Hort Innovation

Final Report

Protecting Australian Citrus Germplasm through Improved Diagnostic Tools

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Protecting Australian Citrus Germplasm through Improved Diagnostic Tools – CT14009

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Content

Summary

Biosecurity is a priority for the Australian citrus industry (Citrus Strategic Investment Plan 2017-2022). Diseases can destroy an industry, with the Australian citrus industry being no exception. 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. For this, we need an experienced biosecurity team with appropriate knowledge and diagnostic tools to protect our industry.

The NSW DPI Citrus Pathology Program 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. Hort Innovation funded project CT14009 'Protecting Australian citrus germplasm through improved diagnostic tools' provided the NSW DPI citrus pathology team, in collaboration with Auscitrus, the opportunity to assess current and new diagnostic tests for graft-transmissible citrus pathogens to ensure we are using the most reliable, sensitive and efficient methods available. The NSW DPI citrus pathogen collection was catalogued and expanded providing a valuable resource to support diagnostic tool development. The team evaluated published methods or developed new tests to improve our capability to detect 15 endemic and 10 exotic graft-transmissible citrus pathogens; including the causal agents of huanglongbing (HLB), the biggest threat to global citrus. New viroid detections were made using improved diagnostic tools. Efficiency of testing was improved by changing from conventional to real-time PCR for most assays, and by multiplexing assays (testing for more than one pathogen in the same assay). Eight viroid assays were successfully combined into three assays, and four viral assays into three. Within the scope of the project, three multiplex assays were adopted by Auscitrus, improving the efficiency of testing and confidence in diagnostic results.

The team contributed to increasing national awareness of biosecurity threats to Australian citrus in collaboration with Auscitrus, Plant Health Australia, Citrus Australia, and the Federal and State governments. Team members participated in surveillance for citrus emergency plant pests in production, urban or high risk areas, and surveillance samples were tested for the causal agents of HLB and citrus variegated chlorosis (CVC) to provide evidence of absence. Project team members delivered the message about the threat of graft-transmissible diseases to Australian citrus via extension publications and presentations at industry forums, including biosecurity workshops in the major citrus growing regions of the Riverina, Sunraysia and Riverland.

Improved diagnostic tools developed and validated through the project have been adopted by the National Citrus Repository program and the Auscitrus propagation scheme to test plant material prior to supply to industry. Development of diagnostic tools, extension publications and surveillance activities have all contributed to building our knowledge base and increasing national awareness of biosecurity issues associated with Australian citrus. 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; detection; graft-transmissible disease; budwood

List of acronyms

ACD	Australian citrus dieback
ACIAR	Australian Centre for International Agricultural Research
CA	Citrus Australia
CCDaV	Citrus chlorotic dwarf associated virus
CCEPP	Consultative Committee on Emergency Plant Pests
CEVd	Citrus exocortis viroid
CiLV	*Citrus leprosis virus*
CLBV	*Citrus leaf blotch virus*
CPsV	*Citrus psorosis virus*
CTLV	*Citrus tatterleaf virus*
CTV	*Citrus tristeza virus*
Ct	cycle threshold
CVC	*Citrus variegated chlorosis*
CVd-I	Citrus viroid I or Citrus bent leaf viroid
CVd-IIa	Citrus viroid IIa
CVd-IIb	Citrus viroid IIb or cachexia
CVd-III	Citrus viroid III or Citrus dwarfing viroid
CVd-IV	Citrus viroid IV or Citrus bark cracking viroid
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*
CYVCV	*Citrus yellow vein clearing virus*
DAWR	Department of Agriculture and Water Resources
DTBIA	Direct tissue blot immunoassay
EMAI	Elizabeth Macarthur Agricultural Institute, NSW DPI
EPP	Emergency Plant Pest
HLB	Huanglongbing
HSVd	Hop Stunt viroid or Citrus viroid II

NAQS	Northern Australian Quarantine Strategy
NGS	Next generation sequencing
NSW DPI	New South Wales Department of Primary Industries
NT DPIR	Northern Territory Department of Primary Industries and Resources
OSP	Orange stem pitting
PCR	Polymerase chain reaction
PHDS	Plant Health Diagnostic Service
PHA	Plant Health Australia
Qld DAF	Queensland Department of Agriculture and Fisheries
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
sRNA	small Ribonucleic Acid
UCR	University of California, Riverside
USDA	United States Department of Agriculture

Introduction

Biosecurity is a priority for the Australian citrus industry (Citrus Strategic Investment Plan 2017-2022). It is important to maintain the high health status of Australian citrus to maximize orchard productivity and maintain market access. Therefore the ability to detect pathogens which pose a risk to the industry is vital. Graft-transmissible diseases are spread by grafting or use of infected plant material, mechanically on infected cutting tools during pruning and hedging, and in some cases by aphids or other insect vectors. There is no cure for these diseases hence they must be prevented through the use of uninfected 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 a number of 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-transmissible diseases include citrus exocortis viroid (CEVd), cachexia (CVd-IIb) and *Citrus tristeza virus* (CTV). Studies recorded yield loss in an orchard infected by CEVd was nearly 50% on citrange and 65% on trifoliata rootstock during the first 9 years of production (Bevington and Bacon 1977). For most graft-transmissible diseases, symptoms will not be seen in nursery trees, the signs will 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 and Water Resources (DAWR) significantly reduces the risk of entry of graft-transmissible diseases into Australia. Graft-transmissible citrus diseases are managed within Australia by:

- surveillance programs for early detection to increase the chance of eradication - mostly undertaken by the DAWR Northern Australia Quarantine Strategy (NAQS) who conduct surveillance and testing in high risk areas of Australia and offshore;
- the DAWR post-entry quarantine system where newly imported citrus varieties are tested for exotic and endemic plant pathogens before release from quarantine;
- the National Citrus Repository Program, where 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 a non-profit industry organisation (Australian Citrus Propagation Association) that supplies high health status, true to type budwood and rootstock seed to nurseries for tree production.

Viroids and viruses are not all pathogenic, some can be beneficial. Inoculating trees with a viroid that induces mild dwarfing is useful for high density plantings and ease of orchard management. Mild strain cross protection is used successfully in Australia to protect white grapefruit trees against severe stem pitting isolates of *Citrus tristeza virus* (CTV) by inoculation with a mild CTV isolate. Whilst some viroids and viruses can be non-pathogenic on their own, in combination their effects can be detrimental to the tree. In either case, it is important that we know what organisms are present in propagation material before its use.

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.

Detecting graft-transmissible pathogens can be difficult because the pathogen particles may be present below detectable levels or unevenly distributed within the tree. It is important that diagnostic tests are specific to the target organism, sensitive (i.e. will detect even at low levels), and efficient (time and cost). Investigating new detection methods and newly described pathogens takes considerable time and resources and was beyond the means of the non-profit Auscitrus propagation scheme. A support project was developed by NSW DPI in consultation with industry to assess current and new diagnostic methods for graft-transmissible pathogens of citrus to ensure we are using the most robust, sensitive and efficient methods available.

The work on graft-transmissible citrus diseases in project 'CT14009 Protecting Australian citrus germplasm through improved diagnostic tools' enhanced the ability of the NSW DPI team to deliver for industry and government by:

- improving our knowledge and ability to detect and manage pathogenic and beneficial graft-transmissible organisms in citrus;
- ensuring the availability of healthy planting material to industry; and
- helping to prepare for an incursion of HLB, the worst disease reported to affect citrus globally, and other exotic disease threats.

Methodology

This project had a national focus of interest to Australian citrus growers and nurserymen, industry organisations (such as Auscitrus and Citrus Australia), industry service providers, diagnostic laboratories (including post-entry quarantine), and regulators and policy makers.

Citrus pathogen collection

The NSW DPI citrus pathology team at EMAI maintains a collection of organisms known to infect citrus; stored in the form of nucleic acid extracts, cultures, and infected citrus trees maintained in controlled environment greenhouses. Graft-transmissible organisms are not culturable and, therefore, are stored in infected trees (endemic organisms only) or as nucleic acid extracts (endemic or exotic organisms). The pathogens in the collection are used as positive controls (i.e. give a positive result in assays to check that the test worked) for the improvement and validation of diagnostic protocols.

During the project, a database was established to catalogue all accessions in the collection and record their test history. New accessions were sourced from around the world and added to the collection. Trees that form part of the positive control collection were given tree identification numbers and a database was established to accurately track the test history of the trees; note that many trees contain more than one organism. Accessions in the collection were used to validate existing and new molecular diagnostic tools. This also confirmed their health status.

National Citrus Repository

The National Citrus Repository maintains high health status citrus trees for supply of propagation material to the Auscitrus propagation scheme and to owners of private varieties.

HLB is one of the most destructive diseases of citrus in the world and one of the major factors limiting citrus production in South East Asia, Florida and Brazil. Three forms of HLB have been described in association with phloem limited bacteria '*Candidatus* Liberibacter asiaticus', '*Ca*. L. americanus' and '*Ca*. L. africanus'; none of which have been recorded in Australia. The pathogen of most concern to Australia, and the one reported to be closest to our shores is the Asian strain ('*Ca*. L. asiaticus').

Australian citrus selections were not tested for HLB prior to entering the repository program because HLB is not known to occur in Australia. Imported varieties were only tested in post-entry quarantine for HLB by biological indexing until very recently when a molecular test was added to the post-entry quarantine requirements. The importance of testing the repository trees for HLB using sensitive molecular techniques was recognised due to their role in supplying high health status material to the Australian citrus industry.

During the project, trees representing all of the public varieties held in the National Citrus Repository were tested for '*Ca*. L. asiaticus', '*Ca*. L. americanus' and '*Ca*. L. africanus' using real-time PCR (Li et al. 2006), the most sensitive and robust technique currently available in our laboratory for detecting these organisms. Leaf samples were collected from each quadrant of each tree and grouped. Nucleic acids were extracted from leaf midribs; extractions and PCR assays were performed in duplicate.

Endemic citrus viroids

The EMAI citrus team tested for citrus viroids using biological indexing on 'Etrog' citron indicator plants and by conventional PCR using individual PCR reactions for each viroid based on Bernad and Duran-Vila (2006), prior to project CT14009.

Biological indexing is laborious and costly. Taking several months to obtain a result, this method does not distinguish between strains, and its efficacy can be reduced when the sample is infected by more than one citrus pathogen (Vidalakis et al. 2004). At EMAI, CTV infection confuses symptom expression on 'Etrog' citron biological indicator plants when testing trees for citrus viroids. Also, symptoms of CVd-II (viz. cachexia and dwarfing viroids) are unlikely to be expressed on 'Etrog' citron indicator plants, requiring additional indicator plants for their detection. Despite this, biological indexing is still considered a useful tool by citrus diagnosticians around the world for detection and biological characterisation of citrus viroids and viruses, including detection of new pathogens or pathogen strains. Indicator plants are also bio-amplification hosts, increasing the ability to detect viroids or viruses that are present at a low titre in the original sample. Biological indexing, when performed in conjunction with serological or molecular assays, allows diagnostic results to be confirmed by more than one method.

Pot trial

A fully replicated and randomized greenhouse trial was established to investigate the effect of *Citrus tristeza virus* (CTV) infection on biological indexing for citrus viroids, i.e. does CTV infection affect symptom expression in the 'Etrog' citron indicator plants. Treatments included combinations of mild and severe strains of CTV and citrus exocortis viroid (CEVd).

'Etrog' citron indicator plants were inoculated with CEVd and CTV isolates from the living pathogen collection (i.e. infected plants with a known health status). The presence of viroid-like symptoms was recorded in 3 flushes on each indicator plant. After each recording, the plants were pruned to encourage the next flush growth.

The plants were also tested using a molecular assay to confirm the presence or absence of the pathogens; CEVd and CTV. Samples were collected for this during the first recording of symptoms.

The pot trial was housed in a controlled environment greenhouse at temperatures best suited to expression of symptoms induced by CEVd infection (30-32°C day / 25-28°C night).

Molecular diagnostics

Wildman (2013) adapted the published method of Wang et al. (2009) to detect citrus viroids I, II, III, IV and CEVd using a multiplex conventional RT-PCR. However, more work was needed before the method could be adopted by diagnostic laboratories. During project CT14009, the Wildman assay was trialled using samples from biological indicator plants and field trees.

A patented, real-time, quantitative reverse transcription PCR (RT-qPCR) (Vidalakis and Wang 2013) was evaluated to detect a number of apscaviroids in one assay (CVd-I, III, V, VI, VII).

A SYBR™ RT-qPCR assay that uses universal primers for citrus non-apsca group viroids (Vidalakis and Wang 2013) was assessed for its ability to detect and differentiate between CEVd and hop stunt viroid (HSVd, also called CVd-II). SYBR™ assays are known to be less sensitive than fluorescent probe-based assays and are limited in their use to differentiate between amplification products; although SYBR™ assays can be more efficient due to reduced assay set up and running costs. A fluorescent probe-based assay (Lin et al. 2015) was also evaluated.

Endemic citrus viruses

Citrus tristeza virus (CTV)

All CTV-infected plants in the living pathogen collection were screened to confirm pathogen presence using conventional RT-PCR with primers (CP1 and CP3) designed to amplify the coat protein gene (Gillings et al. 1993). All trees where CTV was not detected via the RT-PCR assay were then tested by direct tissue blot immunoassay (DTBIA) to determine if CTV was present in the plant. The plants were then re-tested using different primers, also based on the coat protein sequence (Osman et al. 2015) in a conventional RT-PCR assay, and in a SYBR™ RT-qPCR assay (Table 1).

Field samples were collected from the Atherton tablelands in far north Qld (November 2016) from lime and grapefruit trees, and from orange trees in the Central Burnett Qld (May 2017). The source trees were potentially infected with stem pitting strains of CTV. The samples were transported to EMAI (under permit OUT15/7720) for propagation onto biological indicator plants and molecular analyses.

Primers (Herrera-Isidron et al. 2009) that amplify the P13 gene region of CTV were used to determine the sequence of the region in Australian isolates of CTV which induce stem pitting in oranges, with the intention of developing a test specific for Australian OSP-CTV inducing isolates (Table 1).

Another primer set B165/T68-R (Wang et al. 2013) was trialled in an attempt to discriminate OSP-CTV isolates (Table 1).

Sequences were analysed from previous work conducted at EMAI where the helicase region of Australian CTV isolates was cloned and sequenced. This region is being looked at for its potential to discriminate Australian OSP-CTV inducing isolates.

Citrus psorosis virus (CPsV)

Published RT-qPCR assays were based on overseas isolates and did not amplify Australian CPsV isolates. Sequencing of Australian psorosis isolates (from plants in the living pathogen collection) identified differences between our strains and those found overseas. A new probe was designed based on the sequence analysis of

Australian isolates and evaluated in a probe-based RT-qPCR assay (Table 1).

PCR assays were evaluated for a number of other endemic graft-transmissible citrus viruses (Table 1).

Table 1: PCR assays evaluated for detection of endemic graft-transmissible citrus viruses

Target organisms	PCR assays evaluated		
	Assay type	Primers / Probes	Reference
Citrus tristeza virus (CTV)	conventional RT-PCR	CTV CACP16678F & CTV CACP 16679F2/CTV CACP16813	Osman et al. 2015
	SYBR™ RT-qPCR	CTV CACP16678F/CTV CACP16813	
	RT-qPCR	CTV CACP16678F & CTV CACP 16679F2/CTV CACP16813; CTV CA-CP-16763p-TET	
	conventional RT-PCR	IRA5/IRA6	Herrera-Isidron et al. 2009
	conventional RT-PCR	B165/T68-R	Wang et al. 2013
	conventional RT-PCR	CP1/CP3	Gillings et al. 1993
Citrus tatterleaf virus (CTLV)	SYBR™ RT-qPCR	CTLV-ASG-Pf1/CTLV-ASG-Pr	Lui et al. 2011
		6703F/6413R	Unpublished
	probe based RT-qPCR	6308F17/6413R;6340p	Unpublished
Citrus psorosis virus (CPsV)	probe based RT-qPCR	CPsV-792F1 and 791F2/CPsV 946R1 and R2; CPsV-851p-Vic	Osman et al. 2015
		CPsV851p-Aus-CFO560	Unpublished
Citrus leaf blotch virus (CLBV)	probe based RT-qPCR	CLBV CP7711F/CLBV CP7872R; CLBV CP-7738pFAM	Osman et al. 2015
CLBV + CPsV	multiplex probe based RT-qPCR	as above	Osman et al. 2015 + our probe for CPsV
Citrus vein enation virus (CVEV)	conventional RT-PCR	VE5F/VE15R	Vives et al. 2013
Citrus variegation virus (CVV)	conventional RT-PCR	Ilar2F5/2R9 (Ilarvirus genus) Ilar1F5/1R7 (Bromoviridae family)	Untiveros et al. 2010

Australian Citrus Dieback

Australian citrus dieback (ACD) does not have a confirmed causal agent but the disease is believed to be associated with phytoplasma-like organisms. Symptoms consistent with ACD have occasionally been observed in Australian citrus orchards. These symptoms of leaf mottling and dieback are similar to those of HLB; therefore, ACD has the potential to confound HLB surveillance efforts.

New primers that target phytoplasmas (Christensen et al. 2004) were evaluated using plants in the EMAI nursery propagated using material from field trees with ACD symptoms.

Samples collected from citrus surveillance exercises during the course of the project were tested for phytoplasmas using a nested conventional PCR assay (Deng and Hiruki 1991; Lee 1993; Schenider et al. 1995). A subset of positive samples was subject to further analysis by sequencing the amplicon.

Next generation sequencing

Samples known to contain citrus viruses and viroids were sent for Next generation sequencing (NGS) to two sequencing facilities in Sydney (Table 2). High quality total RNA was extracted from plants in the living pathogen collection at EMAI that have previously tested positive for various viruses and viroids.

Table 2: Evaluation of Next Generation Sequencing (NGS) for citrus virus and viroid detection

Sequencing facility	Platform	Run	Total Project Cost (excluding GST):	Turnaround time	Data Yield (gigabase pairs)	Reads (PF) (million)	Quality (% Q≥30)
Service provider 1	Illumina HiSeq2500	10 small RNA (sRNA) samples; 50bp single end reads	$5600 ($560/sample)	4 months	16.6	146	88.34
Service provider 2	Illumina NextSeq	12 total RNA (tRNA) samples; 75bp paired end reads	$7760 ($646/sample)	5 weeks	26.4	340	90.48

The team gained useful advice from Dr Hano Maree (Senior Researcher at Stellenbosch University) on data analysis and future research directions. Data was manually analysed using CLC workbench 6, and Geneious which takes more time than using automated pipelines but provides a greater understanding of the data.

The raw NGS data was analysed using two different pipelines. sRNA run data was analysed using VirFind (Ho and Tzanetakis, 2014) which is a web based pipeline, and data from the NextSeq run was analysed using Truffle software (Visser et al. 2016) which uses e-probes.

Exotic graft-transmissible diseases

Huanglongbing

The conventional PCR assay described in Hocquellet et al. (1999) and the robust, qPCR assay described by Li et al. (2006) are used worldwide to detect Liberibacter species associated with HLB. However, in the real-time assay thresholds vary in the literature and there appears to be an 'inconclusive' region between Ct values of 32 to 40. New diagnostic methods were trialled for the causal agents of HLB, with particular focus on '*Ca*. L. asiaticus'. This included a tissue imprint detection method (Nagesawara-Rao et al. 2013) evaluated in the field in Bhutan. Generic Liberibacter primers (unpublished) were evaluated for their ability to detect '*Ca*. L. asiaticus', '*Ca*. L. africanus', '*Ca*. L. americanus', '*Ca*. L. solanacearum' and *L. crescens* in extracts. Other molecular assays evaluated for their ability to detect '*Ca*. L. asiaticus' are outlined in Table 3

Molecular assays were evaluated for other exotic graft-transmissible pathogens of citrus not known to occur in Australia. *Citrus leprosis virus* (CiLV) which is not considered to be a systemic pathogen and is difficult to transmit via grafting, was included as a significant exotic threat to Australian citrus (Table 3).

Table 3: PCR assays evaluated for detection of citrus pathogens considered to be exotic to Australia

Target organisms	PCR assays evaluated		
	Assay type	**Primers**	**Reference**
'*Candidatus* Liberibacter asiaticus' (huanglongbing)	conventional PCR	rpoBF/rpoBR (expected band size = 111bp)	Ananthakrishnan et al. 2013
	Loop mediated isothermal amplification (LAMP)		Keremane et al. 2015
	LAMP		Rigano et al. 2014
Xylella fastidiosa ssp. *pauca* (citrus variegated chlorosis)	conventional PCR	HL5/HL6 (expected band size = 221bp) - *X. fastidiosa* (not specific to ssp. *pauca*)	Francis et al. 2006
		CVCf/CVCrc (expected band size = 1023bp)	Li et al. 2013
Spiroplasma citri (citrus stubborn disease)	conventional PCR	P58-6f/P58-4r (expected band size = 450)	Yokomi et al. 2008
		Rep3dXbaF/Rep3dBamR (expected band size = 912bp)	Bevan et al. 2012
		Spiro Rep3dF/Spiro Rep3dR1/Spiro Rep3dR2 (expected band size = 912bp)	modified from Bevan et al. 2012
		D/D' (expected band size = 1053bp)	Foissac et al. 1996
		CSF/CSR (expected band size = 269 bp)	Nunan et al. 2004
	real-time PCR	Php-orf1-F/Php-orf1-R (expected band size = 190bp)	Wang et al. 2015
		Php-orf3-F/Php-orf3-R (expected band size = 149bp)	Wang et al. 2015
		p58-3f/p58-4r (expected band size = 119 bp)	Yokomi et al. 2008
Citrus chlorotic dwarf associated virus (CCDaV)	conventional RT-PCR	3202fw/6rev (expected band size = 444 bp)	Loconsole et al. 2012 a
	SYBR™ real-time RT-PCR	1583fw/1650rev (expected band size = 68 bp)	
Citrus yellow mosaic virus (CYMV)	conventional RT-PCR	FP/RP (expected band size = 448bp)	Huang pers. comm.
	conventional RT-PCR	ORFIIIF/ORFIIIR (expected band size = 638bp)	Baranwal et al. 2003
Citrus yellow vein clearing virus (CYVCV)	conventional RT-PCR	1fw/921rev (expected band size = 921bp)	Loconsole et al. 2012 b
	SYBR™ real-time RT-PCR	CV4/CY5 (expected band size = 174bp)	Chen et al. 2016
	SYBR™ real-time RT-PCR	391fw/449rev (expected band size = 59 bp)	Loconsole et al. 2012 b
Citrus leprosis virus (CiLV)	conventional RT-PCR	MPF/MPR (expected band size = 339 bp)	Locali et al. 2003
		RepF/RepR (expected band size = 402 bp)	Locali et al. 2003

Surveillance and awareness

Team members participated in surveillance activities organised by NAQS, or by Stuart Pettigrew (AgDynamics) in his role as the National Citrus Biosecurity Manager on the citrus biosecurity project (CT12022), managed by Plant Health Australia and funded by Hort Innovation. Surveillance samples were tested for '*Ca*. L. asiaticus' (associated with HLB) and *Xylella fastidiosa* (associated with CVC) at EMAI.

The importance of biosecurity and graft-transmissible diseases was discussed at industry forums (meetings, workshops, conferences), in extension publications, and with individuals on their properties during surveillance exercises or industry events.

Outputs

Citrus pathogen collection

- Database established for the NSW DPI citrus pathogen collection held at EMAI in the form of nucleic acid extracts, cultures or infected plants to catalogue each accession and record their test history.
- Accessions in the citrus pathogen collection tested to confirm their health status.
- New accessions sourced and added to the citrus pathogen collection.
- Accessions in the citrus pathogen collection used to validate or develop diagnostic protocols.
- Positive control material provided to the Federal DAWR post-entry quarantine team who test imported citrus varieties.

A database was established to catalogue and record the test history of accessions in the NSW DPI citrus pathogen collection during the project. Trees in the living pathogen collection (infected with unculturable organisms) have been given tree identification numbers and tested to confirm their health status. Some trees were found to contain more than one organism, or different organisms as to what had been previously recorded. Despite maintaining a fairly constant temperature year round in the greenhouses, there appears to be variation in titre and ease of detection during the year for some organisms, possibly due to seasonal changes in day length. Viroid isolates extracted from 26 trees were sequenced to enable strain characterisation.

Nucleic acid extracts containing citrus pathogens have been tested and added to the citrus pathogen collection since the project commenced. This includes samples from the United States, Brazil, Bhutan, Lao PDR and Thailand.

Associate Professor Paul Holford (collaborator from Western Sydney University WSU) undertook a sabbatical in April 2015 at the Federal United States Department of Agriculture (USDA) laboratory at Maryland in Florida. Paul worked with Dr John Hartung, an expert in citrus disease diagnostics, and brought back extracts of a range of citrus pathogens that have been tested and added to the collection. Many of the extracts sourced from the USDA collection did not originate from the United States.

Overseas field visits (Bhutan, Thailand, China, United States) were funded by other sources but gave project team members additional experience with field identification of exotic citrus diseases and their management strategies, and allowed for collection of samples to boost the pathogen collection.

During project CT14009, accessions in the citrus pathogen collection were used as positive controls for the validation and development of diagnostic protocols. Extracts from the collection were provided to the Federal DAWR post-entry quarantine team for use as positive controls when testing imported citrus varieties.

A summary of the NSW DPI citrus pathogen collection is provided in Table 4.

Table 4: Accessions of graft-transmissible organisms in the NSW DPI citrus pathogen collection

Pathogen	No. accessions	Source
Citrus exocortis viroid (CEVd)	5	Australia
Citrus bent leaf viroid (CBLVd or CVd-I)	8	Australia
Hop stunt viroid (HSVd or CVd-II)	19	Australia
Citrus dwarfing viroid (CDVd or CVd-III)	8	Australia
Citrus bark cracking viroid (CBCVd or CVd-IV)	1	California
Citrus viroid V (CVd-V)	1	California
Citrus tristeza virus (CTV)	142	Australia, China, Spain, India, Taiwan, Indonesia, Thailand
Citrus psorosis virus (CPsV)	2	Australia
Citrus tatterleaf virus (CTLV)	3	Australia
Citrus leaf blotch virus (CLBV)	2	Australia
Citrus vein enation virus (CVEV)	2	Australia
Citrus chlorotic dwarf associated virus (CCDaV)	1	Turkey
Citrus yellow mosaic virus (CYMV)	1	India
Citrus yellow vein clearing virus (CYVCV)	1	Pakistan
'*Candidatus* Liberibacter asiaticus'	20	Bhutan, Lao PDR, USA, China
'*Candidatus* Liberibacter americanus'	2	Brazil
'*Candidatus* Liberibacter africanus'	4	South Africa
Spiroplasma citri (stubborn)	3	USA
Xylella fastidiosa ssp. *pauca* (citrus variegated chlorosis CVC strain)	1	Brazil
Citrus leprosis virus (CiLV)*	1	Brazil

* CiLV is difficult to transmit via grafting

National Citrus Repository

- Foundation trees of commercial citrus varieties tested for the causal agents of huanglongbing.

Trees of publicly owned varieties in the National Citrus Repository were tested for the putative causal agents of huanglongbing (HLB); '*Candidatus*' Liberibacter asiaticus', '*Ca*. L. africanus' and '*Ca*. L. americanus' using qPCR (Li et al. 2006). The target pathogens were not detected in the 117 public varieties tested.

Citrus viroids

- Validation of a multiplex, conventional RT-PCR assay for citrus viroids I, II, III and citrus exocortis viroid.
- Real-time RT-qPCR assays evaluated for citrus viroids I, II, III, IV, V, VI, VII and citrus exocortis viroid.
- A probe-based, RT-qPCR assay validated and recommended for citrus exocortis viroid and citrus viroid II.
- A SYBR™, RT-qPCR assay validated and recommended for citrus viroids I, III, V, VI and VII.
- A novel (previously unknown) viroid confirmed in Australian citrus germplasm.
- Two viroids not known to occur in Australia detected in Australian citrus germplasm.
- Progress made with assays to distinguish between non-apscaviroids.
- The use of improved diagnostic tools for citrus viroids recommended to Auscitrus and used to test trees in the National Citrus Repository and Auscitrus programs.

Clones of CVd-IV and V were received from California and sequenced to determine their viability for use as controls. These isolates were used to develop and validate diagnostic protocols for use in Australia and to test citrus germplasm to determine if CVd IV and V are present in Australia.

Work undertaken during this project checked the robustness of a multiplex conventional RT-PCR assay that tests for CEVd, CVd-I, II, and III in one PCR reaction (Wildman 2013 adapted from Wang et al. 2009). The multiplex reaction worked well on fresh extracts from plants in the living pathogen collection at EMAI and using extracts obtained from fresh budwood sampled from field trees and transported via post to EMAI (i.e. to mimic normal sampling procedures). The budsticks were cut from trees in a long-term field trial at Dareton DPI which were inoculated with different viroids, singly and in combination.

The multiplex PCR assay for viroid detection was used to help Auscitrus meet budwood demand for a popular new variety as 120 trees on a grower property were tested for CEVd and CVd-II. The trees were propagated using Auscitrus material, so the risk of infection was negligible. However, the multiplex PCR allowed the trees to be efficiently tested prior to budwood supply in spring 2015.

Universal primers for citrus Apscaviroids (Vidalakis and Wang 2013) were used successfully to detect CVd-I, and III in extracts from fresh plant material, and CVd-VI in a stored extract. The assay detected a new viroid, not previously reported in Australia, in an extract from fresh plant material collected during a surveillance exercise. This viroid has been reported to the Consultative Committee on Emergency Plant Pests (CCEPP) to meet our responsibility under the Emergency Plant Pest Response Deed (EPPRD). This viroid has been added to the test schedule for Australian germplasm in the National Citrus Repository and the Auscitrus propagation scheme.

In an example of end user adoption, the Vidalakis and Wang (2013) assay was used to investigate a diagnostic sample submitted to EMAI for routine testing as part of our work with the Auscitrus scheme. Symptoms indicating viroid presence were expressed on an 'Etrog' citron indicator plant that was inoculated with material from a field Lisbon lemon tree but no known citrus viroids were detected by conventional PCR. Use of the universal primers confirmed the presence of a novel citrus viroid, tentatively called CVd-VII. The distribution of this viroid in Australian citrus germplasm and effect on different hosts is unknown. CVd-VII was reported to the CCEPP, and it was not deemed to be an Emergency Plant Pest (EPP) of citrus. Expert advice was sought from Professor Georgios Vidalakis (University of California Riverside and Californian Citrus Clonal Protection Programme) and further work was conducted in collaboration with the UCR lab. Specific primers were designed for a more sensitive assay to detect CVd-VII which is likely to be used in subsequent surveillance efforts (Chambers et al. 2018, Appendix 1).

SYBR™ assays are known to be less sensitive than fluorescent probe-based assays and are limited in their use to differentiate between amplification products; although SYBR™ assays can be more efficient due to reduced assay set up and running costs. A SYBR™ RT-qPCR assay that uses universal primers for citrus non-apsca group viroids (Vidalakis and Wang 2013) was able to detect and differentiate between CEVd and CVd-II, although with medium specificity and variable sensitivity. A fluorescent probe-based assay (Lin et al. 2015) was trialled and proved more sensitive and specific for detecting and differentiating between CEVd and CVd-II. This assay was able to detect CEVd and CVd-II in extracts from indicator plants at EMAI and field samples transported via post without the need for intermediary indicator plants to increase titre.

The SYBR™ RT-qPCR assay (Vidalakis and Wang 2013) required further optimisation to confirm the presence of CVd-IV in the multiplex, as the samples tested produced high Ct values, i.e., for the CVd-IV samples, the cycle at which the threshold value was passed occurred late in the assay and the pathogen was close to not being detected. The qPCR products from the SYBR™ assay were run on an agarose gel and bands for CVd-IV (65 bp), CVd-II (75 bp) and CEVd (85 bp) could be clearly delineated. As with the SYBR™ assay, there were issues with detecting CVd-IV in a fluorescent probe-based assay (Osman et al. 2017), with high (late) Ct values. It is not known whether these are related to low titre or non-specific amplification.

Greenhouse trials were undertaken to investigate the impact of CTV infection on biological indexing for citrus viroids on 'Etrog' citron indicator plants. Treatments included combinations of CEVd with mild or severe strains of CTV. CEVd symptoms were not masked by co-infection with CTV in this trial, although CTV alone produced symptoms similar to those expressed by infection with mild viroids. Further trials could evaluate the impact of CTV infection on the expression of symptoms by other citrus viroids.

Endemic citrus viruses

- RT-PCR and RT-qPCR assays validated to detect *Citrus tristeza virus*, *Citrus tatterleaf virus*, *Citrus psorosis virus*, *Citrus leaf blotch virus*, *Citrus variegation virus* and ilarviruses (*Citrus variegation virus)*.
- RT-qPCR assay validated to simultaneously detect *Citrus psorosis virus* and *Citrus leaf blotch virus*.
- RT-qPCR assays developed to detect Australian strains of *Citrus tatterleaf virus* and *Citrus psorosis virus*.

- RT-PCR assays evaluated to distinguish between strains of *Citrus tristeza virus*.

Citrus tristeza virus (CTV)

CTV is a complicated virus and exists in hundreds of different forms known as isolates. Most infected trees contain more than one isolate, and each isolate may contain more than one strain.

CTV was not detected using the Gillings et al. (1993) coat protein primers in some plants in the living pathogen collection at EMAI that were expected to contain CTV. These plants were then re-tested using different primers, also based on the coat protein sequence (Osman et al. 2015) in a conventional PCR assay; most of these plants were found to be positive. The results were confirmed using DTBIA. This suggests that the primers designed by Osman et al. (2015) are more sensitive than the Gillings primers but further work is needed to determine specificity and resolve potential false positives. Also, when the Osman primers were used in a RT-qPCR assay, a number of Australian strains were not detected due to a mismatch between the sequence of the probe and Australian strains of CTV; including the mild CTV isolate (PB61) used commercially to protect grapefruit trees from severe stem pitting isolates, and a pathogenic isolate known to induce orange stem pitting (OSP).

Field samples were collected from the Atherton tablelands (November 2016) in Far North Queensland from lime and grapefruit trees, and from orange trees in the Central Burnett (May 2017), potentially infected with stem pitting isolates of CTV. Samples were also collected for CTV and viroid testing from the Qld Department of Agriculture and Fisheries' (Qld DAF) Bundaberg Research Station, with citrus breeder Malcolm Smith. The samples were transported to EMAI (under permit OUT15/7720). RNA was extracted from the budwood samples, and live material was stored by propagating onto maintenance plants in the nursery.

Given the diversity of CTV isolates and their varied impact on citrus trees, it is important to be able to distinguish between CTV strains. There are two strains of CTV believed to be associated with OSP symptoms in Qld, known as PB155 and PB235, although there is evidence to suggest that more isolates exist in Queensland with the ability to induce OSP (Hailstones et al. 2006). Several primer sets (Herrera-Isidron et al. 2009 and Wang et al. 2013) were trialled to see if they were able to differentiate these and other Australian isolates but were not found useful for this purpose. The Herrera-Isidron et al. (2009) primer set amplified CTV, but sequencing was required to differentiate between CTV strains.

Assays using primers designed to amplify the P23 region of PB155 or PB235 OSP inducing strains (Conner 2001) were able to amplify these CTV strains in field samples from Queensland. Some samples had both known OSP strains present.

Melting curve analysis following SYBR™ RT-qPCR using Osman et al. 2015 primers did not clearly delineate between the strains (i.e., there was not enough sequence variation in the amplified region to distinguish between the strains); this may be due to infection with multiple strains.

Sequences were analysed from previous work conducted at EMAI where the helicase region of Australian CTV strains was cloned and sequenced. This region has potential to discriminate Australian OSP-CTV inducing isolates.

NGS data confirmed the existence of multiple strains in each isolate, making strain analysis challenging in the absence of deep sequencing. Information gained from sequencing data may assist with developing a test to effectively differentiate between important Australian strains of CTV.

Citrus tatterleaf virus

Prior to the project, CTLV was detected using a nested conventional PCR assay (Hailstones et al. 2000). Further work determined that the nested element of the published protocol was not necessary; CTLV was able to be detected in extracts diluted 1:1000.

Two primer sets (Liu et al. 2011 and a set that we designed for future use in a probe-based assay see Table 1) were trialled for use in SYBR™ RT-qPCR assays to detect CTLV. Both primer sets successfully detected Australian and overseas strains of CTLV, and sequence analysis revealed several Australian variants using our primers which were more sensitive than the Liu primers. The SYBR™-based assay was found to be more specific (i.e. detect more positive CTLV samples) than the current conventional RT-PCR CTLV diagnostic assay (Hailstones et al. 2000).

Two probes were then designed to work with our primers and evaluated in a probe-based RT-qPCR assay (Table 1). One of the probes successfully detected CTLV in extracts from field trees.

Citrus psorosis virus (CPsV)

Sequencing of Australian psorosis isolates identified differences between our strains and those found overseas. Published RT-qPCR assays were based on overseas isolates and did not amplify Australian CPsV isolates. A new probe was designed based on sequence analysis of Australian isolates (Table 1), and this probe successfully detected CPsV in a RT-qPCR assay.

CPsV could not be detected using the probe-based RT-qPCR assay or by conventional RT-PCR in one Symons sweet orange plant in our living pathogen collection thought to contain CPsV which also exhibits symptoms consistent with CPsV. The plant may be harbouring a CPsV isolate that is sufficiently different to those Australian isolates for which the new probe was designed, or the plant may contain a different organism that induces symptoms similar to those induced by psorosis infection. Further work is needed.

A serological detection method for CPsV, a direct tissue blot immunoassay, has been used in our laboratory in the past as it is a cost-efficient assay for screening large tree numbers, provided it is sensitive and specific. We abandoned the CPsV immunoassay several years ago due to issues with false negatives that we concluded were due to a lack of specificity of the monoclonal antibody (conjugate monoclonal line PS29) used in the assay. There is only one commercial supplier of antisera for CPsV, and the DAS ELISA kit currently available (as of March 2018) uses the same conjugate monoclonal line (PS29) with which we experienced issues several years ago. Therefore, a new antibody source was not available for us to revisit this detection method.

A thorough comparison of the sensitivity and specificity of RT-qPCR and serological detection methods for CPsV determined that a SYBR™ RT-qPCR assay based on the coat protein region was more rapid and three orders of magnitude more sensitive than a TAS-ELISA (triple antibody sandwich enzyme-linked immunosorbent assay) (De Francesco et al. 2015). Given the difference between Australian and overseas CPsV isolates, the lack of a new commercial antibody source, and that development of antibodies specific to Australian CPsV strains was beyond the scope of the current project, it is recommended to use the RT-qPCR assay rather than the immunoassay for detection of CPsV in plant material.

Citrus leaf blotch virus (CLBV)

A probe based RT-qPCR assay (Osman et al. 2015) was found to be more sensitive for detecting CLBV than the conventional RT-PCR assay. The real-time assay detected CLBV in material previously thought to be free from CLBV, based on results from the conventional RT-PCR assay.

A multiplex real-time assay has been successfully trialled to simultaneously detect CPsV and CLBV based on Osman et al. (2015) but using a CPsV probe designed by our group specific for Australian isolates (Table 1). The published assay includes CTV in the multiplex, but we were not able to detect CTV in the multiplex assay despite attempts to adapt the method by replacing the fluorophore on the probe. As outlined above, the Osman probe for CTV (Osman et al. 2015) does not detect all Australian strains.

It was recommended that Auscitrus adopt the multiplex assay for testing their rootstock seed supply trees. These trees have been tested for CPsV approximately every 10 years due to reports of seed transmission (Bridges et al. 1965; Childs and Johnson 1966). Later work contradicts this, providing evidence that CPsV is not seed transmitted (D'Onghia et al. 2000). More information has come to light in recent years, and it is widely suggested that symptoms observed in seedlings from CPsV-infected trees could have been expressing CLBV symptoms, not CPsV. The confusion occurred because CLBV had not yet been discovered. A low rate of seed transmission is reported for CLBV (Guerri et al. 2004). A multi-pathogen assay detecting both CPsV and CLBV is a useful way to test for both pathogens, reducing the risk with little impact on assay cost.

Citrus vein enation virus (CVEV)

Progress has been made with molecular detection of CVEV. In the EMAI nursery, there were two rough lemon plants labelled as infected with CVEV, but no test history had been recorded. Vein enation was successfully detected in these two plants using a PCR assay targeting the conserved, RNA-dependent RNA polymerase region (Vives et al. 2013). Sequence analysis showed differences between these isolates and Spanish CVEV isolates. The two CVEV-infected plants are now included in the living pathogen collection.

Citrus variegation virus (CVV)

CVV is a member of the Ilarvirus genus. There are no CVV accessions in the citrus pathogen collection, but a conventional RT-PCR designed to detect ilarviruses (Untiveros et al. 2010) was successfully trialled using other Ilarvirus species as positive controls. This assay was then used on citrus samples submitted to the NSW DPI Plant Health Diagnostic Service (PHDS) exhibiting symptoms similar to those induced by CVV infection.

Australian Citrus Dieback (ACD)

ACD does not have a confirmed causal agent but the disease is believed to be associated with phytoplasma-like organisms. Symptoms consistent with ACD have occasionally been observed in Australian citrus orchards. These symptoms of leaf mottling and dieback are similar to those of HLB; therefore, ACD has the potential to confound HLB surveillance efforts. Through the course of the project, no samples were found during field work or submitted for diagnostic testing that have symptoms consistent with ACD. However, two suspect samples from the Central Burnett and Sunraysia were collected prior to project CT14009 and are maintained in living plants in the EMAI greenhouse.

Asymptomatic samples collected from citrus surveillance exercises during the course of the project were tested for phytoplasmas. DNA extracted from 45 field samples was tested for phytoplasmas using a nested conventional PCR assay (Deng and Hiruki 1991; Lee 1993; Schenider et al. 1995). Nine samples produced an amplicon of an approximate size indicating the presence of phytoplasma; two of these were further examined by sequencing the amplicon. The sequence data indicated that one phytoplasma was identical to a strain of Australian lucerne yellows phytoplasma and the other was most similar to a number of isolates in Genbank including '*Candidatus* phytoplasma aurantifolia' and Citrus blotchy mottle phytoplasma. Both samples belong to the 16SrII group (peanut witches broom group); the phytoplasma potentially associated with ACD is also a member of this group.

Multi-pathogen assays

Multi-pathogen assays validated through the project and recommended for use are outlined below.

- A conventional RT-PCR assay for citrus exocortis viroid and citrus viroids I, II and III (Wildman 2013 adapted from Wang et al. 2009).
- A probe-based, RT-qPCR assay for citrus exocortis viroid and citrus viroid II (Lin et al. 2015).
- A SYBR™, RT-qPCR assay for citrus viroids I, III, V, VI and VII (Vidalakis and Wang 2013).
- A probe-based, RT-qPCR assay for *Citrus leaf blotch virus* and *Citrus psorosis virus* (Osman et al. 2015) using a probe adapted for Australian psorosis strains (Table 1).

Next generation sequencing (NGS)

- Next generation sequencing evaluated for the ability to detect a range of endemic citrus viruses and viroids.
- Methods of data analysis compared for raw NGS data.
- A new citrus viroid detected in Australia through the analysis of NGS data.
- Two new viroid detections in Australia confirmed by NGS technology.

High quality total RNA was extracted from plants in the living pathogen collection at EMAI that have previously tested positive for various viruses and viroids. Extracts were sent for NGS to two sequencing providers. The raw NGS data was manually analysed using CLC workbench 6 and Geneious; and two automated pipelines, VirFind (Ho and Tzanetakis, 2014) and Truffle (Visser et al. 2016).

Data analysis of both sequencing runs using CLC workbench 6 and Geneious, confirmed the presence of the viroids and viruses that were known to be present in the samples, as well as detecting other viruses and viroids that were not expected to be present. The data enabled almost complete genomes to be generated for a number of viruses and viroids.

A citrus viroid, previously unreported in Australia, was detected through analysis of NGS data in an extract from a tree held in the collection at EMAI. Another viroid, also previously unreported in Australia but detected at the same time in a surveillance sample, was detected in the EMAI tree using the RT-qPCR (Vidalakis and Wang 2013) and its presence was confirmed by analysis of NGS data. Both viroids were reported to the Commonwealth and have been added to the Auscitrus test schedule for Australian citrus germplasm. The presence of a novel citrus viroid, tentatively named CVD-VII, in an Auscitrus sample was also confirmed using NGS.

The Truffle software (Visser et al 2016) once installed was easy to use but is currently only able to detect 11 citrus viruses (no viroids). VirFind (Ho and Tzanetakis, 2014) is a web based pipeline. Both methods produced results comparable to in-house analyses (CLC workbench 6 and Geneious). Further work using NGS as a diagnostic tool would likely use total RNA on the Illumina NextSeq platform or similar to generate high quality data. The end output required dictates the methods chosen for data analysis.

Exotic graft-transmissible pathogens

- Assays evaluated to detect the putative causal agents of huanglongbing; '*Candidatus*' Liberibacter asiaticus, '*Ca*. L. africanus' and '*Ca*. L. americanus'
- Assays validated to detect *Xylella fastidiosa* ssp. *pauca* (CVC), *Citrus chlorotic dwarf associated virus* (CCDaV), *Citrus yellow mosaic virus* (CYMV) and *Citrus leprosis virus* (CiLV)
- Assays evaluated to detect *Spiroplasma citri*, *Citrus yellow vein clearing virus* (CYVCV)
- Data collected providing 'evidence of absence' of emergency plant pathogens in Australian citrus through surveillance.
- Data collected providing 'evidence of absence' of emergency plant pathogens, '*Candidatus*' Liberibacter asiaticus' (causal agent of HLB) and *Xylella fastidiosa* (causal agent of CVC), through testing of surveillance samples and Australian germplasm sources.

Huanglongbing (HLB)

Hocquellet et al. (1999) is a conventional PCR assay and Li et al. (2006) is a robust, qPCR assay used worldwide to detect Liberibacter species associated with HLB. However, in the real-time assay thresholds vary in the literature and there appears to be an 'inconclusive' region between Ct values of 32 to 40, making it less reliable for early detection or when the pathogen is present at low levels.

Real-time PCR (qPCR) is used to determine how much of a target sequence or gene is present in a sample and, hence, the titre of the pathogen. The amplification of targeted DNA is monitored during the PCR, creating an amplification curve. In the procedure, a threshold is set that represents background fluorescence. The Ct (cycle threshold) value is the point at which the amplification curve for a sample intersects the threshold line. Low Ct values mean there are higher amounts of targeted nucleic acid in the sample; and high Ct values mean there are fewer copies. In the literature, thresholds vary for Liberibacter species associated with HLB and there appears to be an 'inconclusive' region between Ct values of 32 to 40. For example, Keremane et al. (2015) reports that Ct values of 34 or above are generally considered inconclusive, whereas the California Department of Food and Agriculture deems results inconclusive for psyllid extracts achieving a Ct value between 32 and 40 and plant extracts scoring values between 37 and 40.

Namgay Om is a Bhutanese plant pathologist who recently completed her PhD at Western Sydney University, under the supervision of Professors Paul Holford and Andrew Beattie, and Nerida Donovan. Project team scientists in the EMAI lab worked with Namgay in an attempt to resolve issues with defining a positive result for '*Ca*. L asiaticus' in the qPCR assay. Samples were analysed using conventional and qPCR. Of these, 54 out of 89 samples had a Ct value greater than 35. Of the 54 with high Ct values, 49 were negative and 5 were positive using the conventional PCR assay. This means that 5 / 89 (5.6%) were potentially false negatives using qPCR — positive results that were missed by qPCR because they were above the set threshold. And 24 / 89 (27%) were positive from the qPCR but were not detected by conventional PCR — potentially false negatives using conventional PCR.

New diagnostic methods were evaluated to detect the causal agents of HLB, with particular focus on '*Ca*. L. asiaticus'.

Tissue imprint method (Nagesawara-Rao et al. 2013) – field samples were collected in October / November 2014 during visits to field sites in Bhutan as part of an aid project funded by the Australian Centre for International Agricultural Research (ACIAR). Samples were collected from citrus trees in the Bhutan National Citrus Repository and commercial nurseries and orchard. Leaf petioles and stem were blotted onto a nylon membrane and midrib samples were placed in ethanol for transport to Australia (permit IP14009959). The processing was performed in hotel rooms in Bhutan due to time constraints but could easily have been conducted in the field by securing the membrane on a clipboard. At EMAI, midrib samples were extracted and tested for '*Ca*. L asiaticus' using real-time PCR. The method for processing the blots was deemed too time consuming and not practical therefore was abandoned.

A conventional PCR assay using primers for the *rpoB* gene (Ananthakrishnan et al 2013) was trialled successfully on extracts from plant material. This assay was useful for detecting Liberibacter species, although did not detect *Liberibacter crescens*.

LAMP (loop-mediated amplification technology) assays were trialled due to reported improved sensitivity and efficiency over other qPCR assays. Positive samples were detected using primers from Keremane et al. (2015) and

Rigano et al. (2014) but there were issues with false negatives and positives. Detection assays were also run using an OptiGene Genie III portable LAMP machine on temporary loan from GeneWorks. This technology has potential for use in a mobile or temporary lab. No samples were amplified using the Optigene Plant Material Lysis kit on the infected plant material that had been stored in ethanol for a considerable period. Further work is needed to determine if the technique is suitable for detecting the causal agents of HLB.

Citrus variegated chlorosis (CVC)

Xylella fastidiosa ssp. *pauca* is associated with CVC. Conventional PCR assays using two different primers successfully detected *X. fastidiosa* (Francis et al. 2006) and *X. fastidiosa* ssp. *pauca* (Li et al. 2013) in one DNA extract of CVC from sweet orange, sourced from the USDA.

Our protocols were then used by the PHDS to test for *X. fastidiosa* in grapevine samples collected during targeted surveillance early in 2016.

Citrus Stubborn Disease (CSD)

Three nucleic acid extracts sourced from the USDA and originally extracted from sweet orange, were used to evaluate conventional and real-time PCR diagnostic assays for *Spiroplasma citri* (causal agent of CSD).

S. citri was detected using primers from Wang et al. (2015) (PhP-orf3 F/orf3 Rev), Yokomi et al. (2008) (P58-6f/P58-4r), Bevan et al. (2012), Foissac et al. 1996, Nunan et al. (2004), and modified from Bevan et al. (2012); but non-specific amplification also occurred. None of the assays that were evaluated are recommended for use at this stage. Further work is needed.

Citrus Chlorotic Dwarf Associated Virus (CCDaV)

One nucleic acid extract of CCDaV from alemow and imported from the USDA was assayed by qPCR (Loconsole et al. 2012a); although the assay was not successful. Non-specific amplification generated false positive results. However, a conventional PCR assay (Lonconsole et al. 2012a) using different primers (Table 3) detected CCDaV without non-specific amplification of non-CCDaV extracts.

Citrus yellow mosaic virus (CYMV)

CYMV was detected using two different primer sets (Huang pers. comm. and Baranwal et al. 2003) in a conventional RT-PCR assay with the results confirmed by sequencing. This virus was detected in extracts imported from the USDA and we were not aware they contained CYMV; they were in the collection because they contained other organisms.

Citrus yellow vein clearing virus (CYVCV)

There is one extract in the EMAI collection originally from Pakistan that was supposed to contain CYVCV, and we had not attempted to detect this virus before. CYVCV was successfully detected in the extract using conventional RT-PCR (Loconsole et al. 2012b); the result was confirmed by sequencing. However, the primers amplified a shorter than expected fragment due to a lack of specificity. The sequence gained showed 80% similarity to CYVCV isolates in Genbank. SYBR™ qPCR assays based on primers from Chen et al. (2016) and Loconsole et al. (2012b) were not able to detect CYVCV in the extract. This is likely due to the low level of sequence similarity between the isolate in our control collection and other CYVCV isolates in Genbank. Further validation of diagnostic assays would benefit from sourcing other positive extracts for use as positive controls.

Citrus leprosis virus (CiLV)

CiLV is not considered to be a systemic virus and is reported to be difficult to transmit via grafting of symptomatic tissue. The pathogen was studied in this project because it is an exotic virus with the potential to have a significant impact if introduced, exacerbated by the presence in Australia of known vectors like the false spider mite (*Brevipalpus phoenicis*).

There is one extract in the EMAI collection containing CiLV. Primers from Locali et al. (2003) both worked well and were specific for CiLV; the authors recommend the use of both sets of primers in the one assay to minimise false negatives due to genetic variation existing amongst isolates. The amplicon amplified with the Rep primers was sequenced to confirm the presence of CiLV in our positive control extract.

Surveillance

Team members participated in surveillance activities for citrus EPP's (Table 5) organised by NAQS or by the

National Citrus Biosecurity Manager (Stuart Pettigrew) as part of the citrus biosecurity project CT12022 (managed by Plant Health Australia and funded by Hort Innovation).

Citrus and *Murraya* species were inspected for the Asian citrus psyllid (ACP) which is associated with transmission of '*Ca*. L. asiaticus'. Citrus species were also inspected for citrus canker (*Xanthomonas citri* ssp. *citri*), powdery mildew, and symptoms consistent with HLB (or the endemic Australian Citrus Dieback).

Surveillance samples were tested for '*Ca*. L. asiaticus' (HLB) and *X. fastidiosa* ssp. *pauca* (CVC), at EMAI by qPCR with no positive detections. Samples were also tested for phytoplasmas, although no symptoms resembling ACD were observed.

The project leader participated in the National Citrus Surveillance workshop in 2016 and the 2nd Annual Surveillance Workshop in 2018. A National Plant Biosecurity Surveillance Network is working to standardise data capture systems and develop surveillance protocols to improve our ability to respond to incursions.

Project team members attended 'Exercise Yellow Dragon' held at EMAI in 2015. Exercise Yellow Dragon was a simulation exercise run by Plant Health Australia to test the effectiveness of Australia's planned eradication strategies for ACP and HLB. The exercise emphasised the need to improve our capacity to deal with the large numbers of diagnostic samples that may be received during an incursion by ensuring that we adequately road test high throughput processing methods.

Table 5: Surveillance activities for citrus emergency plant pests involving members of the CT14009 project team

Location	Timing	Area	Participants from
Ord River irrigation area WA	2015	production (orchards), peri-urban, urban	NAQS, PHA, NSW DPI, WA DPIRD
Sydney NSW	2015	production (nurseries), peri-urban, urban	PHA, NSW DPI
Central coast NSW	2016	production (orchards, nurseries), peri-urban, urban	PHA, NSW DPI
Darwin, Katherine NT	2016	production (nurseries, orchards), peri-urban, urban	NAQS, PHA, NSW DPI, NT DPIR
Atherton Tablelands Qld	2016	production (orchard, nurseries), peri-urban, urban	NAQS, PHA, NSW DPI, Qld DAF, CA
Torres Strait islands Qld	2017	5 island communities	NAQS, NSW DPI

NAQS = Northern Australia Quarantine Strategy; PHA = Plant Health Australia; NSW DPI = NSW Department of Primary Industries; WA DPIRD = WA Department of Primary Industries and Regional Development; NT DPIR = NT Department Primary Industries and Resources, Qld DAF = Qld Department of Agriculture and Fisheries; CA = Citrus Australia

Communication

Communication activities listed below were delivered by project team members to disseminate the message about the importance of citrus biosecurity and graft-transmissible diseases.

Refereed scientific journal publication

Chambers GA, Donovan NJ, Bodaghi S, Jelinek SM, Vidalakis G. 2018. A novel citrus viroid found in Australia, tentatively named citrus viroid VII. *Archives of Virology* **163**(1), 215-218. DOI 10.1007/s00705-017-3591-y (Appendix 1)

A number of journal manuscripts are in preparation by the project team.

Conference publications

Donovan N, Herrmann T, Jelinek SM, Chambers GA, Englezou A. Supply of healthy propagating material to Australian citrus nurseries. XI Congress of the International Society of Citrus Nurserymen, Mildura Australia 24-28th July 2017

Donovan N, Englezou A, Chambers G, Jelinek S, Tan M, Chapman T, Holford P. 2017. Protecting Australian citrus germplasm through improved diagnostic tools. Citrus Technical Forum and Field Day, Mildura Australia 1-2 March

2017 (Appendix 2)

Chambers GA, Donovan NJ, Jelinek SM, Vidalakis G. A novel citrus viroid found in Australia, tentatively named citrus viroid VII. 20th International Organisation of Citrus Virologists Conference, Chongqing China 10-15th April 2016

Donovan NJ, Englezou A, Chambers GA, Jelinek SM, Tan MK, Chapman TA, Holford P. Protecting Australian citrus germplasm through improved diagnostic tools. International Citrus Congress, Foz Do Iguacu, Brazil 18-23 September 2016 (Appendix 3)

Donovan N. Ready to respond to disease threats. Citrus Australia Technical Forum and Field Day. Mildura Vic 16-17/3/2015 (Appendix 4)

Extension publications

Donovan N, Holford P, Chambers G. 2018. Citrus Viruses in Australia. Auscitrus fact sheet (Appendix 5)

Donovan N, Holford P, Chambers G. 2018. *Citrus tristeza virus in Australia*. Auscitrus fact sheet (Appendix 6)

Donovan N, Chambers G, Holford P. 2018. Viroids in Australian Citrus. Auscitrus fact sheet – confidential until new viroid detections published (Appendix 7)

Donovan N, Sanderson G, Falivene S. 2017. Budwood and graft-transmissible disease. In: Citrus Plant Protection and Management Guide 2017 pp 52-53. Eds: Falivene S, Creek A. State of New South Wales through NSW Department of Industry. ISSN – 2208-5963 (print) ISSN – 2208-5971 (online)

Donovan N, Creek A. 2017. Diseases and disorders. In: Citrus Plant Protection and Management Guide 2017 pp 35-46. Eds: Falivene S, Creek A. State of New South Wales through NSW Department of Industry. ISSN – 2208-5963 (print) ISSN – 2208-5971 (online)

Ong R. 2017. Congress key message: don't risk disease. Australian Citrus News Spring 2017 p 33 (N.B. content and editing provided by N Donovan)

Donovan N. 2016. Huanglongbing (HLB) worldwide update. Citrus Connect December 2016

Plant Biosecurity and Product Integrity. 2016. Exotic Plant Pests and Diseases of Citrus. NSW DPI booklet

Donovan N. 2015. Untested budwood can cost you thousands. Citrus Connect December 2015

Donovan N, Herrmann T. 2015. Preventing disease at the source. Australian Citrus News Summer 2015/16 p24

Presentations

Donovan N. NSW DPI Citrus R&D Roadshows. Perth WA 12/9/17, Griffith NSW 16/10/17, Mildura Vic 18/10/17, Loxton SA 19/10/17

Donovan N. The dangers of using untested budwood. Advances in Disease Management and Detection Workshop, Citrus Technical Forum and Field Day. Mildura Vic 1-2 March, 2017

Donovan N. Citrus Pathology Program – Protecting Australian Citrus Germplasm through Improved Diagnostic Tools. Citrus Strategic Investment Advisory Panel, Horticulture Innovation Australia, Central Coast Primary Industries Institute, Ourimbah, NSW Australia 1st February 2017

Donovan N. Management of citrus diseases and disorders of concern to industry. Leeton and Griffith NSW, 9/3/16

Donovan N. The dangers of using untested budwood / Yellow Dragon: A worldwide citrus epidemic – our greatest threat / The Citrus Repository and Indexing Program. 3 presentations at 'Preparing the Australian citrus nursery industry for Huanglongbing / Asian Citrus Psyllid Workshop'. Dareton NSW 19/8/15

Donovan N. Citrus Disease Threats. Citrus Biosecurity Workshops. Griffith NSW 3/3/15, Loxton SA 19/5/15, Dareton NSW 20/5/15

Donovan N. Huanglongbing. Biosecurity and Fruit Fly Technology workshop, Citrus Australia Technical Forum and Field Day. Mildura Vic 16-17/3/15

Reports

Donovan N. 2017. International Research Conference on Huanglongbing, Florida - March 2017. Report submitted to industry

Donovan N, Sanderson G. 2016. International Citriculture Congress, Brazil – September 2016. Report submitted to industry

Donovan N, Chambers G. 2016. International Organisation of Citrus Virologists, China – April 2016. Report submitted to industry

Halling L, Pettigrew S, Ray J, Anderson S, Donovan N, Broughton S, Henshaw B, Arthur T, Peck D, Walker J. 2016. Final report: Industry Citrus Survey, ORIA, 2015. Australian Government Department of Agriculture and Water Resources

Outcomes

- Improved knowledge and access to positive control material for developing and validating diagnostic assays.
- Confirmation of the high health status of public varieties held in the National Citrus Repository which supplies propagation material to industry.
- Strengthened Australian diagnostic networks (NSW DPI and DAWR) through exchange of knowledge and materials.
- Improved preparedness by increasing diagnostic capabilities of NSW DPI and, consequently, the Australian industry, for graft-transmissible pathogens of citrus:
    - improved detection methods identified for Citrus bent leaf viroid (CBLVd or CVd-I), Hop stunt viroid (HSVd or CVd-II), Citrus dwarfing viroid (CDVd or CVd-III), Citrus bark cracking viroid (CBCVd or CVd-IV), Citrus viroid V (CVd-V), Citrus viroid VI (CVd-VI), novel viroid tentatively called Citrus viroid VII (CVd-VII), Citrus exocortis viroid (CEVd), *Citrus tatterleaf virus* (CTLV), *Citrus psorosis virus* (CPsV), *Citrus leaf blotch virus* (CLBV), *Citrus tristeza virus* (CTV) and *Citrus vein enation virus* (CVEV);
    - multiplex assay recommended to simultaneously detect CPsV and CLBV to improve efficiency of testing budwood supply trees in the Auscitrus propagation scheme.
- Improved preparedness by increasing diagnostic capabilities of NSW DPI, and consequently the Australian industry, to handle an incursion of an emergency plant pest:
    - new accessions added to the citrus pathogen collection of '*Candidatus* Liberibacter asiaticus' (huanglongbing), '*Ca*. L. americanus', *Citrus chlorotic dwarf associated virus* (CCDaV), *Citrus yellow mosaic virus* (CYMV), *Citrus yellow vein clearing virus* (CYVCV), *Citrus leprosis virus* (CiLV), *Spiroplasma citri* (Citrus stubborn disease);
    - detection methods identified for '*Candidatus* L. asiaticus', '*Ca*. L. africanus' and '*Ca*. L. americanus' (associated with huanglongbing), *Xylella fastidiosa* ssp. *pauca* (citrus variegated chlorosis), *Citrus chlorotic dwarf associated virus* (CCDaV), *Citrus yellow mosaic virus* (CYMV), *Citrus leprosis virus* (CiLV);
    - detection methods evaluated for *Citrus yellow vein clearing virus* (CYVCV) and *Spiroplasma citri* (citrus stubborn disease)
- 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 by publications, and presentations at industry forums.

Monitoring and evaluation

Biosecurity is a priority for the Australian citrus industry (Citrus Strategic Investment Plan 2017-2022). Diseases can threaten industry survival and the ability to effectively detect these diseases is important for reducing their impact. For this we need industry awareness of the threats and robust diagnostic tools. Hort Innovation funded project CT14009 'Protecting Australian citrus germplasm through improved diagnostic tools' is relevant and important because it aimed to improve our ability to detect graft-transmissible diseases in citrus, and build our knowledge on these disease threats. The work was conducted efficiently (within budget) and was effective in delivering improved knowledge and diagnostic tools for graft-transmissible citrus pathogens (milestones met or exceeded). The project benefited from linking with the existing portfolio and networks of the NSW DPI Citrus Pathology Program which enhanced project outcomes.

To support diagnostic tool development, the NSW DPI citrus pathogen collection was catalogued and expanded providing a valuable resource. The team evaluated published methods or developed new tests to improve our capability to detect 15 endemic and 10 exotic graft-transmissible citrus pathogens; including the causal agents of huanglongbing (HLB), the biggest threat to global citrus. New viroid detections were made using improved diagnostic tools. Efficiency of testing was improved by changing from conventional to real-time PCR for most assays, and by multiplexing assays (testing for more than one pathogen in the same assay). Eight viroid assays were successfully combined into three assays, and four viral assays into three. Within the scope of the project, three multiplex assays were adopted by Auscitrus, improving the efficiency of testing and confidence in diagnostic results.

The team contributed to increasing national awareness of biosecurity threats to Australian citrus in collaboration with the Auscitrus, Federal and State governments, Plant Health Australia (PHA) and Citrus Australia (CAL). Team members participated in surveillance for emergency plant pests in production, urban and high risk areas and surveillance samples were tested for the causal agents of HLB and citrus variegated chlorosis to provide evidence of absence data. Project team members delivered the message about the threat of graft-transmissible diseases to Australian citrus via extension publications and presentations at industry forums, including biosecurity workshops in the major citrus growing regions of the Riverina, Sunraysia and Riverland.

Project findings were communicated via milestone reports (every six months), the final report, articles in Australian Citrus News and Citrus Connect, and presentations to industry and the scientific community at meetings, workshops and conferences.

Project progress was reported to Auscitrus via quarterly updates throughout the project term. Tim Herrmann (Auscitrus Manager) and the Auscitrus Executive Committee provided industry perspective and advice, helping to keep the project on track. Project updates were also given annually at the Auscitrus General meetings to the entire Auscitrus Board and visiting industry representatives (e.g. Citrus Australia), and in February 2017 to the Hort Innovation Strategic Investment Advisory Panel.

Recommendations

Diagnostic tools recommended for adoption

- A conventional RT-PCR assay for citrus exocortis viroid (CEVd), Citrus bent leaf viroid (CBLVd or CVd-I), Hop stunt viroid (HSVd or CVd-II) and Citrus dwarfing viroid (CDVd or CVd-III) (Wildman 2013 adapted from Wang et al. 2009).
- A probe-based, RT-qPCR assay for CEVd and HSVd (Lin et al. 2015).
- A SYBR™, RT-qPCR assay for citrus viroids I, III, V, VI and VII (Vidalakis and Wang 2013).
- A probe-based, RT-qPCR assay for *Citrus leaf blotch virus* (CLBV) and *Citrus psorosis virus* (CPsV) (Osman et al. 2015) using a probe adapted for Australian CPsV strains.

Future research to improve diagnostic capability

- Identify methods to successfully detect those pathogens which we are not confident in our ability to detect.

  e.g. Citrus bark cracking viroid (endemic) and *Spiroplasma citri* (exotic)

- Identify methods to effectively differentiate between viroid and viral strains.

  e.g. between strains of Hop stunt viroid, also called Citrus viroid II, either the mildly dwarfing CVd-IIa or the pathogenic CVd-IIb

  e.g. between strains of *Citrus tristeza virus* which range from mild cross protecting isolates to severe disease-inducing isolates

- Multiplex more tests to further improve efficiency.

  e.g. include a third virus in the multiplex assay for *Citrus leaf blotch virus* and *Citrus psorosis virus*

- Evaluate the diagnostic potential of new sequencing technologies.
- Develop field detection assays.
- Trial newly published diagnostic methods for endemic and exotic pathogens of citrus to improve specificity, sensitivity and efficiency 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 (as outlined above) in collaboration with Auscitrus and DAWR post-entry quarantine, 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 host range, symptom expression and distribution of newly discovered graft-transmissible organisms (including the tentatively named citrus viroid VII) to determine the potential impact on the citrus industry.
- 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 disease).
- 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 to add value to activities and reduce duplication.

Refereed scientific publications

Journal article

Chambers GA, Donovan NJ, Bodaghi S, Jelinek SM, Vidalakis G. 2018. A novel citrus viroid found in Australia, tentatively named citrus viroid VII. *Archives of Virology* **163**(1), 215-218. DOI 10.1007/s00705-017-3591-y

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Intellectual property, commercialisation and confidentiality

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

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 CT14009 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

Appendix 1: Chambers GA, Donovan NJ, Bodaghi S, Jelinek SM, Vidalakis G. 2018. A novel citrus viroid found in Australia, tentatively named citrus viroid VII. *Archives of Virology* **163**(1), 215-218. DOI 10.1007/s00705-017-3591-y

Appendix 2: Donovan N, Englezou A, Chambers G, Jelinek S, Tan M, Chapman T, Holford P. 2017. Protecting Australian citrus germplasm through improved diagnostic tools. Citrus Technical Forum and Field Day, Mildura Australia 1-2 March 2017 – abstract and poster

Appendix 3: Donovan NJ, Englezou A, Chambers GA, Jelinek SM, Tan MK, Chapman TA, Holford P. Protecting Australian citrus germplasm through improved diagnostic tools. International Citrus Congress, Foz Do Iguacu, Brazil 18-23 September 2016 – abstract and poster

Appendix 4: Donovan N. Ready to respond to disease threats. Citrus Australia Technical Forum and Field Day. Mildura Vic 16-17/3/2015

Appendix 5: Donovan N, Holford P, Chambers G (2018) Citrus Viruses in Australia. Auscitrus fact sheet

Appendix 6: Donovan N, Holford P, Chambers G (2018) *Citrus tristeza virus in Australia*. Auscitrus fact sheet

Appendix 7: Donovan N, Chambers G, Holford P (2018) Viroids in Australian Citrus. Auscitrus fact sheet – confidential until approval given by the CCEPP to release information on new viroid detections

Appendix 1: Chambers GA, Donovan NJ, Bodaghi S, Jelinek SM, Vidalakis G. 2018. A novel citrus viroid found in Australia, tentatively named citrus viroid VII. *Archives of Virology* **163**(1), 215-218. DOI 10.1007/s00705-017-3591-y

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BRIEF REPORT

A novel citrus viroid found in Australia, tentatively named citrus viroid VII

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Abstract A novel citrus viroid was discovered in a non-symptomatic Lisbon lemon (*Citrus x limon* L. Burm.f.) tree in New South Wales, Australia. Bioindexing, molecular detection and characterization involving sequencing combined with *in silico* analysis for the identification of the viroid-RNA hallmark properties of transmissibility and autonomous replication as well as specific sequence and structural motifs suggest that this viroid is a member of a new species in the genus *Apscaviroid*, family *Pospiviroidae*, which we have tentatively named "citrus viroid VII" (CVd-VII).

Citrus cultivars in Australia have been found to be infected with a number of viroids, including the pathogenic citrus exocortis viroid (CEVd) and the cachexia-inducing citrus variants of hop stunt viroid (HSVd), citrus viroid IIb (CVd-IIb) and citrus viroid IIc (CVd-IIc), as well as the non-cachexia variant CVd-IIa, citrus bent leaf viroid (CBLVd), and citrus dwarfing viroid (CDVd) [7]. Citrus viroids that have not been reported in Australia include citrus bark cracking viroid (CBCVd), CVd-V and CVd-VI. Programmes are in place to test imported and local citrus germplasm for graft-transmissible pathogens, including viroids, to prevent nursery and orchard infections. Routine biological indexing of a Lisbon lemon (*Citrus x limon* L. Burm.f.) field tree located in Dareton, New South Wales, Australia, was initiated in February 2015. Bark pieces from the symptomless Lisbon lemon were grafted onto four 'Etrog' citron Arizona 861-S-1 (*C. medica* L.) indicator plants grown from cuttings and maintained in a temperature-controlled greenhouse at 32 °C. Subsequent growth flushes on all of the indicator plants over a six-month period expressed epinasty symptoms (leaf bending and curling) indicative of citrus viroid infection (Fig. 1).

Total RNA was extracted from fresh bark tissue samples from one uninoculated plant and the four symptomatic 'Etrog' citron indicator plants using a VioTotal Plant RNA extraction miniprep system (Viogene, Taiwan) following the manufacturer's instructions. All samples tested negative by conventional reverse transcription polymerase chain reaction (RT-PCR) with primers designed to detect CEVd, HSVd, and CDVd [1], CBLVd [9], CVd-I-low sequence similarity (CVd-I-LSS) [8, 9], CBCVd [1, 2], CVd-V [12] and CVd-VI [9] (Table S1). A SYBR Green RT-qPCR assay using a degenerate primer pair Apsca-F-3-25/Apsca-R-232-212 (Table S1), which was designed for the universal detection of citrus apscaviroids (CBLVd, CDVd, CVd-V and CVd-VI) [16], produced an amplicon with a larger size and a different melting temperature from the known apscaviroid controls, but only in extracts from the symptomatic 'Etrog' citron plants. The 279-bp PCR product was purified using an Isolate II PCR and Gel Kit (Bioline, Australia) and directly sequenced in both directions (Australian Genome Research Facility-AGRF, Sydney) using the degenerate apscaviroid primers. BLASTn analysis of the 279-bp sequence showed a

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Fig. 1 Symptoms of leaf epinasty in new flush (left) and mature leaves (right) of 'Etrog' citron (*Citrus medica* L.) Arizona 861-S-1 plants inoculated with bark pieces from a field-grown non-symptomatic Lisbon lemon tree (*C. x limon* L. Burm.f.) are shown

low level similarity to the apscaviroid, Australian grapevine viroid (AGVd).

The sequence-specific overlapping primers VIIF1 (5′-CTTGCCTTGAGAAGCGAAGC-3′) and VIIR3 (5′-AAGCAGTTCCAGTTACAG-3′) were designed based on the 279-bp PCR product sequence for the amplification of the full circular genome of the potentially novel viroid. One-step RT-PCR was carried out using a SuperScript™ III One-step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen, Australia) according to the manufacturer's instructions. RT-PCR cycling conditions comprised reverse transcription at 55 °C for 30 minutes followed by 2 minutes at 94 °C and 35 cycles of denaturation (20 s at 94 °C), annealing (15 s at 56 °C) and extension (20 s at 72 °C), followed by a final extension step of 5 minutes at 72 °C. The resulting RT-PCR product was purified using an Isolate II PCR and Gel Kit (Bioline, Australia) and cloned using the pGEM-T Easy Vector System (Promega, Australia). Sequencing of selected clones in both directions using SP6 and T7 primers (AGRF, Sydney) revealed a 368-nucleotide insert, thus supporting the circular nature of the amplified RNA.

BLASTn analysis of three variants of these circular RNAs that were deposited in the GenBank database, LD2 (KX013549), LD3 (KX013550) and LD4 (KX013551), revealed sequences almost identical to each other and significantly different from all other known viroids. The highest sequence identity (59-59.5%) was observed with AGVd (FJ746829). The LD4 variant, selected here as the type variant, consisted of 89 A (24.2%), 87 U (23.6%), 96 C (26.1%), and 96 G (26.1%) nucleotides and had a G + C content of 52.2%. The other two variants displayed nucleotide substitutions at position 77, A to G (LD2 - KX013549 and LD3 - KX013550) and at position 231, U to A (LD3 - KX013550) (Fig. S1). The secondary structure of minimal free energy of LD4, as predicted by MFOLD software [17], was of a rod-like conformation with 69% paired nucleotides (Fig. 2a). More importantly, however, the secondary structure contained a terminal conserved region (TCR) and upper and lower central conserved regions (CCR) characteristic of members of the genus *Apscaviroid*. The TCR was found to have two mismatches in comparison to the core sequence (CNNGNGGUUCCUGUGG) of the apscaviroid type reference sequence (NC_001340) of apple scar skin viroid (ASSVd) [3]. Such mismatches in the TCR are observed in other proposed apscaviroids such as apple fruit crinkle viroid (NC_003777) and persimmon viroid 2 (NC_021720) [10]. Both the upper and lower central conserved regions were identical to the apscaviroid type reference sequence (Fig. S1). A thermodynamically stable hairpin I (HPI) structure similar to that described in other apscaviroids including citrus dwarfing viroid IIIa variant [13] and citrus viroid V [12], was present between nucleotides 88 and 120 (Fig. 2b), flanking the upper CCR, with a terminal tetraloop with a 3-bp stem followed by an extended stem at the base. On the lower strand, a hairpin II (HPII)-like metastable structure, with a 7-bp stem and a 71-bp loop containing the lower CCR was present, resembling that described in the apscaviroid CVd-V [12]. The stem of the HPII-like structure in viroid-like RNA did not have a high GC content, unlike the HPII structures present in other viroids [14] (Fig. 2b).

In addition to RT-PCR and sequence analysis, the total RNA extracted from the symptomatic graft-inoculated 'Etrog' citrons was slash inoculated into four new 'Etrog' citrons. The plants were maintained at 32 °C, and the viroid-like RNA was first detected six weeks post-inoculation in new growth above the inoculation site by RT-PCR using the primers VIIF1 and VIIR3, which are homologous and complementary to the nucleotide positions 233-252 and 218-235, respectively, of the type variant LD4 (KX013551). The PCR amplicons were sequenced, verifying their identity to

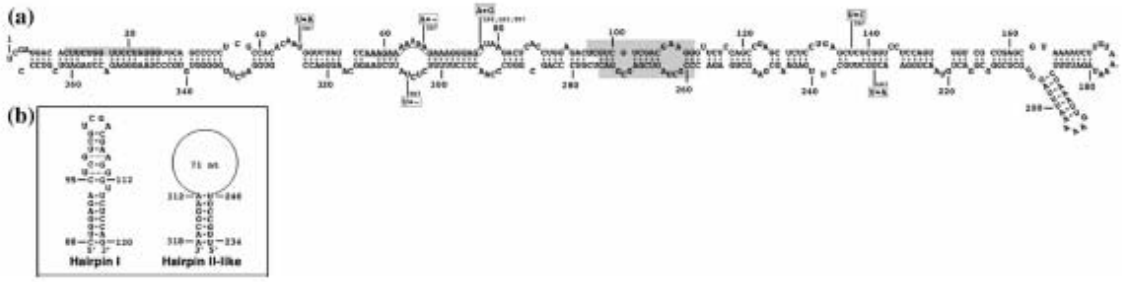

Fig. 2 **a.** Nucleotide sequence and proposed secondary structure of citrus viroid VII (CVd-VII) type variant (LD4). Structures are represented as the minimum free energy form at 24 °C predicted by MFOLD. The changes observed in the four additional variants (LD2, LD3, TD7, and TD8) are shown in marked boxes. The terminal conserved region (TCR) is contained within the shaded box (nucleotides 10-25), and the upper and lower central conserved regions (CCR) are also shaded. The upper CCR is represented by nucleotides 96-111, and the lower CCR is between nucleotides 259 and 275. The oligopurine region (nucleotides 55-77) is underlined. **b.** Schematic representation of the predicted hairpin I and hairpin II-like structures

the LD4 variant. Prominent symptoms of leaf bending and curling developed in all slash-inoculated 'Etrog' citrons at 12 weeks post-inoculation. Sequential polyacrylamide gel electrophoresis (sPAGE) followed by northern blot hybridization analysis [11] of the graft- and slash-inoculated 'Etrog' citrons, using a full-length DIG-labelled LD4 probe, revealed both circular and linear viroid-like RNA forms of the expected size (Fig. 3).

To further investigate the biological hallmarks of the newly identified viroid-like RNA, namely transmissibility and autonomous replication, a recombinant plasmid containing a single copy of the LD4 type variant (positive orientation) was linearized with *Nde*I and transcribed *in vitro* using an Ambion MEGAscript T7 Kit (Life Technologies, Australia). The monomeric RNA transcript, free of DNA, was slash inoculated into six uninfected 'Etrog' citron indicator plants. Four months after inoculation, samples of new leaf growth, above the inoculation site, were tested by RT-PCR with the VIIF1/VIIR3 primers and by RT-qPCR with the universal Apsca-F-3-25/Apsca-R-232-212 primers. The viroid-like RNA was detected by both assays, and the full-length RT-PCR amplicon was cloned and sequenced in both directions, confirming the presence of the full-length LD4 type variant in two of the transcript-inoculated plants. Further evidence of the autonomous replication and biological activity of the viroid-like RNA was obtained by the detection and characterization of the negative-sense RNA in one of the LD4 transcript-inoculated 'Etrog' citrons. More specifically, reverse transcription using the VIIF1 primer, which anneals to the negative RNA viroid strand, followed by amplification with the VIIF1/VIIR3 overlapping primer pair, amplified a product that was directly sequenced and determined to be a negative-sense full-length LD4-type variant. Based on the above biological, molecular, and *in silico* analysis findings,

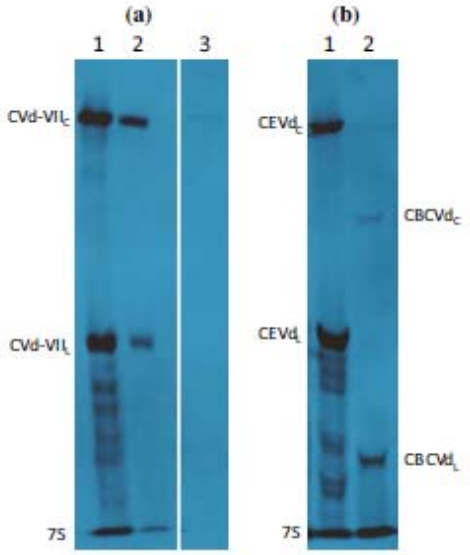

Fig. 3 Sequential polyacrylamide gel electrophoresis in a gel containing 5% acrylamide and 8 M urea, followed by northern-blot hybridization analysis [10] using (a) a full-length DIG-labelled LD4 type variant probe of the putative 368-nt citrus viroid VII (CVd-VII) and (b) full-length DIG-labelled probes of the 371-nt citrus exocortis viroid (CEVd) and the 284-nt citrus bark cracking viroid (CBCVd). Migration of circular (C) and linear (L) forms of viroid RNA and plant 7S RNA is indicated. **a** Lane 1, graft-inoculated 'Etrog' citron with bark pieces from the original Lisbon lemon tree; lane 2, slash-inoculated 'Etrog' citron with total RNA extracts from the graft-inoculated 'Etrog' citron (lane 1); and lane 3, uninoculated 'Etrog' citron. **b** Lanes 1 and 2, CEVd and: CBCVd used as viroid RNA mobility markers

Appendix 2: Donovan N, Englezou A, Chambers G, Jelinek S, Tan M, Chapman T, Holford P. 2017. Protecting Australian citrus germplasm through improved diagnostic tools. Citrus Technical Forum and Field Day, Mildura Australia 1-2 March 2017

PROTECTING AUSTRALIAN CITRUS GERMPLASM THROUGH IMPROVED DIAGNOSTIC TOOLS

Donovan NJ[1], Englezou A[1], Chambers GA[1], Jelinek SM[1], Tan MK[1], Chapman TA[1], Holford P[2]
[1]New South Wales Department of Primary Industries (NSW DPI), Narellan NSW 2567, Australia;
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Healthy and true-to-type propagation material is critical for the Australian citrus industry to remain competitive in the marketplace. A number of graft-transmissible diseases occur in Australian citrus, leading to yield loss and tree decline. There is no cure for these diseases so they must be managed using healthy (pathogen tested) budwood and rootstock seed. Programs are in place to test imported and local citrus germplasm for graft-transmissible diseases to prevent orchard infections. The supply of high health status propagation material depends upon accurate disease identification. Australian quarantine and the Auscitrus propagation scheme have been highly successful at reducing the impact of graft-transmissible diseases on the local industry. However, in recent years, market pressure has led to an increase in the number of varieties imported into the country, increasing the risk of introducing diseases at the same time. Diagnostic tests are currently under review to ensure that they are sensitive and cost effective; via a project funded by Horticulture Innovation Australia, Auscitrus and NSW DPI. Targets not only include diseases found in Australia, but also exotic diseases like huanglongbing (HLB) so that we are prepared if these potentially devastating diseases enter the country. An integrated, diagnostic approach incorporating biological, serological and molecular techniques is used to protect the health status of the Australian citrus industry.

Department of Primary Industries

PROTECTING AUSTRALIAN CITRUS GERMPLASM THROUGH IMPROVED DIAGNOSTIC TOOLS

Donovan NJ[1], Englezou A[1], Chambers GA[1], Jelinek SM[1], Tan MK[1], Chapman TA[1], Holford P[2]

[1]New South Wales Department of Primary Industries, Narellan NSW 2567, Australia; [2]Western Sydney University, Richmond NSW 2753, Australia. E-mail: nerida.donovan@dpi.nsw.gov.au

Healthy and true-to-type propagation material is critical for the Australian citrus industry to remain competitive in the marketplace. A number of graft-transmissible diseases have been reported to occur in Australian citrus, leading to yield loss, tree decline and death. In the absence of a cure, their management is through the use of pathogen-tested propagation material from Auscitrus, a not-for-profit industry organisation. The distribution and impact of graft-transmissible viruses and viroids on national production is likely to be low and detections are rare due to the availability of healthy material from Auscitrus; except for *Citrus tristeza virus* which is also aphid transmitted. Diagnostic tests for graft-transmissible citrus pathogens are currently under review via an industry funded project to ensure that they are robust, sensitive and cost effective. Targets include diseases found in Australia and exotic diseases like huanglongbing (HLB).

Viroids

Citrus exocortis viroid (CEV), and citrus viroids I, II, III and VII are reported to occur in Australia. The main pathogens of concern are CEV and cachexia (CVd-IIb).

Graft-transmissible dwarfing budwood containing a dwarfing strain of CVd-III is commercially available for the establishment of high density plantings, although this has not been widely adopted by growers.

Viruses

Citrus tristeza virus (CTV), *Citrus psorosis virus*, *Citrus leaf blotch virus* and *Citrus tatterleaf virus* are known to occur in Australian citrus. CTV is widespread, with hundreds of strains causing different diseases in different varieties. Mild strain cross protection is used commercially to protect grapefruit varieties against CTV grapefruit stem pitting. Orange stem pitting strains of CTV are thought to be limited to Queensland; movement restrictions exist to protect the orange industry in other states.

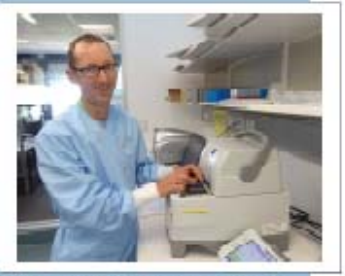
Diagnostic tools

An integrated, diagnostic approach is used to protect the health status of Australian citrus; incorporating advanced molecular technologies with biological and serological methods. A novel citrus viroid (CVd-VII) was recently discovered in Australian citrus germplasm using traditional biological indexing.

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

Acknowledgements

We would like to thank Horticulture Innovation Australia and the Australian Citrus Propagation Association (Auscitrus) for funding support.

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Appendix 3: Donovan NJ, Englezou A, Chambers GA, Jelinek SM, Tan MK, Chapman TA, Holford P. Protecting Australian citrus germplasm through improved diagnostic tools. International Citrus Congress, Foz Do Iguacu, Brazil 18-23 September 2016

PROTECTING AUSTRALIAN CITRUS GERMPLASM THROUGH IMPROVED DIAGNOSTIC TOOLS

Donovan NJ[1], Englezou A[1], Chambers GA[1], Jelinek SM[1], Tan MK[1], Chapman TA[1], Holford P[2]
[1]New South Wales Department of Primary Industries (NSW DPI), Narellan NSW 2567, Australia;
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Healthy and true-to-type propagation material is critical for the Australian citrus industry to remain competitive in the marketplace. A number of graft-transmissible diseases occur in Australian citrus, leading to yield loss and tree decline. In the absence of a cure, their management is through the use of pathogen-tested propagation material. Programs are in place to test imported and local citrus germplasm for graft-transmissible pathogens to avoid orchard infections, and the supply of high health status material is dependent upon accurate identification of pathogens in propagation material and their elimination. Australian quarantine and the Auscitrus propagation scheme have been highly successful at reducing the impact of graft-transmissible pathogens on the local industry. However, in recent years, market pressure has led to an increase in the number of varieties imported into the country increasing the risk of graft-transmissible diseases being brought in at the same time. Diagnostic assays are currently under review to ensure that they are robust, cost effective and sensitive. An integrated, diagnostic approach incorporating biological, serological and molecular techniques is used to protect the health status of the Australian citrus industry. Traditional tools, like biological indexing, are not overlooked given the recent discovery of a new viroid in Australian citrus germplasm found using indicator plants.

Keywords: citrus, germplasm, graft-transmissible disease.

Financial support: Horticulture Innovation Australia, NSW DPI.

NSW GOVERNMENT Department of Primary Industries

Horticulture Innovation Australia

Auscitrus

WESTERN SYDNEY UNIVERSITY

PROTECTING AUSTRALIAN CITRUS GERMPLASM THROUGH IMPROVED DIAGNOSTIC TOOLS

Donovan NJ[1], Englezou A[1], Chambers GA[1], Jelinek SM[1], Tan MK[1], Chapman TA[1], Holford P[2]

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Healthy and true-to-type propagation material is critical for the Australian citrus industry to remain competitive in the marketplace. A number of graft-transmissible diseases have been reported to occur in Australian citrus, leading to yield loss, tree decline and death. In the absence of a cure, their management is through the use of pathogen-tested propagation material from Auscitrus, a not-for-profit industry organisation. The distribution and impact of viroids and viruses on national production is likely to be low and detections are rare (except for aphid transmitted *Citrus tristeza virus*) due to the availability of healthy material from Auscitrus. Diagnostic assays for graft-transmissible citrus pathogens are currently under review to ensure that they are robust, sensitive and cost effective. An integrated, diagnostic approach, incorporating biological, serological and molecular techniques, is used to protect the health status of Australian citrus.

Viroids

Citrus exocortis viroid, and citrus viroids I, II, III and VII are reported to occur in Australia. The main pathogens of concern are exocortis and cachexia (CVd-IIb).

Graft-transmissible dwarfing budwood containing a dwarfing strain of CVd-III is commercially available for the establishment of high density plantings, although this has not been widely adopted by growers.

Viruses

Citrus tristeza virus (CTV), *Citrus psorosis virus*, *Citrus leaf blotch virus* and *Citrus tatterleaf virus* are known to occur in Australian citrus. CTV is endemic throughout Australian orchards. Mild strain cross protection is used commercially to protect grapefruit varieties against CTV strains which induce grapefruit stem pitting. Orange stem pitting strains of CTV are thought to be limited to the Australian state of Queensland; movement restrictions exist to protect the orange industry in other states.

Diagnostic tools

Traditional diagnostic tools, like biological indexing, are not overlooked. A novel citrus viroid (tentatively called CVd-VII) was recently discovered in Australian citrus germplasm using biological indexing on Etrog indicator plants.

Healthy citrus orchard in Australia

Stunted tree (L) due to CEV infection

CTV stem pitting symptoms in grapefruit

Symptoms induced by CVd-VII on Etrog

Acknowledgements

We would like to thank Horticulture Innovation Australia and the Australian Citrus Propagation Association (Auscitrus) for funding support.

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Appendix 4: Donovan N. Ready to respond to disease threats. Citrus Australia Technical Forum and Field Day. Mildura Vic 16-17/3/2015

READY TO RESPOND TO DISEASE THREATS

Nerida Donovan and Toni Chapman

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Australian citrus enjoys a high health status relative to many parts of the world due to the success of our quarantine system and the Auscitrus propagation scheme. However, exotic diseases like huanglongbing (HLB) and citrus canker are ever present threats. Our ability to combat new diseases depends on our preparedness and the ability of industry and government to collaborate effectively.

State government diagnostic laboratories have the ability to diagnose a range of citrus diseases. Nationally endorsed diagnostic protocols are available for the exotic citrus diseases of HLB, canker and mal secco. However it is important that Australian diagnostic laboratories validate or develop new methods using the latest technologies available and continue to build reference collections of disease agents to confirm the results of diagnostic tests. The NSW DPI citrus pathology team is currently working on projects (funded by Horticulture Innovation Australia, CRC for Plant Biosecurity and Plant Health Australia) to ensure that Australia is using the most sensitive and reliable diagnostic techniques for a number of citrus diseases including HLB and canker.

Government aid projects such as those funded by the Australian Centre for International Agricultural Research provide valuable opportunities for Australian scientists to learn about pests and diseases that are not found in Australia. It also means that scientists are able to bring samples back to Australia to boost reference collections.

The Australian industry and government departments have made biosecurity and emergency management the focus of many local activities and events in order to increase general awareness of potential risks. Industry representatives and citrus scientists have also attended international and local conferences to obtain the latest knowledge on pest and disease threats and to develop international linkages that prove invaluable when preparing for the arrival of new pests or diseases.

Study tours have helped to build the capacity of industry members, service providers, scientists and policy makers. The most recent of which was a study tour to California and Florida in 2014 led by Citrus Biosecurity Manager Stuart Pettigrew. The majority of participants were decision makers from Federal and State government departments to extend the awareness of the dangers of HLB beyond members of the citrus and nursery industries and encourage a more coordinated effort between industry and government.

Australia has been pro-active in managing citrus pests and diseases and preparing for new threats that may reach our shores. However we must continue this vigilance so that in the face of increasing biosecurity risks, we are ready to respond.

Appendix 5: Donovan N, Holford P, Chambers G (2018) Citrus Viruses in Australia. Auscitrus fact sheet

Citrus viruses in Australia

Virus infection can lead to reduced yield and tree decline

Graft-transmissible viruses are a serious economic threat to citrus production. Virus diseases can cause stunting, yield loss, developmental abnormalities and even death in particular scion and rootstock combinations – yet other varieties may be symptomless carriers.

Symptoms may not be seen in nursery trees, but may appear a few years later in the orchard. By this time, the disease is likely to have spread to surrounding trees via root grafting, aphid vectors or through the use of cutting tools. Nothing can be done to rid infected orchard trees of viruses; infected trees need to be pulled out and replanted. The only way to be sure of the health of your budwood and rootstock seed is to purchase them from a tested source such as Auscitrus.

Citrus tristeza virus (CTV), *Citrus tatterleaf virus* (CTLV), *Citrus psorosis virus* (CPsV), *Citrus leaf blotch virus* (CLBV) and *Citrus vein enation virus* (CVEV) are found in Australia.

Virus diseases have NO CURE

Prevention is the ONLY OPTION

ONLY use healthy budwood and seed from Auscitrus

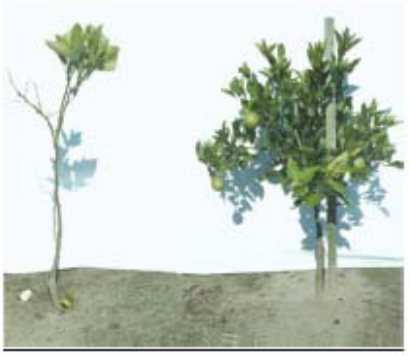
There is no cure for virus infections; management is through the use of pathogen-free rootstock seed and budwood from the Auscitrus propagation scheme. The small cost for using Auscitrus-tested material is negligible when compared to the cost of orchard establishment.

Viruses can be spread via infected budwood, infected rootstock seed (e.g. *Citrus leaf blotch virus*), on cutting tools (such as budding knives, secateurs or hedging machines) and by root grafts between trees in the orchard. The only disinfectant recommended for treating cutting tools is chlorine bleach (1.25% or 12,500 ppm sodium hypochlorite solution). Tristeza and vein enation viruses are also transmitted by aphids.

Sterilise cutting tools with bleach to kill viruses

NSW Primary Industries | Horticulture Innovation Australia | Auscitrus | Western Sydney University

Citrus tristeza virus: Most species of citrus are hosts for tristeza. Many strains causing mild to severe symptoms can be found in Australian citrus trees. Strains exist that can cause a quick decline of infected orange and mandarin scions on sour orange rootstocks. Other severe strains cause grapefruit or orange stem pitting that leads to tree decline and reduced yield and fruit size. Orange stem pitting strains are thought to be limited to Queensland.



A healthy nursery tree (R) and one infected with a quick decline strain of tristeza (L)

A healthy sweet orange tree (L) and one infected with orange stem pitting (R)

Do not move citrus propagation material from Queensland to other states to avoid spreading ORANGE STEM PITTING

Citrus tatterleaf virus: This virus causes stunting and chlorosis in infected scions when grafted onto susceptible rootstocks such as *Citrus* (*Poncirus*) *trifoliata*, citrange or Swingle citrumelo. A yellow ring is seen at the bud union of symptomatic trees and may be mistaken for horticultural incompatibility.

Scions infected with tatterleaf virus on tolerant (L) and susceptible (R) rootstocks

Citrus psorosis virus infection leads to tree decline and bark scaling on limbs and trunks.

Citrus leaf blotch virus causes a bud union disorder of susceptible scions (such as Nagami kumquat and calamondin) on trifoliate type rootstocks.

Citrus vein enation virus can cause swellings or woody galls on rough lemon or Mexican lime rootstocks.

Prepared by: Nerida Donovan, Paul Holford and Grant Chambers

The strategic levy investment project *Protecting Australian citrus germplasm using improved diagnostic tools* (CT14009) is part of the Hort Innovation Citrus Fund.

Appendix 6: Donovan N, Holford P, Chambers G (2018) *Citrus tristeza virus in Australia*. Auscitrus fact sheet

Citrus tristeza virus in Australia

Citrus tristeza virus (CTV) is the most devastating viral disease affecting citrus globally.

CTV is a complicated virus and exists in hundreds of different forms known as isolates. Most trees are infected with more than one isolate, and each isolate may contain more than one strain of CTV. Individual isolates can cause different symptoms in different citrus varieties, and many isolates of CTV can be found in Australian citrus trees that cause no disease symptoms. However, the symptoms caused by other isolates can vary from mild to severe. Two economically significant symptoms caused by CTV in the field are stem pitting and decline.

CTV infection can lead to reduced yield and tree decline

Infection with decline-inducing isolates causes an incompatibility between orange or mandarin scions when grafted onto sour orange rootstocks. The virus-induced incompatibility leads to slow or quick decline and eventual death of affected trees. Other symptoms include nutritional deficiencies and defoliation.

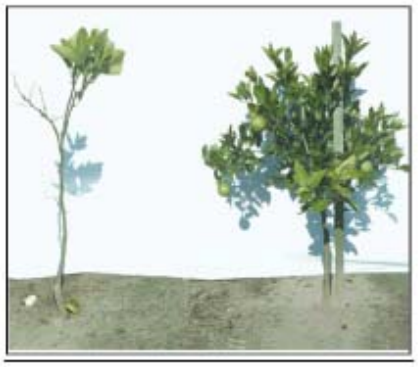
In Australia, CTV can cause stem pitting in lime, grapefruit or sweet orange cultivars and some rootstocks, but with a high degree of specificity. For example, isolates that induce severe stem pitting symptoms in oranges do not cause symptoms in mandarins. Trees affected by stem pitting have poor growth, brittle branches, and reduced yield and fruit size. Pits range in size from large grooves to small indentations; growth is more severely affected by smaller pits. Orange stem pitting isolates of CTV have only been reported in Queensland.

Stem pitting symptoms on a grapefruit tree

Healthy sweet orange tree (L) and a tree infected with orange stem pitting (R)

Do not move citrus propagation material from Queensland to other states to avoid spreading ORANGE STEM PITTING

NSW Primary Industries | Horticulture Innovation Australia | Auscitrus | Western Sydney University



A healthy nursery tree (R) and one infected with a quick decline isolate of CTV (L)

Tree protected by a mild CTV isolate (L) and an unprotected tree infected with severe CTV (R)

Citrus virus diseases have NO CURE

Citrus tristeza virus is spread in infected budwood (but not seed); therefore, it is important that propagation material is not taken out of Queensland nor imported illegally. Aphids also spread CTV, and the most efficient aphid vector is the brown citrus aphid (*Toxoptera citricida*) which is found in Australia.

There are no cures for virus infections. To reduce the impact of severe CTV isolates, manage aphid vector populations and use high health status budwood from Auscitrus. Grapefruit budwood supplied by Auscitrus has been sourced from trees inoculated with a mild protective isolate of CTV to protect trees against infection from severe grapefruit stem pitting isolates. In Australia, there is no commercially available mild isolate of CTV to protect oranges against severe stem pitting.

Use healthy budwood from Auscitrus

Brown citrus aphid (*Toxoptera citricida*) can transmit CTV

Prepared by: Nerida Donovan, Paul Holford, Grant Chambers

The strategic levy investment project *Protecting Australian citrus germplasm using improved diagnostic tools* (CT14009) is part of the Hort Innovation Citrus Fund.

Appendix 7: Donovan N, Chambers G, Holford P (2018) Viroids in Australian Citrus. Auscitrus fact sheet – confidential until approval given by the CCEPP to release information on new viroid detections

Viroids in Australian citrus

Viroid infection can lead to stunting and tree decline

Graft-transmissible viroids are a serious economic threat to citrus production. Viroid diseases can cause stunting, yield loss and even the death of particular scion and rootstock combinations – yet other varieties may be symptomless carriers.

Symptoms will not be seen in nursery trees, they will appear a few years later in the orchard. By this time, the disease is likely to have spread to surrounding trees through the use of cutting tools or potentially by root grafting. Nothing can be done to rid infected trees of viroids; infected trees need to be pulled out and replanted. The only way to be sure of the health of your budwood is to purchase it from a tested source such as Auscitrus.

Eight viroids are known to infect citrus around the world and there are different viroid strains within each of those viroid types. Citrus exocortis viroid, citrus bent leaf viroid, hop stunt viroid, citrus dwarfing viroid, and citrus viroids [redacted] and VII are found in Australia.

Viroids that are known to be most devastating to citrus production are **exocortis** and a strain of hop stunt viroid that causes **cachexia**. Citrus bark cracking viroid is a minor pathogen of citrus.

Exocortis infection has been found to reduce yields by nearly 50% on citrange and 69% on *Citrus* (*Poncirus*) *trifoliata* rootstock during the first 8 years.

Viroid diseases have NO CURE

Prevention is the ONLY OPTION

Be sure to use healthy budwood from Auscitrus

There is no cure for viroid infections; management is through the use of pathogen-free budwood from the Auscitrus propagation scheme. The small cost for using Auscitrus-tested material is negligible when compared to the cost of orchard establishment.

Viroids can be spread via infected buds, on cutting tools (like budding knives, secateurs or hedging machines) and by root grafts between trees in the orchard. The only disinfectant recommended for treating cutting tools is chlorine bleach (1.25% or 12,500 ppm sodium hypochlorite solution). Viroids have been known to survive on cutting tools for 12 months when left untreated.

Sterilise cutting tools with bleach to kill viroids

Prepared by: Nerida Donovan, Grant Chambers, Paul Holford

The strategic levy investment project *Protecting Australian citrus germplasm using improved diagnostic tools* (CT14009) is part of the Hort Innovation Citrus Fund.

Cachexia affects some mandarins, tangelos, kumquats and Rangpur lime. Many citrus species are symptomless hosts. Infection leads to severe stunting and tree decline, with characteristic gumming and pitting.

Healthy (L) and cachexia-infected (R) trees

Symptoms of gumming (L) and pitting (R) on trees infected with cachexia

Exocortis disease symptoms develop when infected budwood is grown on susceptible rootstocks such as trifoliata, citrange, Swingle citrumelo and Rangpur lime. This disease can infect all varieties of citrus but is symptomless in many hosts. Affected trees can exhibit stunting and decline; and bark scaling may be observed on trifoliata rootstocks.

Bark scaling on *Citrus trifoliata* rootstock caused by exocortis infection

Exocortis-infected tree showing stunting (L) and a healthy uninfected tree (R)

Viroid dwarfing: Viroids that induce mild to moderate dwarfing in trees without a negative impact on yield or quality can be used to create high density plantings, and a strain of viroid III that induces mild dwarfing is used commercially in Australia, mainly for oranges. Graft-transmissible dwarfing requires a high level of management. If trees are stressed or poorly managed, the dwarfing effect is increased and could result in undersized trees which do not fill their allotted space, reducing the overall production of the block.