI in Australia infecting Citrus. Plant Disease DOI 10.1094/PDIS-12-19-2662-PDN

Appendix 14: Scientific journal paper – Donovan NJ, Chambers GA, Englezou A, Phanthavong S, Daly A, Wildman O, Holford P, Burgess LW (2020) First report of citrus exocortis viroid, citrus bent leaf viroid, hop stunt viroid and citrus dwarfing viroid in Lao PDR. Australasian Plant Pathology 49: 661-663 DOI 10.1007/s13313-020-00740-6

Appendix 15: Scientific journal paper – Donovan NJ, Englezou A, Chambers GA, Phanthavong S, Daly A, Saleh F, Holford P, Burgess LW (2021) First report of citrus tristeza virus in Lao PDR. Australasian Plant Pathology DOI:10.1007/s13313-021-00818-9

Appendix 16: Scientific journal paper – Chambers GA, Geering ADW, Holford P, Vidalakis G, Donovan NJ. 2022. Development of a one-step RT-qPCR for the newly described citrus viroid VII. Journal of Virological Methods DOI: 10.1016/j.viromet.2021.114330

Appendix 17: Scientific journal paper – Osman F, Dang T, Bodaghi S, Haq R, Lavagi-Craddock I, Polek M, Rawstern A, Rodriguez E, Wulff N, Pietersen G, Englezou A, Donovan N, Vidalakis G. An updated multiplex qPCR detection assay for the detection of three *Candidatus* Liberibacter species associated with citrus Huanglongbing. Phytofrontiers DOI: 10.1094/PHYTOFR-04-22-0046-FI

Appendix 18: Scientific journal paper – Donovan NJ, Englezou A, Phanthavong S, Chambers GA, Dao HT, Phitsanoukane P, Daly A, Cowan S, Holford P, Beattie GAC, Vilavong S, Burgess LW. Investigating the impact of huanglongbing in citrus in southern Lao PDR. Journal of Citrus Pathology 9 DOI:10.5070/C49155026

Appendix 19: Other NSW DPI Citrus Pathology Program publications on graft-transmissible citrus threats

Appendix 1: Summary of outputs and diagnostic or research gaps for graft- and sap-transmissible agents affecting citrus

Name	Endemic to Australia?	Can we test for it?		To be done
		Do we have a control?	Do we have a recommended assay?	
Viroid				
citrus exocortis viroid (CEVd)	Y	Y	Y	Continue biological characterisation - collect data from pot/field trials
citrus bent leaf viroid (CBLVd)	N	Y	Y	Continue biological characterisation - collect data from pot/field trials, source additional controls
citrus bent leaf viroid low sequence similarity (CBLVd – LSS)	Y	Y	Y	Continue biological characterisation - collect data from pot/field trials
hop stunt viroid (HSVd)	Y	Y	Y	Continue biological characterisation - collect data from pot/field trials. Validate near-field assay
citrus dwarfing viroid (CDVd)	Y	Y	Y	Continue biological characterisation - collect data from pot/field trials
citrus bark cracking viroid (CBCVd)	Y	Y	Y	Continue biological characterisation - collect data from pot/field trials
citrus viroid V (CVd-V)	Y	Y	Y	Continue biological characterisation - collect data from pot/field trials
citrus viroid VI (CVd-VI)	Y	Y	Y	Continue biological characterisation - collect data from pot/field trials
citrus viroid VII (CVd-VII)	Y	Y	Y	Continue biological characterisation - collect data from pot/field trials, transmission, and host studies. Continue genomic analyses.
Virus				
citrus tristeza virus (CTV)	Y	Y	Y	Improve detection assays to include Aust. variants. More information needed on variants – genotype vs phenotype. Mild cross protective variants needed for management of stem pitting in oranges and limes.
citrus psorosis virus (CPsV)	Y	Y	Y	
citrus virus A (CiVA)	Y	Y	Y	
citrus concave gum associated virus (CCGaV)	Y	Y	Y	
citrus leaf blotch virus (CLBV)	Y	Y	Y	
citrus tatterleaf virus (CTLV)	Y	Y	Y	
citrus vein enation virus (CVEV)	Y	Y	Y	
citrus variegation virus (CVV)	Y	N	Y (genus specific)	Source controls. Evaluate or develop a specific assay.
citrus leprosis virus cytoplasmic (CiLV-C)	N	Y	Y	Source additional controls.
citrus leprosis virus C2 (CiLV-C2)	Y	Y	Y	Only hibiscus strain of citrus leprosis virus C2 (CiLV-C2H) detected in Australia but not detected in citrus in Australia. Source additional controls. Evaluate assays.
hibiscus green spot virus 2 (HGSV-2)	N	N	N	Source controls. Evaluate assays.
orchid fleck virus (OFV)	N	N	N	Source controls. Evaluate assays.
citrus leprosis virus N *sensu novo* (CiLV-N)	N	N	N	Source controls. Evaluate assays.
citrus chlorotic spot virus (CiCSV)	N	N	N	Source controls. Evaluate assays.
citrus bright spot virus (CiBSV)	N	N	N	Source controls. Evaluate assays.
citrus yellow vein clearing virus (CYVCV)	N	Y	Y	Evaluate high throughput RT-qPCR assays.
citrus yellow mosaic virus (CYMV)	N	Y	Y	Evaluate high throughput RT-qPCR assays.
citrus chlorotic dwarf virus (CCDaV)	N	Y	Y	Evaluate high throughput RT-qPCR assays.
satsuma dwarf virus (SDV)	N	N	N	Source controls. Evaluate assays.
Indian citrus ringspot virus (ICRSV)	N	N	N	Source controls. Evaluate assays.
citrus leaf rugose virus (CLRV)	N	N	N	Source controls. Evaluate assays.
citrus sudden death associated virus (CSDaV)	N	N	N	Source controls. Evaluate assays.

Appendix 1 continued

Name	Endemic to Australia?	Can we test for it?		To be done
		Do we have a control?	Do we have a recommended assay?	
citrus virus B (CiVB)	N	N	N	Source controls. Evaluate assays.
citrus virus C (CiVC)	N	N	N	Source controls. Evaluate assays.
citrus associated ampelovirus 1 (CaAV-1)	N	N	N	Source controls. Evaluate assays.
citrus associated ampelovirus 2 (CaAV-2)	N	N	N	Source controls. Evaluate assays.
citrus associated rhabdovirus (CiaRV)	N	N	N	Source controls. Evaluate assays.
citrus yellow mottle associated virus (CiYMaV)	N	N	N	Source controls. Evaluate assays.
citrus leaf blotch virus 2 (CLBV-2)	N	N	N	Source controls. Evaluate assays.
citrus yellow spot virus (CiYSV)	N	N	N	Source controls. Evaluate assays.
citrus yellow vein associated virus (CYVaV)	N	N	N	Source controls. Evaluate assays.
Bacteria and bacterial-like				
Australian Citrus Dieback (ACD)	Y	Y	Y (generic phytoplasma)	Source infected plants. Identify causal agent. Develop specific detection assays. Build awareness of symptom similarity to HLB
'*Candidatus* Liberibacter asiaticus' (huanglongbing)	N	Y	Y	Develop early detection assay. Validate new multiplex assay.
'*Candidatus* Liberibacter africanus' (huanglongbing)	N	Y	Y	Develop early detection assay. Validate new multiplex assay.
'*Candidatus* Liberibacter americanus' (huanglongbing)	N	Y	Y	Source additional controls. Develop early detection assay. Validate new multiplex assay.
Spiroplasma citri (citrus stubborn disease)	N	Y	Y	Source additional controls. Validate new multiplex assay.
Xylella fastidiosa subsp. *pauca* (citrus variegated chlorosis)	N	Y	Y	Source additional controls. Evaluate new assays for National Diagnostic Protocol.
'*Candidatus* Phytoplasma phoenicium 16SrIX (HLB-like phytoplasma)	N	N	Y (generic phytoplasma)	Source controls. Evaluate specific assays.
'*Candidatus* Phytoplasma aurantifolia' 16SrII (Witches broom)	Y	N	Y (generic phytoplasma)	Source controls. Evaluate specific assays.
'*Candidatus* Phytoplasma asteris' 16SrI	N	N	Y (generic phytoplasma)	Source controls. Evaluate specific assays.
'*Candidatus* Phytoplasma pruni' 16SrIII	N	N	Y (generic phytoplasma)	Source controls. Evaluate specific assays.
'Candidatus Phytoplasma' 16SrIV	N	N	Y (generic phytoplasma)	Source controls. Evaluate specific assays.
'*Candidatus* Phytoplasma ulmi' 16SrV	N	N	Y (generic phytoplasma)	Source controls. Evaluate specific assays.
'Candidatus Phytoplasma' 16SrVI	N	N	Y (generic phytoplasma)	Source controls. Evaluate specific assays.
'*Candidatus* Phytoplasma fraxini' 16SrVII	N	N	Y (generic phytoplasma)	Source controls. Evaluate specific assays.
'*Candidatus* Phytoplasma' 16SrX	N	N	Y (generic phytoplasma)	Source controls. Evaluate specific assays.
'*Candidatus* Phytoplasma' 16SrXI	Y	N	Y (generic phytoplasma)	Source controls. Evaluate specific assays.
'*Candidatus* Phytoplasma solani' 16SrXII	Y	N	Y (generic phytoplasma)	Source controls. Evaluate specific assays.
'*Candidatus* Phytoplasma hispanicum' 16SrXIII	N	N	Y (generic phytoplasma)	Source controls. Evaluate specific assays.
'*Candidatus* Phytoplasma ' 16SrXIV	N	N	Y (generic phytoplasma)	Source controls. Evaluate specific assays.
'*Candidatus* Phytoplasma ' 16SrXV	Y	N	Y (generic phytoplasma)	Source controls. Evaluate specific assays.
'*Candidatus* Phytoplasma ' 16SrXXXII	N	N	Y (generic phytoplasma)	Source controls. Evaluate specific assays.

N.B. Exotic strains / variants exist for endemic viruses and viroids

Appendix 2: New pathogen accessions added to the NSW DPI citrus pathogen collection

Country of origin	Pathogen
Australia	hop stunt viroid (HSVd)*
	citrus dwarfing viroid (CDVd)
	citrus viroid V and VI (Appendix 12)
	citrus tristeza virus (CTV)
	citrus leaf blotch virus (CLBV)
	citrus virus A (CiVA)
	citrus concave gum associated virus (CCGaV)
	citrus vein enation virus (CVEV)
Lao People's Democratic Republic	'*Candidatus* Liberibacter asiaticus' (Appendix 17)
	CTV (Appendix 14)
	citrus tatterleaf virus (CTLV)
	citrus exocortis viroid (CEVd), citrus bent leaf viroid (CBLVd), HSVd (Appendix 13)
United States	'*Ca*. L. asiaticus'
	CTV
	citrus concave gum containing citrus virus A (CiVA)
	CEVd
	Spiroplasma citri
Pakistan	'*Ca*. L. asiaticus'
Timor Leste	'*Ca*. L. asiaticus'
	CTV
	CVEV
Bhutan	'*Ca*. L. asiaticus'

* from citrus and hops

Appendix 3: Diagnostic assays evaluated for detection of graft-transmissible citrus viroids

Target	Assays evaluated			Recommended
	Assay type	Details/primers	Reference	
CEVd	RT-qPCR	CEVd-161F / CEVd-258R / CEV-187P, multiplex assay	Osman et al. 2017	Y
		CEVd-F1 / CEVd-R1 for sequencing genome	Bernad and Duran-Vila 2006	Y
	RT-LAMP		Chambers et al. submitted	Y
CBLVd	RT-PCR -apsca generic	PBCV100C / PBCV194H, requires confirmatory test	Sano et al. 2004	Y
	RT-qPCR - apsca generic	ApscaF3-25 / ApscaR232-212, SYBR assay	Vidalakis and Wang 2013	N
	RT-PCR	CBLVd-F / CBLVd-R for sequencing genome	Donovan et al. 2020	Y
CBLVd - LSS	RT-PCR - apsca generic	PBCV100C / PBCV194H, requires confirmatory test	Sano et al. 2004	Y
	RT-PCR	CBLVd-F / CBLVd-R for sequencing genome	Donovan et al. 2020	Y
	RT-qPCR	ApscaF3-25 / ApscaR232-212, SYBR assay	Vidalakis and Wang 2013	N
HSVd	RT-qPCR	HSVd-208F / HSVd-295R/HSVd-226P, multiplex assay	Osman et al. 2017	Y
	RT-PCR	HSVd-F / HSVd-R for sequencing genome	Wang et al. 2009	Y
	High resolution melt	HSVd hrm-f / HSVd-hrm-r, differentiates cachexia / non-cachexia	Loconsole et al. 2013	N
	Agdia AmplifyRP XRT kit	Isothermal RT-RPA assay (XCS64200/0048)		N
	RT-LAMP	differentiates cachexia and non-cachexia variants	Chambers unpublished	Y
CDVd	RT-qPCR	CDVd-IIIb-F / CDVd-IIIb-R / CDVd3b probe	Vidalakis et al. 2011	Y
	RT-PCR	CDVd-F1 / CDVd-R1 for sequencing genome	Bernad and Duran-Vila 2006	Y
	RT-qPCR	ApscaF3-25 / ApscaR232-212, SYBR assay	Vidalakis and Wang 2013	N
	RT-PCR - apsca generic	PBCV100C / PBCV194H, requires confirmatory test	Sano et al. 2004	Y
CBCVd	RT-qPCR	CBCVd-44F / CBCVd-133R / CBCVd-77P, multiplex assay	Osman et al. 2017	Y
	RT-PCR	CBCVd-For68-89 / CBCVd-Rev71-48, for sequencing genome	Wang et al. 2013	Y
CVd-V	RT-PCR - apsca generic	PBCV100C/PBCV194H, requires confirmatory test	Sano et al. 2004	Y
	RT-qPCR - apsca generic	ApscaF3-25 / ApscaR232-212, SYBR assay	Vidalakis and Wang 2013	N
	RT-PCR	CVdV-F / CVdV-R, for sequencing genome	Serra et al. 2008	Y
CVd-VI	RT-PCR - apsca generic	PBCV100C / PBCV194H, requires confirmatory test	Sano et al. 2004	Y
	RT-qPCR - apsca generic	ApscaF3-25 / ApscaR232-212, SYBR assay	Vidalakis and Wang 2013	N
	RT-PCR	CVd-VI-F / CVd-VI-R for sequencing genome	Cao et al. 2017	Y
CVd-VII	RT-qPCR	VII-F6 / VII-R6 / VII-6p	Chambers et al. 2022	Y
	RT-PCR	VIIF1 / VIIR3 for sequencing genome	Chambers et al. 2018	Y
	RT-qPCR - apsca generic	ApscaF3-25 / ApscaR232-212, SYBR assay	Vidalakis and Wang 2013	N
	RT-PCR - apsca generic	PBCV100C / PBCV194H, requires confirmatory test	Sano et al. 2004	Y
	RT-PCR - apsca generic	Apsca7F / Apsca7R, SYBR assay	Chambers et al. unpublished	N

Appendix 4: Diagnostic assays evaluated for detection of graft-transmissible citrus viruses

Target	Assays evaluated			Recommended
	Assay type	Details	Reference	
CTV	RT-PCR	IRA5 / IRA6 primers	Herrera-Isidron et al. 2009	Y
	RT-qPCR	CTV generic primers – SYBR assay	Cook et al. 2016	N
	RT-PCR	CTV strain-specific primers	Cook et al. 2016, Roy et al. 2010	Y
CPsV	RT-qPCR	CPsV-792 F1 / CPsV-946 R1 / CPsV-851p-Aus* Multiplex assay with CLBV and CTLV	Osman et al. 2015 *probe amended for Aust sequence	Y
CiVA	RT-PCR	112F / 612R	Chambers unpublished	Y
	RT-PCR	Ka1 / Ka3 primers	Navarro et al. 2018b	Y
	RT-qPCR	RdRp-qF3581 / RdRp-qR3688 SYBR assay	Beris et al, 2021	Y
	RT-qPCR	CiVA-F / CiVA-R / CiVA-P	Diaz-Lara et al. 2022	Y
CCGaV	RT-PCR	Bunya-cit.1F / Bunya-cit.4R	Navarro et al. 2018	Y
		CG19 / CG18	Navarro et al. 2018	Y
		CG15 / CG20	Navarro et al. 2018	Y
	RT-qPCR	CCGaV-F / CCGaV-R / CCGaV-P	Diaz-Lara et al. 2022	Y
CLBV	RT-qPCR	CLBV-CP-7711 F / CLBV-CP-7872R / CLBV CP-7738p-FAM Multiplex assay with CPsV and CTLV	Osman et al. 2015	Y
CTLV	RT-qPCR	Multiplex assay with CLBV and CPsV CTLV6308F-17 / CTLV6413R / CTLV6340p	Chambers unpublished	Y
	RT-PCR	CTLVF3 (one base modified to M(A+C)) / CTLVR3	Su et al. 2008	Y
CVEV	RT-PCR	VE5F/VE15R	Vives et al. 2013	Y
	RT-qPCR	UC-Riverside	unpublished	N
CVV	RT-PCR -Ilarvirus generic	Ilar2F5 / Ilar2R9	Untiveros et al. 2010	Y
CiLV	RT-PCR	MPF / MPR or RepF / RepR	Locali et al. 2003	Y
CiLV-C2	RT-PCR	CiLV-C2-CPG-F / CiLV-C2-CPG-R	Roy et al. 2013	needs validation with a control
CiLV-C2H	RT-PCR	CiLV-C2H-F/ CiLV-C2H-R	Roy et al. 2018	Y

Appendix 5: Diagnostic assays evaluated for the detection of graft-transmissible bacteria and bacterial-like organisms infecting citrus

Target	Assays evaluated			Recommended
	Assay type	Details	Reference	
Australian Citrus Dieback causal agent not identified beyond phytoplasma	Nested PCR	Nested assay and sequencing R16F2 / R16R2 and P1 / P7	Deng and Hiruki 1991; Lee 1993; Schenider et al. 1995	Y
'*Candidatus* Liberibacter asiaticus'	qPCR	RNRf / RNRr / RNRp Preferred assay due to greater sensitivity and specificity	Zheng et al. 2016	Y
	qPCR	HLBas.2 / HLBas.3 / HLBr2 / HLBp2	Osman et al. 2022	Y
	qPCR[#]	HLBas_c / HLBr / HLBp	Coy et al. 2014 / Li et al. 2006	Y
	conventional PCR	A2 / J5	Hocquellet et al. 1999	Y
'*Candidatus* Liberibacter africanus'	qPCR	HLBaf.2 / HLBr2 / HLBp2	Osman et al. 2022	Y
	qPCR[#]	HLBaf / HLBr / HLBp	Li et al. 2006	Y
	conventional PCR	A2 / J5	Hocquellet et al. 1999	Y
	conventional PCR	Laf F / Laf R	Roberts et al. 2017	Y
'*Candidatus* Liberibacter americanus'	qPCR	HLBam.2 / HLBr2 / HLBp2	Osman et al. 2022	Y
	qPCR	HLBam / HLBr / HLBp	Li et al. 2006	Y
	conventional PCR	GB1 / GB3	Teixeira et al. 2005	Y
Liberibacter spp.	conventional PCR	484F / 1124R	Morrow et al. 2020	Y
Spiroplasma citri	qPCR	Php-orf1 F / Php-orf1 R Php-orf3 F / Php-orf3 R	Wang et al. 2015	Y
	conventional PCR	P58-6f / P58-4r	Yokomi et al. 2008	Y
Xylella fastidiosa subsp. *pauca* CVC strain	qPCR	XFP-F / XFP-R / XFP-p	Dupas et al. 2019	Y

[#] HLBaf assay may cross react with HLBas positive samples, and vice versa

Appendix 6: Multiplex assays for detection of graft-transmissible citrus pathogens

Targets	Assays evaluated			Recommended
	Assay type	Details	Reference	
citrus exocortis viroid / hop stunt viroid / citrus bark cracking viroid	RT-qPCR	CEVd-161F / CEVd-258R / CEV-187P HSVd-208F / HSVd-295R/HSVd-226P CBCVd-44F / CBCVd-133R / CBCVd-77P	Osman et al. 2017	Y
citrus viroid VII / plant RNA control	RT-qPCR	VII-F6 / VII-R6 / VII-6p Cox_multi-32F / Cox_multi-194 R / Cox_multi-96p	Chambers et al. 2022 Osman et al. 2015	Y
citrus psorosis virus (CPsV) / citrus leaf blotch virus (CLBV) / citrus tatterleaf virus (CTLV)	RT-qPCR	CPsV-792 F1 / CPsV-946 R1 / CPsV-851p-Aus CTLV6308F-17 / CTLV6413R / CTLV6340p CPsV probe and CTLV assay re-designed to enable detection of Australian isolates	Osman et al. 2015 Chambers unpublished	Y
'*Candidatus* Liberibacter asiaticus' and '*Xylella fastidiosa* subsp. *pauca*	RT-qPCR	RNRf / RNRr / RNRp/ XFP-F / XFP-R / XFP-p	Zheng et al. 2016 Dupas et al. 2019	Y

Appendix 7: Incidence of graft-transmissible pathogens detected in Australian citrus surveillance samples from October 2018 to August 2022

Pathogen	no. positive detections / no. samples	Incidence %
Viroids		
citrus bent leaf viroid (CBLVd)	0 / 271	0
hop stunt viroid (HSVd)	30 / 265	11
citrus dwarfing viroid (CDVd)	23 / 271	9
citrus bark cracking viroid (CBCVd)	0 / 259	0
citrus viroid V (CVd-V)	3 / 294	1
citrus viroid VI (CVd-VI)	2 / 294	0.7
citrus viroid VII (CVd-VII)	8 / 307	3
citrus exocortis viroid (CEVd)	18 / 265	7
Viruses		
citrus tristeza virus (CTV)	166 / 260	64
citrus tatterleaf virus (CTLV)	1 / 241	0.4
citrus psorosis virus (CPsV)	0 / 242	0
citrus virus A (CiVA)	0 / 227	0
citrus concave gum associated virus (CCGaV)	2 / 127	1.6
citrus leprosis virus C (CiLV-C)	0 / 83	0
hibiscus strain of citrus leprosis virus C2 (CiLV-C2H)	0 / 83	0
Bacteria		
'*Candidatus* Liberibacter asiaticus' ('*Ca*. L. asiaticus') associated with huanglongbing (HLB)	0 / 248	0
'*Candidatus* Liberibacter africanus' ('*Ca*. L. africanus') associated with huanglongbing (HLB)	0 / 248	0
'*Candidatus* Liberibacter americanus' ('*Ca*. L. americanus') associated with huanglongbing (HLB)	0 / 248	0
Xylella fastidiosa subsp. *pauca (X. fasitidiosa* subsp. *pauca)* causal agent of citrus variegated chlorosis (CVC)	0 / 247	0

Appendix 8: Extension article – The team of scientists on the frontline of disease prevention. Australian Citrus News, Spring 2021 p 31 – find entire article in a separate attachment

INNOVATION

The team of scientists on the frontline of disease prevention

On the edge of south-western Sydney, a team of dedicated scientists are working hard to protect the Australian citrus industry from disease.

The New South Wales Department of Primary Industries (NSW DPI) citrus pathology team is based at the Elizabeth Macarthur Agricultural Institute (EMAI), a 1600 ha property within the Camden Park Estate, one of the oldest farms in Australia.

EMAI was established in 1990 and is now a world-renowned biosecurity facility for plant and animal health. The citrus team has access to world class laboratories and a biosecure nursery facility. The labs include a quarantine facility for working on new diseases during an outbreak.

The scientists working with the NSW DPI Citrus Pathology Program include molecular biologists, Grant Chambers and Anna Englezou, who undertake citrus diagnostic research, and Wendy Forbes and Adrian Dando who carry out the commercial activities for the Auscitrus propagation scheme.

Grant and Anna are highly experienced after working with graft-transmissible citrus pathogens for 20 and 14 years respectively; before this Anna worked in medical research for more than ten years.

Key points

- Enhanced testing for diseases
- Australian-developed technology
- World class citrus pathology team

Wendy and Adrian joined the team three years ago but prior to this, Wendy spent two decades building a diverse technical capability in agricultural research and diagnostic laboratories and Adrian brings a wealth of nursery experience to the role.

Vipawee Iamsa-at (Noi) and George Haizer provide valuable nursery assistance. The team is led by Nerida Donovan, who has been working as a citrus pathologist for 22 years.

"The program aims to enhance the ability of the Australian citrus industry to combat disease threats by having the capability to test for all described citrus diseases, understanding the threats within our borders and making sure industry has access to disease-free propagation material to give our orchards the best start," Nerida said.

There is a strong focus on graft-transmissible diseases because they can kill trees and there is no cure.

"These diseases are spread in infected planting material, on cutting tools and a few are also spread by insects.

"They may be present in plants without symptoms, but the infected plants pose a risk to healthy plants.

"Therefore, it is essential that we can detect these diseases soon after they enter Australia or in propagation material before its use."

Detecting graft-transmissible diseases can be difficult because field symptoms may be confused with other disorders, and the pathogen may be present below detectable levels or unevenly distributed within a tree and missed during sampling.

Nerida said it is important that diagnostic tests are specific to the target organism, sensitive (i.e. will detect even at low levels), and efficient in terms of time and cost.

"The team is working on a Hort Innovation project focussed on making sure we know how to test for any graft-transmissible diseases that the industry may face," Nerida said.

"This includes exotic diseases that are not found here yet, such as the devastating bacterial disease huanglongbing (HLB), currently wreaking havoc in citrus orchards around the globe, plus other endemic diseases that do exist in Australian orchards and backyards."

Past work on international aid projects in Bhutan and Lao PDR, has provided

Anna Englezou, Nerida Donovan, Adrian Dando, Wendy Forbes, and Grant Chambers.

continued page 32

Appendix 9: Industry forum – poster – Donovan N, Chambers G, Englezou A, 2019. Protecting Australian citrus germplasm through improved diagnostic tools. Citrus Technical Forum, Adelaide 6-7 March 2019

NSW GOVERNMENT Department of Primary Industries

Auscitrus

Hort Innovation CITRUS FUND

PROTECTING AUSTRALIAN CITRUS GERMPLASM THROUGH IMPROVED DIAGNOSTIC TOOLS

Donovan NJ, Chambers GA, Englezou A

Elizabeth Macarthur Agricultural Institute, NSW DPI, Menangle NSW, Australia. E-mail: nerida.donovan@dpi.nsw.gov.au

PREVENT DETECT ERADICATE MANAGE

- PREVENT – stop diseases from entering Australia, or from entering production systems
- DETECT - find diseases soon after they enter Australia or in plant material before its use
- ERADICATE – stop a disease from becoming established
- MANAGE – reduce the impact of established diseases

Diseases can destroy an industry. Graft -transmissible diseases, mostly spread by the use of infected plant material, are of most concern as they can kill trees and there is no cure. The disease-causing agents may be present in plants without symptoms, but these plants are a source of future infections. Therefore, it is important that we have the ability to detect these diseases soon after they enter Australia or in propagation material before its use. A number of graft-transmissible viruses and viroids are present in Australia, such as citrus exocortis viroid (CEV). Diseases not found in Australia but posing a significant threat to our industry include huanglongbing (HLB) and citrus variegated chlorosis (CVC - Xylella).

THERE IS NO CURE FOR GRAFT-TRANSMISSIBLE DISEASES

Graft-transmissible diseases are managed using healthy budwood and rootstock seed from Auscitrus where the source trees have been tested for disease. The NSW DPI citrus pathology team assessed current and new diagnostic tests for graft -transmissible citrus pathogens to make sure we are using the most reliable and efficient methods available.

Tests were evaluated for 15 established and 10 exotic graft -transmissible citrus diseases.

PROJECT IMPACT

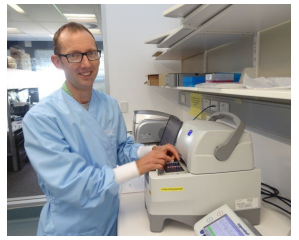
- Improved ability to detect disease threats – these methods have been adopted by Auscitrus to test plant material before supply to industry.
- Improved confidence that key emergency plant pests are not found in Australia through surveillance and testing.
- Greater awareness within industry and government of domestic and international biosecurity issues affecting citrus

The strategic levy investment project *Protecting Australian citrus germplasm using improved diagnostic tools* (CT14009) was part of the Hort Innovation Citrus Fund. The authors acknowledge funding support from Hort Innovation, Auscitrus and NSW DPI.

HLB kills trees - Florida citrus orchard

Stunted tree (L) due to CEV infection

CTV stem pitting symptoms in grapefruit



NSW DPI Citrus Pathology Laboratory – molecular testing for citrus pathogens

Appendix 10: Industry forum – poster – Donovan N, Herrmann T, Chambers G, Englezou A, Forbes W, Dando A (2022) Protecting our industry from incurable diseases. Poster. Citrus Technical Forum, Sunshine Coast, Australia 8-9 March 2022

NSW GOVERNMENT Department of Primary Industries

auscitrus Hort Innovation CITRUS FUND

PROTECTING OUR INDUSTRY FROM INCURABLE DISEASES

N Donovan[1], T Herrmann[2], G Chambers[1], A Englezou[1], W Forbes[1], A Dando[1]

[1] Elizabeth Macarthur Agricultural Institute, NSW DPI, Menangle NSW Australia. E-mail: nerida.donovan@dpi.nsw.gov.au

[2] Auscitrus, Dareton NSW Australia

Diseases can destroy an industry. The NSW DPI Citrus Pathology Program aims to protect the health of the Australian citrus industry by minimising the impact of established diseases and preparing for the arrival of new diseases.

- **PREVENT** – stop diseases from entering Australia, or from entering production systems
- **DETECT** - find diseases soon after they enter Australia or in plant material before its use
- **ERADICATE** – stop a disease from becoming established
- **MANAGE** – understand established diseases to reduce their impact

PREVENT DETECT ERADICATE MANAGE

The best protection is prevention – Auscitrus and NSW DPI work together to safeguard the supply of disease-free budwood and rootstock seed to industry. A citrus tree may look healthy but there may be an incurable disease lurking within. The tree will not show symptoms if the variety is tolerant or resistant to the disease but if buds from that tree are propagated onto a susceptible rootstock, the new trees will show symptoms of reduced yield or fruit quality, tree decline or death. Graft-transmissible diseases are mostly spread through infected propagation material. Therefore, infections can be prevented by establishing new orchards with healthy plants propagated using disease-free material supplied by Auscitrus.

These diseases can also be spread mechanically on infected cutting tools during pruning and hedging, and in some cases by aphids or other vectors. Several graft-transmissible viruses and viroids are present in Australia, such as citrus exocortis viroid (CEV) and citrus tristeza virus (CTV). Diseases not found in Australia but posing a significant threat to our industry include huanglongbing (HLB) and citrus variegated chlorosis (CVC - Xylella).

THERE IS NO CURE FOR GRAFT-TRANSMISSIBLE DISEASES

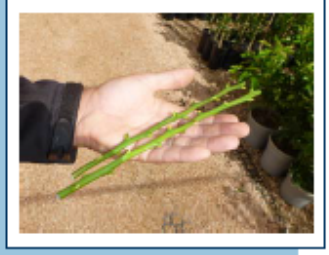
The Auscitrus source trees are tested for disease by the NSW DPI citrus pathology team who, with support from Hort Innovation, identify better methods to detect endemic diseases found in Australian orchards and backyards, as well as exotic diseases that are not found here yet. As we learn more about these pathogens, we improve our ability to prevent, detect, eradicate and manage citrus diseases.

INDUSTRY IMPACTS

- Improved ability to detect disease threats.
- Improved confidence that key emergency plant pests are not found in Australia.
- Greater awareness within industry and government of domestic and international biosecurity issues affecting citrus.

HLB kills trees - Florida citrus orchard

Stunted tree (L) due to CEV infection



You need to know what's in your budwood

NSW DPI Citrus Pathology Laboratory – molecular testing for citrus pathogens

Funding support is acknowledged from Auscitrus and Hort Innovation, using the citrus research and development levy and contributions from the Australian government under project 'CT17007 Improving diagnostics and biosecurity for graft-transmissible diseases in citrus. Hort Innovation is a grower owned, not-for-profit research and development corporation for Australian horticulture.

www.dpi.nsw.gov.au

Appendix 11: Scientific conference – abstract – Chambers G, Englezou A, Webster J, Bogema D, Donovan N, 2019. Using Next generation sequencing (NGS) to characterize Australia's living pathogen collection. Joint Conference of the International Organization of Citrus Virologists and the International Research Conference on Huanglongbing, Riverside California, United States, 10-15 March 2019

IOCV-O1-05

Using Next generation sequencing (NGS) to characterize Australia's living pathogen collection

Grant CHAMBERS[1], Anna ENGLEZOU[1], John WEBSTER[1], Daniel BOGEMA[1], Nerida DONOVAN[1]

[1]NSW Department of Primary Industries, Menangle, Australia

Abstract: Major graft-transmissible citrus diseases, such as huanglongbing (HLB), are not known to occur in Australia. However, there are several endemic graft-transmissible viruses and viroids which are managed using uninfected propagation material. NSW DPI maintains a living pathogen collection to evaluate and develop diagnostic protocols, and as controls for testing Australian citrus germplasm. To evaluate the potential of NGS, high quality small or total RNA extracts from this collection were sequenced using Illumina HiSeq or NextSeq technologies. The NGS data was manually analysed in-house, using CLC workbench 6, Geneious, and an adaption of the *VirusFinder* pipeline (Wang *et al*. 2013); and also analysed with web-based automated pipelines. Data analysis confirmed the presence of 7 viroid and 6 virus species, including several Citrus tristeza virus strain variants. Some viruses and viroids detected were known to be present within the samples, whereas others were only revealed using NGS. Citrus viruses and viroids previously unreported in Australia were detected, and the presence of a novel citrus viroid, tentatively named CVd-VII, was confirmed. The data enabled the generation of draft genomes for a number of viruses and viroids; some potentially unique to Australia.

Non-technical summary: Endemic viruses and viroids of citrus, and new species not known to be present in Australia, were confirmed in an Australian living pathogen collection using advanced sequencing methods. This technology greatly enhances the diagnostic tool kit for robust testing of citrus germplasm before supply to industry.

Citations:

Wang Q, Jia P, Zhao Z (2013) VirusFinder: software for efficient and accurate detection of viruses and their integration sites in host genomes through next generation sequencing data. PLOS ONE 8(5)

Appendix 12: Scientific conference – abstract – Chambers GA, Geering A, Holford P, Vidalakis G, Donovan N. 2021. Determining the potential economic impact of the newly discovered citrus viroid VII. Proceedings of the 23rd Biennial Australasian Plant Pathology Conference, 23-26th November 2021 p 73 online

Determining the potential economic impact of the newly discovered Citrus viroid VII

Mr Grant Chambers[1], A/Prof Andrew Geering[2], Paul Holford[3], Georgios Vidalakis[4], Dr Nerida Donovan[1]

[1]New South Wales Department of Primary Industries, Menangle, Australia, [2]Queensland Alliance for Agriculture and Food Innovation, University of Queensland, Dutton Park, Australia, [3]Western Sydney University, Penrith, Australia, [4]University of California, Riverside, USA

Biography:

Grant Chambers has worked for New South Wales Department of Primary Industries for more than 20 years, primarily in plant disease diagnostics and biosecurity. He has extensive experience working with exotic and endemic graft-transmissible pathogens of citrus. Grant is a PhD candidate with the University of Queensland researching viroids in Australian citriculture, with a focus on citrus viroid VII.

Abstract:

Citrus viroid VII (CVd-VII) is a recently discovered viroid that has been detected in a small number of lemon trees in Australia but nowhere else in the world. It is important to better characterise this viroid to establish the biosecurity risk that it poses for the citrus industry in Australia and elsewhere in the world, including potential economic impacts to growers. Host range studies are being done to examine whether the viroid is able to infect a broader range of citrus cultivars other than lemon, as well as non-citrus species. To assist with these studies, a highly sensitive RT-qPCR has been developed, which supersedes the published diagnostic test for CVd-VII, a conventional RT-PCR. Furthermore, potential synergisms between CVd-VII and other citrus-infecting viroids in Australia are being investigated. Several viroids are known to infect citrus, with some causing stunting, yield loss and even death, while others induce mild symptoms. To determine the impact of co-infection of CVd-VII with other endemic viroids, greenhouse and field trials have been established.

Appendix 13: Scientific journal paper – Chambers GA, Bogema DR, Englezou A, Donovan NJ (2020) First Report of Citrus viroid V and Citrus viroid VI in Australia infecting Citrus. Plant Disease DOI 10.1094/PDIS-12-19-2662-PDN

DISEASE NOTES

OPEN

First Report of Citrus Viroid V and Citrus Viroid VI in Australia Infecting Citrus

G. A. Chambers, D. R. Bogema, A. Englezou, and N. J. Donovan

Affiliations

Authors and Affiliations
G. A. Chambers †
D. R. Bogema
A. Englezou
N. J. Donovan
NSW Department of Primary Industries, Elizabeth Macarthur Agricultural Institute, Menangle, NSW 2568, Australia

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TOOLS SHARE

Citrus viroid V (CVd-V) and citrus viroid VI (CVd-VI) are members of the *Apscaviroid* genus found in citrus growing areas worldwide but previously unreported from Australia. Other viroids, including the pathogenic citrus exocortis viroid and hop stunt viroid, have previously been reported in Australian citrus orchards. A *Citrus reticulata* Blanco field sample was submitted for routine pathogen screening. Total RNA extracted with the VioTotal Plant RNA Extraction Miniprep system (Viogene, U.S.A.) tested positive for CVd-V using a SYBR-Green RT-qPCR for apscaviroids (Vidalakis and Wang 2013). To examine this result further, high-throughput sequencing was performed. Total nucleic acid was extracted using the mirVana miRNA Isolation kit (Life Technologies, Australia). Following rRNA depletion, a cDNA library was prepared using the Truseq RNA Sample Prep Library (Illumina, U.S.A.) and sequenced using an Illumina NextSeq500 sequencing platform. Sequencing generated 13,985,690 × 75 bp paired-end reads, which were trimmed and analyzed using the VirusFinder 2.0 pipeline (Wang et al. 2013). This involved preprocessing of reads by aligning to the *Citrus sinensis* genome (AJPS01000000) with Bowtie2 version 2.3.4.1 (Langmead and Salzberg 2012). Nonhost reads were extracted with SAMtools version 0.1.18 (Li et al. 2009) and mapped to NCBI viroid/virus genome reference databases using BLAT version 34 (Kent 2002). Viral and viroid reads were extracted and were de novo assembled using Trinity version 2012-06-08 (Grabherr et al. 2011). Assembled contigs were matched to the above viroid/virus databases with BLASTn version 2.2.26+. Two contigs, 548 nucleotide (nt) and 338 nt, made of 173 and 86 reads, aligned with 96.6 and 93.6% identity to respective reference sequences of the 294-nt CVd-V (EF617306) and the 330-nt CVd-VI (AB019508). No other viroids or viruses were detected in this sample. Reverse transcription PCR (RT-PCR) specific for CVd-V (Serra et al. 2008) and CVd-VI (Cao et al. 2017) confirmed the presence of each viroid. These full-length amplicons were cloned using pGEM-T Easy vector system (Promega, Australia), and at least five clones of each were sequenced in both directions, producing complete viroid genomes. Four 294-nt sequence variants of CVd-V (MN640600 to MN640603) were present in the sample, each with 2- to 3-nt substitutions compared with AB560862 (TS1 isolate, Japan).

For CVd-VI, two 331-nt variants were observed (MN640604 to MN640605) with a single substitution, showing 100 and 99% respective sequence similarity to the Japanese TS isolate (AB054601). Subsequent testing by RT-PCR detected both CVd-V and CVd-VI in two of 23 field samples of the same mandarin cultivar. 'Etrog' citron Arizona 861-S-1 (*C. medica* L.) cuttings were graft-inoculated with the original sample and maintained at 30°C. CVd-V and CVd-VI were detected in new growth by RT-PCR. Although symptoms exhibited by CVd-V and CVd-VI in citron are reported to be mild (Ito et al. 2001; Serra et al. 2008), no symptoms were observed on the indicator plants in the 12 months postinoculation. These detections in nonsymptomatic material highlight the importance of budwood certification programs to reduce the spread of viroids. To our knowledge, this is the first detection of CVd-V and CVd-VI in Australia. Field symptoms may be augmented when other viroids coinfect with CVd-V and CVd-VI (Ito et al. 2002; Serra et al. 2008).

The author(s) declare no conflict of interest.

Funding: This work was supported by the not-for-profit citrus industry organization Auscitrus. The project was also funded by Horticulture Innovation, using the citrus research and development levy and contributions from the Australian Government. Hort Innovation is the grower owned, not-for-profit research and development corporation for Australian horticulture.

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Appendix 14: Scientific journal paper – Donovan NJ, Chambers GA, Englezou A, Phanthavong S, Daly A, Wildman O, Holford P, Burgess LW (2020) First report of citrus exocortis viroid, citrus bent leaf viroid, hop stunt viroid and citrus dwarfing viroid in Lao PDR. Australasian Plant Pathology 49: 661-663 DOI 10.1007/s13313-020-00740-6 – find entire paper in a separate attachment

Australasian Plant Pathology
https://doi.org/10.1007/s13313-020-00740-6

RESEARCH NOTE

First report of citrus exocortis viroid, citrus bent leaf viroid, hop stunt viroid and citrus dwarfing viroid in Lao PDR

N. J. Donovan[1] · G. A. Chambers[1] · A. Englezou[1] · S. Phanthavong[2] · A. Daly[1] · O. Wildman[1] · P. Holford[3] · L. W. Burgess[4]

Received: 30 August 2020 / Accepted: 8 September 2020

Abstract
Citrus exocortis viroid, citrus bent leaf viroid, hop stunt viroid and citrus dwarfing viroid were detected for the first time in Lao PDR. Samples were collected from citrus trees across southern Lao PDR for laboratory testing in Australia. RNA was extracted and amplified using quantitative reverse transcription polymerase chain reaction (qRT-PCR); viroid identities were confirmed by sequencing.

Keywords Citrus · Lao · *Pospiviroid* · *Hostuviroid* · *Apscaviroid* · Graft-transmissible

Viroids are small, circular, naked, single stranded RNA molecules that infect a broad range of host plants (Flores et al. 2011). Eight viroids are known to infect citrus (Ito et al. 2001; Durán-Vila and Semancik 2003; Serra et al. 2008b; Chambers et al. 2018), and different variants or strains are present within some of these species. Viroid diseases are a serious economic threat to citrus production, causing stunting, yield loss and sometimes death in susceptible varieties (Roistacher et al. 1996). Viroids on their own may be symptomless in some citrus varieties, but co-infection can alter plant responses (Vernière et al. 2006; Serra et al. 2008a). Viroids may also be beneficial when they induce mild to moderate dwarfing with no reduction of fruit size or yield per canopy surface area, proving useful for planting orchards at high density to increase productivity (Hutton et al. 2000). Therefore, it is important to know what graft-transmissible agents are present in a citrus tree before using it as a source of material to propagate new trees, and for understanding the factors affecting tree health in an orchard or region.

Citrus trees are in serious decline across Lao People's Democratic Republic (Lao PDR); a landlocked, mountainous country in southeast Asia. To investigate the cause of the decline, citrus trees were inspected in nurseries, orchards and backyards in Champasak and Sekong provinces in southern Lao PDR in 2018 and 2020. Samples consisting of 5–10 citrus leaves were collected from each of 59 trees and kept cool until they were processed. Mid-ribs were excised, preserved in 70% ethanol, and transported under permit to Australia for testing for a range of graft-transmissible pathogens known to infect citrus, including viroids.

Total RNA was extracted from each sample using the ISOLATE II Plant RNA Kit (Bioline Meridian Bioscience®) following the manufacturer's instructions. Samples were screened using qRT-PCR for citrus exocortis viroid (CEVd, *Pospiviroid*), hop stunt viroid (HSVd, *Hostuviroid*), (Lin et al. 2015; Osman et al. 2017) and citrus bark cracking viroid (CBCVd, *Cocadviroid*) (Osman et al. 2017); and citrus bent leaf viroid (CBLVd), citrus dwarfing viroid (CDVd), citrus viroid V (CVd-V), and citrus viroid VI (CVd-VI) using a SYBR Green qRT-PCR for apscaviroids (Vidalakis and Wang 2013). Conventional, one-step RT-PCR was conducted with primers amplifying CEVd (Wang et al. 2009), HSVd (Wang et al. 2009), CBLVd (CBLVd-F: 5'-GGTCGTCGTCGACGAAGGCTCSTCAGC-3' and CBLVd-R: 5'-AGGAGCCCTCAGGGGTTC-3') or CDVd

✉ N. J. Donovan
nerida.donovan@dpi.nsw.gov.au

[1] NSW Department of Primary Industries, Elizabeth Macarthur Agricultural Institute, Private Bag 4008, Narellan, NSW 2567, Australia

[2] Agriculture Section, Provincial Agriculture and Forestry Office, Pakse, Champasak Province, Lao People's Democratic Republic

[3] School of Science, Western Sydney University, LB 1797, Penrith, NSW 2752, Australia

[4] Institute of Agriculture, Faculty of Science, The University of Sydney, Sydney, NSW 2006, Australia

Published online: 18 September 2020

Appendix 15: Scientific journal paper – Donovan NJ, Englezou A, Chambers GA, Phanthavong S, Daly A, Saleh F, Holford P, Burgess LW (2021) First report of citrus tristeza virus in Lao PDR. Australasian Plant Pathology DOI:10.1007/s13313-021-00818-9 – find entire paper in a separate attachment

Australasian Plant Pathology (2021) 50:683–685
https://doi.org/10.1007/s13313-021-00818-9

RESEARCH NOTE

First report of citrus tristeza virus in Lao PDR

N. J. Donovan[1] · A. Englezou[1] · G. A. Chambers[1] · S. Phanthavong[2] · A. Daly[1] · F. Saleh[1] · P. Holford[3] · L. W. Burgess[4]

Received: 8 July 2021 / Accepted: 23 August 2021 / Published online: 6 September 2021

Abstract
Citrus tristeza virus was detected for the first time in the Lao People's Democratic Republic. Samples were collected from citrus trees across the southern provinces for testing in Australia. RNA was extracted and tested using conventional and real-time reverse transcription polymerase chain reactions with the virus detected in 12 of 59 samples tested. Viral identities were confirmed by sequencing. Additional confirmation was obtained by an enzyme-linked immunosorbent assay. The implications of the presence of this virus for citrus production in Lao are discussed briefly.

Keywords Citrus · *Closterovirus* · Graft-transmissible · Aphid vector

Introduction

Graft-transmissible viruses of citrus are a serious threat to production world-wide, as they can cause stunting, yield loss, developmental abnormalities and even death in specific scion and rootstock combinations. Symptoms of infection may not be evident in some varieties at the nursery stage but appear a few years later in the orchard; other varieties may be symptomless carriers throughout the life of the tree. The most destructive of these viruses is citrus tristeza virus (CTV), a member of the genus *Closterovirus* that occurs in nearly all citrus-producing countries around the world. CTV is highly variable; several strains (genotypes) are recognised based on their genomes, although viral variants belonging to the same genotype can have different phenotypes. Citrus trees infected with CTV typically contain mixtures of sequence variants and these variants can be members of more than one strain (Harper et al. 2013). Two economically significant syndromes induced by CTV in the field are stem pitting and quick decline (Bar-Joseph et al. 1989; Moreno et al. 2008). However, not all CTV isolates are associated with severe symptoms and may be virtually asymptomatic with some of these mild isolates applied as cross-protection sources to protect against infection by virulent isolates.

The Lao People's Democratic Republic (Lao PDR) is a landlocked, mountainous country in South East Asia. As part of an investigation of the health status of citrus trees in Lao PDR, plants were inspected in nurseries, orchards and backyards in the southern provinces of Champasak and Sekong in 2018 and 2020. Samples consisting of 5–10 citrus leaves were collected from 59 trees and kept cool until they were processed. Leaf mid-ribs were excised, preserved in 70% ethanol, and transported under permit to Australia for testing. Total RNA was extracted from the samples using an ISOLATE II Plant RNA Kit (Bioline Meridian Biosciences) following the manufacturer's instructions.

Samples were screened for CTV using conventional one-step RT-PCR (Herrera-Isidrón et al. 2009). Confirmation of identity was obtained by bidirectional sequencing the amplicons from selected samples. RNA extracts, in which CTV was detected, were tested using conventional RT-PCR with strain specific primers targeting the T68, T3, VT and T30 strains (Roy et al. 2010), the RB1 and RB2 sub-groups of the RB strain, and the T36 and HA16-5 strains (Cook et al. 2016). Variations from the published methods included the use of a SuperScript® III One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase and lowering the annealing temperature to 59 °C in the assay for strain T68.

✉ N. J. Donovan
nerida.donovan@dpi.nsw.gov.au

[1] NSW Department of Primary Industries, Elizabeth Macarthur Agricultural Institute, Private Bag 4008, Narellan, NSW 2567, Australia

[2] Agriculture Section, Provincial Agriculture and Forestry Office, Pakse, Champasak Province, Lao PDR

[3] School of Science, Western Sydney University, LB 1797, Penrith, NSW 2752, Australia

[4] Faculty of Science, Institute of Agriculture, The University of Sydney, Sydney, NSW, Australia

Appendix 16: Scientific journal paper – Chambers GA, Geering ADW, Holford P, Vidalakis G, Donovan NJ. 2022. Development of a one-step RT-qPCR for the newly described citrus viroid VII. Journal of Virological Methods DOI: 10.1016/j.viromet.2021.114330 – find entire paper in a separate attachment

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Short communication

Development of a one-step RT-qPCR detection assay for the newly described citrus viroid VII

Grant A. Chambers [a,b,*], Andrew D.W. Geering [b], Paul Holford [c], Georgios Vidalakis [d], Nerida J. Donovan [a]

[a] NSW Department of Primary Industries, Elizabeth Macarthur Agricultural Institute, Private Bag 4008, Narellan, NSW, 2567, Australia
[b] Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, GPO Box 267, Brisbane, Queensland, 4001, Australia
[c] Western Sydney University, School of Science, LB 1797, Penrith, NSW, 2752, Australia
[d] Department of Microbiology and Plant Pathology, University of California, Riverside, Riverside, CA, United States

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ABSTRACT

An apscaviroid, tentatively named citrus viroid VII (CVd-VII), was recently discovered in citrus in Australia. A diagnostic assay using real-time reverse transcription polymerase chain reaction was developed and validated to detect the viroid in citrus plants. The assay showed a high level of sensitivity, reliably detecting 2000 plasmid copies per reaction, while down to 20 plasmid copies per reaction were occasionally detected. The assay showed high specificity, producing no false positives or cross-reactivity with a range of other citrus graft-transmissible pathogens, including viroids, viruses and bacteria. The real-time assay was also found to be more sensitive than the available end-point reverse transcription polymerase chain reaction assay by a factor of 100,000 and could be a useful tool for the rapid detection of CVd-VII in diagnostic and research environments.

Viroids are the smallest known plant pathogens with genomes ranging in size from 246 to 434 nucleotides (nt). They are naked, covalently-closed, circular, single stranded, non-coding RNA molecules that rely entirely on the host plant for replication and movement (Flores et al., 2005). Viroids are very infectious and are stable outside of the host cell, persisting on hard surfaces for as long as 24 h (Mackie et al., 2015). They are mechanically-transmitted through sap-contaminated pruning, hedging or grafting tools, and are often seed-transmitted (Barba et al., 2007; Card et al., 2007). Some viroids can be insect-vectored, such as tomato apical stunt viroid and tomato chlorotic dwarf viroid, which are carried as contaminants on the bodies of bumblebees (*Bombus terrastris* L.) and mechanically-transmitted during the process of buzz pollination (Antignus et al., 2007; Van Bogaert et al., 2016). However, there are no vectors of any of the citrus viroids reported (Duran-Vila and Semancik, 2003). There are currently 33 recognised viroid species (Di Serio et al., 2020), seven of which infect citrus species and their hybrids. The symptoms of infection in citrus are variable, and range from no discernible impact on growth to severe stunting, bark scaling and gumming in the vascular tissue induced by some variants of citrus exocortis viroid (CEVd) and hop stunt viroid (HSVd) on sensitive citrus species.

A novel viroid was discovered in citrus in New South Wales (NSW), Australia, which was detected following graft-inoculation of 'Etrog' citron plants (*C. medica* L.) with bark chips from an asymptomatic cultivated lemon tree that was submitted for routine pathogen testing (Chambers et al., 2018). Epinasty, curling and midvein necrosis of the leaves were observed on the inoculated 'Etrog' plants, suggesting transmission of an infectious agent from the lemon tree, but the inoculated plants tested negative for all citrus viroids that were known at that time. It was determined that a novel viroid, 368 nt-long and containing characteristic apscaviroid features, was present, which had less than 60 % nucleotide sequence identity with any other viroid, confirming its status as a new species (Di Serio et al., 2014; Chambers et al., 2018). This viroid, named citrus viroid VII (CVd-VII), has only been found in a small number of citrus trees planted at a research station at Dareton, NSW, but it is likely that it has a broader geographic distribution both within Australia and elsewhere in the world.

Real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR) is a widely used method of detection of viroids because of its speed and sensitivity, ability to quantify the viroid and, most importantly, the risk of PCR contamination is reduced as amplification and detection occur simultaneously in the same tube (Navarro et al.,

* Corresponding author at: NSW Department of Primary Industries, Elizabeth Macarthur Agricultural Institute, Private Bag 4008, Narellan, NSW, 2567, Australia.
E-mail address: grant.chambers@dpi.nsw.gov.au (G.A. Chambers).

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Appendix 17: Scientific journal paper – Osman F, Dang T, Bodaghi S, Haq R, Lavagi-Craddock I, Polek M, Rawstern A, Rodriguez E, Wulff N, Pietersen G, Englezou A, Donovan N, Vidalakis G. An updated multiplex qPCR detection assay for the detection of three *Candidatus* Liberibacter species associated with citrus Huanglongbing. Phytofrontiers DOI: 10.1094/PHYTOFR-04-22-0046-FI – find entire paper in a separate attachment

OPEN

Update and validation of the 16S rDNA qPCR assay for the detection of three 'Candidatus' Liberibacter species following current MIQE guidelines and workflow

Fatima Osman, Tyler Dang, Sohrab Bodaghi, Rukhama Haq, Irene Lavagi-Craddock, Amanda Rawstern, Esteban Rodriguez, Marylou Polek, Nelson Arno Wulff, Ronel Roberts, Gerhard Pietersen, Anna Englezou, Nerida Donovan, Svetlana Y. Folimonova, and Georgios Vidalakis

Published Online: 19 Jul 2022 | https://doi.org/10.1094/PHYTOFR-04-22-0046-FI

Abstract

An updated real-time multiplex quantitative polymerase chain reaction (qPCR) assay was designed and validated for the simultaneous detection of three 'Candidatus Liberibacter species' (CLsp), Ca. Liberibacter asiaticus (CLas), africanus (CLaf) and americanus (CLam), associated with the Huanglongbing (HLB) disease of citrus. The multiplex assay was designed based on the previously published qPCR assay by Li et al., 2006, taking into consideration all available CLsp 16S rRNA gene sequences in the GenBank and the MIQE guidelines and workflow for qPCR optimization, which became available after 2006. When using the updated multiplex CLsp qPCR assay compared to singleplex qPCR, no significant increase in Cq values was detected. The specificity and sensitivity of the updated qPCR assay was optimal and measuring the intra and inter assay variations confirmed the reproducibility and repeatability of the assay. The assay was also successfully used with a large number of diverse samples, at independent laboratories in four countries, thus demonstrating its transferability, applicability, practicability, and robustness as different qPCR reaction conditions or instruments had a minor effect on Cq values. This updated multiplex CLsp qPCR assay can be used in a variety of citrus surveys, germplasm, or nursery stock programs that require different pathogen detection tools for their successful operation. Keywords: Citrus greening disease, COX internal DNA control; validation; citrus germplasm; budwood; citrus nursery, citrus survey, regulatory diagnostics, Citrus Clonal Protection Program (CCPP), National Clean Plant Network (NCPN)

First Look

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Appendix 18: Scientific journal paper – Donovan NJ, Englezou A, Phanthavong S, Chambers GA, Dao HT, Phitsanoukane P, Daly A, Cowan S, Holford P, Beattie GAC, Vilavong S, Burgess LW. Investigating the impact of huanglongbing in citrus in southern Lao PDR. Journal of Citrus Pathology 9 DOI:10.5070/C49155026 – find entire paper in a separate attachment

Research Article

Investigating the Impact of Huanglongbing in Citrus in Southern Lao PDR

N.J. Donovan[1,*], A Englezou[1], S Phanthavong[2], GA Chambers[1], HT Dao[3], P Phitsanoukane[2], A Daly[1], S Cowan[4], P Holford[5], GAC Beattie[5], S Vilavong[2], LW Burgess[6]

[1]NSW Department of Primary Industries, Elizabeth Macarthur Agricultural Institute, Woodbridge Road, Menangle NSW 2568, Australia; [2]Agriculture Section, Provincial Agriculture and Forestry Office, Pakse, Champasak Province, Lao PDR; [3]Plant Protection Research Institute, Ha Noi, Viet Nam; [4]Northern Australian Quarantine Strategy, Department of Agriculture, Water and the Environment, Cairns, Qld, Australia; [5]School of Science, Western Sydney University, Penrith, NSW 2752, Australia; [6]Institute of Agriculture, Faculty of Science, The University of Sydney, Sydney, 2006 NSW, Australia

*Correspondence to: nerida.donovan@dpi.nsw.gov.au

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Abstract

Citrus has been promoted in the Lao People's Democratic Republic (Lao PDR) as a poverty reduction strategy for at least two decades. However, citrus trees have been in widespread decline for no less than ten years. Since 2010, the authors have observed symptoms on citrus trees consistent with the bacterial disease huanglongbing (HLB). These symptoms included asymmetric leaf mottle, small lopsided fruit, poor fruit production and tree decline. The authors then initiated a long-term study on the occurrence of HLB in southern Lao PDR. Samples of leaf mid-ribs were collected from citrus trees in orchards, nurseries, and backyards across four provinces: Champasak, Sekong, Salavan, and Savannakhet. The presence of '*Candidatus* Liberibacter asiaticus', the putative causal agent of the Asiatic form of HLB, was confirmed in 59 of 109 samples collected in all four provinces. The Asian citrus psyllid, the vector of '*Ca*. L. asiaticus', was also observed on citrus trees and tested positive for the pathogen. The implications of these findings for citrus production in Lao PDR are discussed.

Keywords: graft-transmissible, Asian citrus psyllid, smallholder

Introduction

The Lao People's Democratic Republic (Lao PDR) is a mountainous country in southeast Asia bordered by Việt Nam, Cambodia, Thailand, Myanmar and China (Figure 1). The Mekong River runs through the country from north to south, and the land is prone to damaging storms, floods, landslides, and drought. There are encouraging trends in economic growth and development and the Lao PDR is expected to graduate from Least Developed Country status in 2026 (United Nations 2021). However, poverty, hunger, and malnutrition remain a challenge for the Lao PDR (World Food Programme 2021). Food insecurity is exacerbated by poverty, volatile food prices, land availability, weather extremes and poor productivity. Rain-fed rice is the main staple crop grown on the relatively flat land along the Mekong and in mountainous areas. Horticultural crops have been promoted and have made a significant contribution to poverty alleviation in some areas. However, plant diseases have constrained productivity (Ireland et al. 2014; Ireland et al. 2016; Callaghan et al. 2016; Callaghan et al. 2016).

Citrus has been promoted as a poverty alleviation crop by the government of Lao PDR for at least two decades. Smallholder orchards and backyard citrus are found in the north near Vang Vieng and Luang Prabang, and in the south mainly in the provinces of Champasak, Sekong and Salavan (Figure 1). There is a new citrus area in the Vilabouly District, eastern Savannakhet, and another on the Bolavan Plateau in Paksong District, Champasak Province. Fruit is exported fresh or dried to Việt Nam and Thailand, but there are no accurate records on total production and domestic consumption. There is limited support for the industry, and little is known about citrus disease issues faced by farmers.

The widespread decline of citrus trees in the major growing areas led to a request for assistance to the Australian authors by the Lao PDR Department of Agriculture in 2010. Subsequent investigations found citrus trees with asymmetric leaf mottle that crosses leaf veins, small upright chlorotic leaves with symptoms resembling nutrient deficiencies, raised corky leaf veins, small lopsided fruit, poor fruit production and tree decline. All these symptoms are consistent with the devastating bacterial disease huanglongbing (HLB) (Bové 2006; Gottwald et al. 2007) and were observed in mandarin, lime and pomelo trees affected by severe decline near Vang Vieng in northern Lao PDR in 2010, and in the south in Champasak Province in 2013.

HLB is the most significant threat to global citrus production and is well established throughout southeast Asia (Beattie and Holford 2008). Citrus and other Rutaceae

Appendix 19: NSW DPI Citrus Pathology Program Publications

Published on graft-transmissible citrus threats during the project term but not as direct project outputs.

Refereed scientific publications

Rani A, Donovan N, Mantri N. 2019. Review: The future of plant pathogen diagnostics in a nursery production system. Bionsensors and Bioelectronics 145 doi:10.1016/j.bios.2019.111631

Morrow JL, Om N, Beattie GAC, Chambers GA, Donovan NJ, Liefting LW, Riegler M, Holford P. 2020. Characterization of the bacterial communities of psyllids associated with Rutaceae in Bhutan by high throughput sequencing. BMC Microbiology 20:215 doi:10.1186/s12866-020 -01895-4

Om N, Beattie GAC, Holford P, Donovan NJ. 2021. Incidence of psyllids and '*Candidatus* Liberibacter asiaticus' in mandarin orchards at different elevations in Tsirang, Bhutan. Tropical Plant Pathology doi:10.1007/s40858-020-00413-1

Chambers GA, Dodds K, Donovan NJ. 2021. Hop stunt viroid detection in hops (*Humulus lupulus*) in Australia. Australasian Plant Disease Notes 16(3) doi:10.1007/s13314-021-00419-x

Om N, Beattie GAC, Donovan NJ, Holford P. 2021. *Diaphorina communis*: molecular identification, development on Citrus reticulata and acquisition and transmission of '*Candidatus* Liberibacter asiaticus'. Journal of Applied Entomology 00: 1-12 doi:10.1111/jen.12937

Holford P, Om N, Donovan NJ, Beattie GAC, Subandiyah S, Gunardi R, Poerwanto ME. 2021. High altitudes limit the incidence of huanglongbing and its vector, *Diaphorina citri*, in citrus orchards. IOP Conference Series: Earth and Environmental Science doi:10.1088/1755-1315/1018/1/012019

Book chapter

Donovan NJ, Chambers GA, Cao M. 2022. Detection of Viroids by RT-PCR. In: Viroids: Methods and Protocols. Eds: A Rao, I Lavagi-Craddock, G Vidalakis. Springer US pp 143-151 doi:10.1007/978-1-0716-1464-8_13 eBook ISBN 978-1-0716-1464-8 Hardcover ISBN 978-1-0716-1463-1

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Donovan N, Herrmann T, Hancock N. 2019. On the frontline: preparing for the arrival of HLB in Australia [abstract]. Journal of Citrus Pathology 6(1): 60

Smith M, Newman T, Gultzow D, Innes D, Donovan N, Chambers G, Barkley P, Miles A, Stein B. 2019. Wide segregation of stem pitting and other CTV symptoms in a hybrid population derived from West Indian lime [abstract]. Journal of Citrus Pathology 6(1): 41-42

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Donovan N. 2022. What happens after an incursion. Citrus Technical Forum, Sunshine Coast, Australia 8-9 March 2022

Extension publications

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Presentations

Donovan N. Citrus biosecurity. WA Citrus Industry Day and Citrus Australia Technical Forum. Hervey and Bunbury Western Australia, Australia, 18-19th October 2018

Donovan N. Surveillance for established and exotic target pathogens. Field Triage for Citrus Pests Workshop. Mildura, Victoria, Australia, 24-25 September 2019

Donovan N. Case study: Managing graft-transmissible diseases in Australian citrus. Guest lecture presented to 3rd year Plant Disease students in the Faculty of Agriculture and Environment, University of Sydney, NSW Australia. Annually from 2001-2019

Donovan N. Viruses and viroids as plant pathogens. Guest lecture presented to 3rd year Plant Disease students in the Faculty of Agriculture and Environment, University of Sydney, NSW Australia. Annually from 2001-2019

Donovan N. Plant Health and Biosecurity. Guest lecture presented to 3rd year students in the Hawkesbury Institute for the Environment, Western Sydney University, NSW Australia. Annually from 2017-2022

Donovan N. Viroid research in citrus – from discovery to dwarfing trials. Online webinar 21st July 2021

Donovan N. Viroids in production citriculture. Invited presentation at the Program Team Forum for the National Tree Crop Intensification in Horticulture Program (Hort Innovation project AS18000) 12th July 2022

Resources developed

Subcommittee on Plant Health Diagnostics. National Diagnostic Protocol for '*Candidatus* Liberibacter asiaticus', the putative causal agent of huanglongbing (HLB) – NDP 25 V2. Authors Donovan NJ, Englezou A, Chambers G, Holford P. Submitted to Plant Health Australia for review

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