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For Public Release

Part 1 - Summary Details

Please use your TAB key to complete Parts 1 & 2.

CRDC Project Number: **DAQ1405**

Project Title: Surveillance for exotic cotton viruses: multiple targets in and nearby Australia

Project Commencement Date: **1/07/2013** **Project Completion Date:** 30/06/2016

CRDC Research Program: 2 Industry

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Signature of Research Provider Representative: ______________________________

Date Submitted: __________________

Background

1. Outline the background to the project.

Cotton leaf curl disease (CLCuD) presents a major biosecurity threat to the Australian industry. The exotic Cotton blue disease (CBD) is also of concern. The industry has invested in preparedness for CLCuD and CBD through project activities commencing in 2008 including DAQ001, DAQ1201, and DAQ1202. These projects delivered surveillance of Australian cotton crops, a draft contingency plan for each exotic disease and a surveillance strategy for detection of CLCuD through trapping its whitefly vector.

The 2012 workshop on whitefly transmitted viruses (DAQ1202) highlighted a need for ongoing investment in this area and a need for improved testing of imported plant material. The detection of *Bemisia tabaci* Biotype B in northern Australia is a concern given the close proximity with Indonesia. Results from DAQ1202 indicate Indonesia has many different begomoviruses, although the full diversity is unknown. The diversity of these viruses and satellites in other areas north of Australia is also unknown. The detection of an exotic aphid-transmitted virus of vegetables in Kununurra and Darwin in 2011 demonstrates a potential pathway into northern Australia. A structured surveillance system for exotic cotton viruses both pre- and post-border was the major aim of this project, DAQ4105.

Objectives

2. List the project objectives and the extent to which these have been achieved, with reference to the Milestones and Performance indicators.

The project objectives are listed in Table 1. The table also lists the milestones and performance indicators for each objective and provides guidance of potential further research for each objective. Further details on the achievements of these objectives are provided in addition to the table.

Table 1. A list of project objectives, including milestones and their performance indicators. The extent of achievement of each objective is listed, along with indications if further research required.

Obj No.	Objective Description	N O.	Milestone Description	N O.	*Performance Indicator*	*Achievement*	*Further research*
1	Establish a whitefly trapping system at ports of entry.	**1.1**	Obtain permission from state and local government to trap whitefly adjacent to ports of entry	**1.1**	*Permission granted and traps established.*	***Entry pathway re-evaluated***	***Not required***
		1.2	Monitor traps for whitefly and index for begomovirus.	**1.2**	*Traps processed and numbers of whitefly and viruses recorded*	***Not pursued***	***An urban surveillance strategy may be useful***
2	Evaluation of establishment potential for whitefly around post entry quarantine facilities (PEQ)	**2.1**	Evaluate environmental conditions and host range around PEQ facilities in NSW and VIC for suitability for whitefly establishment	**2.1**	*Surrounds of PEQ facilities evaluated and traps established if required.*	***Completed***	***Investigation of private PEQ facilities would be beneficial***
3	Survey of cotton in Ayr and Kununurra	**3.1**	Survey cotton at least twice during the project	**3.1**	*Cotton in both districts surveyed twice and results reported to*	***No crops present during the project***	***Surveillance required if future crops are grown in these areas***

					industry		
4	Northern Australian surveys	**4.1**	To accompany NAQS on two surveys in northern Australia	**4.1**	*Two surveys completed and results reported to industry*	***Multiple surveys completed***	***Cotton disease surveys in collaboration with NAQS recommended every 3-5 years***
5	Survey of Indonesia	**5.1**	To accompany ACIAR team on surveys in Indonesia	**5.1**	*Surveys of Indonesia completed and samples indexed*	***Multiple surveys completed***	***Further surveys of southern Indonesian provinces would be useful, particularly West Papua. Similarly, surveys of commercial cotton on Java and other islands would be beneficial***
6	Survey of Papua New Guinea and/or East Timor	**6.1**	To survey countries to the north of Australia	**6.1**	*Surveys completed and samples indexed*	***Multiple surveys completed***	***Cotton disease surveys in collaboration with NAQS recommended every 3-5 years***
7	Index ornamental imports	**7.1**	Obtain permission from biosecurity agencies to access and test ornamental imports	**7.1**	*Permission granted and samples collected*	***Permission not granted and thus no samples collected or indexed. Import conditions for CLCuD high risk material changed to include indexing***	***Introduction of mandatory indexing of high risk hosts would be beneficial***
		7.2	High risk ornamental imports indexed.	**7.2**	*Samples indexed for exotic viruses*		
8	Transmission of Australian begomoviruses	**8.1**	Test endemic begomoviruses for whitefly transmissibility	**8.1**	*Transmission studies completed*	***Completed***	***Testing of newly introduced begomoviruses is required.***
9	CLCuD contingency plan	**9.1**	New information on CLCuD epidemiology and management obtained.	**9.1**	*Updated draft of the CLCuD contingency plan.*	***Completed***	***Ongoing as new information becomes available***

Milestone 1 - Establish a whitefly trapping system at ports of entry.

Initial investigations were centred on identifying ports of entry considered as high risk for establishment of exotic viruses, particularly begomoviruses and poleroviruses. Ports considered as high risk were those where plant material is landed and have environmental conditions conducive for establishment and spread of silverleaf whitefly or aphids.

Prior to starting a trapping program at ports of entry, the value of such a program was further evaluated through discussions about entry pathways with James Walker, Director Plant Surveillance, Department of Agriculture and Water Resources, in April 2015. It was subsequently considered a low value surveillance strategy and thus not pursued further. It was considered ports of entry were low risk for introduction, establishment and spread of viruliferous whitefly. Instead, there is growing evidence that wind is an active entry pathway for both insects and pathogens into northern Australia. Surveillance efforts in northern Australia in combination with the ongoing crop surveys, is considered a more effective preparedness strategy for CLCuD. Additionally, it is recommended the Cotton Industry approaches the Department of Agriculture and Water Resources to discuss further options to mitigate the risk of co-importation of CLCuD with high risk entry pathways such as nursery stock and the postal service. This could include a structured surveillance strategy for urban areas where there is a very high proportion of ornamental CLCuD hosts and poorly managed whitefly populations.

Milestone 2 - Evaluation of establishment potential for whitefly around post entry quarantine facilities (PEQ)

Since the commencement of this project, the Department of Agriculture and Water Resources consolidated the existing federal government Post Entry Quarantine (PEQ) facilities into a single site at 135 Donnybrook Road, Mickleham, Victoria. This newly opened PEQ facility was inspected in March 2016. The containment level of the facility is high and the risk of insect escape from this facility is negligible. Furthermore, the likelihood of insect populations establishing within the facility is very low. Import conditions on live plants require treatment to remove live insects prior to importation or on arrival and plants are monitored regularly during grow-out periods to maintain an insect-free environment.

Evaluation of PEQ facilities within this project was limited to government run facilities. There are a number of private PEQ facilities in operation in various states. It is recommended future projects on preparedness to insect-vectored viruses of cotton aim to investigate private PEQ facilities as a potential entry pathway.

Milestone 3 - Survey of cotton in Ayr and Kununurra

Surveys of commercial cotton crops in Ayr and Kununurra were not completed as no cotton was planted in either area during the project. A small number of volunteer cotton plants in the Kununurra area were inspected and sampled during a survey in April 2015. No samples showed signs of CLCuD. These plants tested negative for begomovirus by molecular indexing.

Milestone 4 - Northern Australian surveys

A survey of potential begomovirus hosts in public areas and commercial vegetable production areas from Townsville to Bowen, QLD was completed in October 2014. This was done in collaboration with Dr Van Brunschot (CRDC project UQ1305) who collected whitefly samples for identification and virus indexing. Plant samples collected during the survey were molecularly indexed for begomovirus and poleroviruses. No exotic begomoviruses were detected from any of the samples. The endemic begomovirus, *Tomato yellow leaf curl virus* (TYLCV), was detected in two tomato samples from Gumlu. There were three distinct poleroviruses detected from the samples. None of the poleroviruses detected had high similarity to those infecting cotton and cause cotton bunchy top or cotton blue disease.

Plant health surveys were completed in collaboration with the Department of Agriculture and Water Resources, Northern Australian Quarantine Strategy (NAQS) staff in three distinct northern Australian regions between March and May 2015. Surveyed areas included communities around Weipa, Mapoon and Bamaga in QLD, Kununurra, Wyndham and Kalumbaru in WA and Darwin in the NT. Random samples were collected of the known CLCuD malvaceous host, ornamental hibiscus. Additionally, other known CLCuD hosts were sampled if showing any virus symptoms. Whiteflies were also sampled during the surveys.

No begomoviruses were detected from any plant samples from these collaborative plant health surveys. The majority of whiteflies collected were identified as *B. tabaci* AUSII, Australian native whitefly. No *B. tabaci* Middle East Asia Minor 1 (MEAM; B biotype) or *B. tabaci* AUS1 (Australian native whitefly 1) were identified from the samples. The whitefly collected from cucurbit plants at a survey site in Wyndham belong to the *B. tabaci* ASIAII species grouping. Whiteflies with highly divergent mitochondrial cytochrome oxidase 1 gene (COI) sequences were detected at a second Wyndham survey site. It is likely these belong to a previously unidentified *Bemisia* spp.

Additionally, NAQS staff submitted 16 plant samples from other surveillance activities in northern Australia and none of these sampled tested positive for begomoviruses or poleroviruses.

Milestone 5 - Survey of Indonesia

Visits to Indonesia as part of an Australian Centre for International Agricultural Research (ACIAR) funded project entitled the 'Sustainable productivity improvements in allium and solanaceous vegetable crops in Indonesia and sub-tropical Australia (HORT-2009-056)' commenced in 2013 and have continued until February 2016. Multiple plant and whitefly samples were collected during visits.

In summary, through testing both whitefly and symptomatic plant samples, four begomoviruses were detected in Indonesia. These were *Ageratum yellow vein virus* (AYVV), *Tomato leaf curl New Delhi virus* (ToLCNDV), *Squash leaf curl virus* (SqLCV) and *Pepper yellow leaf curl virus* (PYLCV). None of these species are known to cause CLCuD. ToLCNDV was recently reported in association with CLCuD in Pakistan.

The Indonesian collaborators on the ACIAR project have identified regions in Indonesia where cotton is cultivated commercially. This includes areas around Yogyakarta on Java and in areas of Sulawesi which is located between Borneo and the Maluku Islands. Inspections of the crops was not possible during this project, however, it would be beneficial to do in future CLCuD preparedness projects.

Milestone 6 – Survey of Papua New Guinea and/or East Timor

Plant health surveys were conducted in the Papua New Guinea (PNG) lowland and highland provinces in collaboration with staff from the Australian Department of Agriculture and Water Resources, National Agriculture Quarantine Strategy (NAQS), and those from the PNG National Agriculture Quarantine Inspection (NAQI) group. These regions of PNG are mostly subsistence farming, with very small village gardens. The area surveyed ranged in elevation from sea level to 1539 m above sea level. The area experiences a wet and dry season, similar to that of northern Australia.

A range of plant and whitefly samples were collected from PNG during surveys in 2014 and 2016. The species of whitefly detected included *Trialeurodes vaporariorum* (Glasshouse whitefly) and a *Bemisia tabaci* with closet match to AUS I (Australian native whitefly 1) in blastn searchs. No begomoviruses were detected during the 2014 surveys but were detected during 2016 surveys. Preliminary identification of these viruses does not indicate the presence of CLCuD. The sequence fragments obtained so far are most closely related to AYVV and *Tomato leaf curl Java virus* and full-genome sequencing of the viruses in underway in collaboration with NAQS to accurately identify the viruses.

Due to other project commitments it was not possible to accompany NAQS staff on surveys of Timor Leste. Instead, NAQS staff inspected high risk CLCuD hosts and collected plant on behalf of the project. The NAQS staff submitted five plant samples from 2012 surveillance activities in Timor Leste. None of the 2012 samples tested positive for begomoviruses, however, one tested positive for polerovirus. The virus was subsequently identified as a causal agent of Cotton Blue Disease. This detection expands the geographical distribution of this disease and importantly heightens the risk rating for introduction into Australia. A further 19 plant samples from 2015 surveys in Timor Leste and Papua New Guinea were submitted and again no samples tested positive for begomoviruses.

Milestone 7 - Index ornamental imports

A review of quarantine procedures in relation to imported 'medium risk nursery stock (MRNS)' was completed by the Department of Agriculture and Water Resources in August, 2012. Plants categorised as "Medium Risk" are grown in post entry quarantine for a minimum of 3 months and released if showing no signs of diseases or pest infestation. The trigger for the review in quarantine procedures was increasing importation of large volume commercial consignments of nursery stock for direct retail sale. There were two important outcomes of the review in relation to risk of co-importation of exotic viruses affecting cotton. The amendment to import conditions to manage the unpacking and inspection of full container load consignments will lower risk of escape of virus vectors if present in the consignment. The second outcome was the requirement for formalised import notifications detailing the consignment size and where the plants will undergo post entry quarantine (PEQ). The improved knowledge of the volume of imported plants, their source and arrival location will assist in risk evaluation of potential escape and simplify post-incursions if escape occurs. A further outcome was a trial to evaluate the value of a sample-based inspection regime on arriving MRNS genera. This is very useful for mitigating exotic cotton diseases if adopted. The species evaluated in the trial are not known hosts of cotton diseases thus direct results of the trial are not relevant.

Although it was not possible to obtain permission for sampling and indexing potential CLCuD hosts grown in PEQ, it was possible to alter import conditions for a high risk host. The conditions for importation of *Hibiscus* spp. from countries known to have CLCuD now require mandatory testing for this disease, irrespective of the presence of symptoms.

Milestone 8 - Transmission of Australian begomoviruses

Australia has two endemic monopartite begomoviruses present in commercial production areas. These are *Tomato yellow leaf curl virus* (TYLCV) and *Tomato leaf curl virus* (ToLCV). Transmission experiments in this project support previously published research which showed TYLCV can infect cotton. Although the virus infects cotton it doesn't induce disease symptoms. Previous work has shown that ToLCV does not infect cotton. These two viruses are rarely found as co-infections in tomato thus there is a very low likelihood of virus recombination between the two species. The geographic distribution of ToLCV has diminished, possibly through displacement by TYLCV and there is now a reduced overlap in the geographic distribution of the two viruses thus reducing chances of co-infection and potential recombination. There is also a bipartite begomovirus, *Abutilon mosaic virus* (AbMV) widespread in Australia. AbMV is non-transmissible by whitefly and is used by the ornamental industry as a variegated form of Chinese Lantern. Currently, the risk of impact from endemic begomoviruses to cotton production is considered as negligible.

Milestone 9 - CLCuD contingency plan

The draft CLCuD contingency plan was updated to include findings from this project and key research outcomes published in peer-reviewed literature. The plan is available for endorsement by industry and Plant Health Australia. Similarly, the national diagnostic protocol for the detection of CLCuD was reviewed and the final version provided to the Subcommittee of Plant Health Diagnostic Standards (SPHDS) for endorsement.

Methods

3. Detail the methodology and justify the methodology used. Include any discoveries in methods that may benefit other related research.

Survey protocol

Surveys were mostly completed in collaboration with the Northern Australian Quarantine Strategy staff, Department of Agriculture and Water Resources. Surveys in Papua New Guinea (PNG) was also in collaboration with staff from the PNG National Agriculture Quarantine Inspection (NAQI) group Surveys in northern Australia (2015) and PNG (2014 and 2016) consisted of plant inspections of residential backyards and public areas such as roadsides and parks. No commercial crops were inspected. A further survey of north QLD from Townsville to Bowen was conducted in collaboration with Dr Van Brunschot (CRDC project UQ1305), in October 2014.

The survey protocol was to inspect any known or likely CLCuD hosts and subsequently sample those plants with symptoms of begomovirus infection. Plant hosts were also inspected for whitefly and where possible insect collections made. Additionally, random samples of high-risk known hosts of CLCuD, such as *Hibiscus* spp. were collected. Begomovirus infection in *Hibiscus* spp. can produce either no or very mild symptoms, thus random sampling was needed. The number of plants inspected was recorded at each site with GPS co-ordinates so locations can contribute to host distribution mapping.

Molecular indexing of samples

Molecular indexing of plant samples collected during surveys were mostly processed according to the national diagnostic standard (NDS, Appendix 1) using the Accore and Avcore begomovirus generic primers and NADH internal control primers for detection of plant DNA. Some samples were tested using the generic polerovirus primers, Gen1/Gen2, published by Knierim, et al (2013).

Briefly, this included CTAB extraction of total nucleic acids from all *Malvaceae* spp. using a protocol modified from Ghosh et al (2009) and described in the NDS. Extractions for all other plant species used the Biosprint™ kit (Qiagen) as per manufacturer's instructions. The begomovirus PCR conditions are described in Appendix 1. For the generic polerovirus assays, each PCR contained 10 pmol of Gen1 and Gen2, 5 × reaction buffer, 1.75 mM $MgCl_2$, 10 mM dNTPs and 2 units of MangoTaq™ (Bioline). The reactions were incubated at 95 °C for 1 min, then 30 cycles of 95 °C for 20 sec, 50 °C for 20 sec and 72 °C for 20 sec, followed by a final incubation at 72 °C for 5 min

Whiteflies were molecularly indexed for begomovirus and molecular identity. They were extracted as individuals or in composite samples of up to 10-100 individuals. The method is essentially as described by de Barro, P.J. and Driver, F. (1997). Briefly, the insect(s) are macerated in 25 µL of extraction buffer (50 mM KCL, 10 mM Tris-HCL pH 8, 0.45% Tween 20 and 0.45% Triton-X, containing proteinase K added fresh at 1 µL of 10 mg/ml per 25 µL of extraction buffer) and incubated at 65 °C for 30 min then 95 °C for 10 min. After incubation 25 µL of sterile distilled water is added to each extract and 1-2 µL used for PCR. For whitefly identification by PCR, samples were either tested with the *Trialeuroides vaporariorum* specific primers, TV981F/TV1251R (Campbell, this study) or with the generic insect primers for mitochondrial Cox I gene, LCO1490/HCO219 (Folmer et al 1994).

For whitefly identification using the *T. vaporariorum* specific primers, each PCR assay contained 5 pmol of TV981F and TV1251R, 5 × reaction buffer, 1.5 mM $MgCl_2$, 10 mM dNTPs and 2.5 units of MangoTaq™ (Bioline). The reactions were incubated at 95 °C for 1 min, then 35 cycles of 95 °C for 10 sec, 56 °C for 20 sec and 72 °C for 20 sec, followed by a final incubation at 72 °C for 5 min. Samples were considered positive as *T. vaporariorum* if a DNA fragment of 270 bp was amplified. As this was a new protocol, the identity of whiteflies in 14 samples was confirmed by sequencing the TV981F/TV1251R amplified fragments. PCR conditions were essentially the same for whitefly identification using the generic Cox I primers, except each assay contained 25 pmol of LCO1490 and HCO219 and 2.5 mM $MgCl_2$. These reactions were incubated at 95 °C for 1 min, then 35 cycles of 95 °C for 20 sec, 40 °C for 20 sec and 72 °C for 45 sec, followed by a final incubation at 72 °C for 5 min.

Whitefly collected from two sites in Indonesia on the island of Java, were tested by accore PCR for the presence of begomovirus. For each site, 100 whitefly individuals were combined in a sample for extraction and subsequent PCR. The amplicons were then cloned and 100 clones per site evaluated by restriction fragment length polymorphism (RFLP) to evaluate virus diversity. The amplicons were digested with Dde I and EcoRV in the same reaction. RFLP patterns were evaluated by gel electrophoreses and amplicons with representative patterns subsequently sequenced. There were 4-5 unique RFLP patterns observed from the two collection sites; however, subsequent sequencing only identified two begomoviruses. These were *Tomato yellow leaf curl New Delhi Virus* and *Ageratum yellow vein virus*. This technique was deemed too labour intensive for the virus diversity it identified, and other molecular assays are needed for evaluating future survey samples.

References:

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R, 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular marine biology and biotechnology 3, 294-9.

Ghosh, R., S. Paul, S. K. Ghosh, and A. Roy. 2009. "An Improved Method of DNA Isolation Suitable for PCR-Based Detection of Begomoviruses from Jute and Other Mucilaginous Plants." Journal of Virological Methods 159: 34–39.

De Barro PJ, Driver F, 1997. Use of RAPD PCR to distinguish the B biotype from other biotypes of Bemisia tabaci (Gennadius) (Hemiptera:Aleyrodidae). Australian Journal of Entomology 36, 149–52.

Knierim, D., W-S. Tsai, and L. Kenyon. 2013. "Analysis of Sequences from Field Samples Reveals the Presence of the Recently Described Pepper Vein Yellows Virus (Genus Polerovirus)." Archives of Virology 158: 1337–41.

Results

4. Detail and discuss the results for each objective including the statistical analysis of results.

Milestone 1 - Establish a whitefly trapping system at ports of entry.

Initial investigations were centred on identifying ports of entry considered as high risk for establishment of exotic viruses, particularly begomoviruses and poleroviruses. Ports considered as high risk were those where plant material is landed and have environmental conditions conducive for establishment and spread of silverleaf whitefly (SLW) or aphids. For example, certain ports located in QLD, NT and WA.

These investigations discovered a number of Australian ports where imported plant material may generally be landed detailed in the 2014 version of the Quarantine Proclamation 1998 legislation (http://www.comlaw.gov.au/Series/F1998B00158/Compilations). These include:

- Australian Capital Territory : Canberra International Airport; Royal Australian Air Force Base, Fairbairn
- New South Wales: Kingsford Smith Airport, Sydney; Newcastle, Port of Botany Bay, Sydney
- Victoria: Geelong, Melbourne, Tullamarine Airport, Melbourne
- Queensland: **Brisbane**, **Brisbane Airport**, **Cairns**, **Cairns Airport**, **Gladstone**, **Port Alma**, **Townsville**, **Townsville Airport**
- South Australia: Adelaide Airport, Port Adelaide
- Western Australia: **Broome**, **Broome Airport**, Bunbury, **Dampier**, Fremantle, Geraldton, Perth Airport, **Port Hedland**, **Port Hedland Airport**
- Tasmania: Burnie, Devonport, Hobart, Hobart Airport, Launceston, Stanley
- Northern Territory: **Darwin**, **Darwin Airport**
- **Christmas Island** and **Cocos Island** are listed in the March 2014 unincorporated amendment recommendations

Of the above listed ports, those highlighted in bold text are considered a high risk, given their tropical or sub-tropical locations, for the establishment of exotic viruses if co-imported with infected plant material.

In addition to commercial ports where imported plant material is landed, there are also many ports where maritime travellers dock. A comprehensive list of Australian ports and requirements for travellers to report to Customs and Border Protection is available on the 'Maritime Travellers Processing Committee' website (http://mtpc.govspace.gov.au). These ports represent a much lower risk for co-importation of exotic viruses as the volume of plant and/or insect material, if inadvertently introduced, is considerably less than commercial importations.

The value of trapping and testing whitefly at ports of entry was further evaluated through entry pathway discussions with federal quarantine staff. A risk evaluation for CLCuD was conducted in collaboration with James Walker, Director Plant Surveillance, Department of Agriculture and Water Resources, in April 2015. Likely entry pathways for any pest or pathogen were divided into seven broad categories and the likelihood of CLCuD entering via these pathways either in live adult whitefly or host plant material was evaluated (Table 1). High risk pathways identified were transfer of viruliferous whitefly via wind and infected plant material entering through the largely unregulated postal service or as nursery stock. By contrast, it was considered ports of entry were low risk for introduction, establishment and spread of viruliferous whitefly. Additionally, there is growing evidence that wind is an active entry pathway for both insects and pathogens into northern Australia.

Table 1 Risk evaluation for entry of Cotton leaf curl disease (CLCuD) via live viruliferous adult whitefly or infected host plant material for the different major pathways.

Category	Whitefly	Plant Material	Comments
Wind	**high**	unlikely	cyclones – very relevant to northern areas
Postal service	unlikely	**high**	unregulated parcels – largely unscreened
Airport baggage	unlikely	unlikely	low volume –some screening of baggage for plant material

Nursery stock	unlikely	**high**	usually fumigated, nymphs don't transmit CLCuD
Fresh fruit and vegetables	unlikely	unlikely	disinfestation treatments on most commodities & very low volume infected plant material would entry this way
Seed	n/a	very unlikely	begomoviruses not known to be seed transmitted[1]
Vessels	unlikely	unlikely	very low volume of plant material entry on vessels

[1] the research published by (Kil et al. 2016) reports seed transmission of the begomovirus *Tomato yellow leaf curl virus*, however, this requires verification.

Following this evaluation, the trapping and indexing of whitefly at ports of entry was considered a low value survey option for early detection of CLCuD. Instead, surveys of northern Australia in combination with ongoing crop surveys are considered more cost effective. Additionally, it is recommended the Cotton Industry approaches the Department of Agriculture and Water Resources to discuss further options to mitigate the risk of co-importation of CLCuD with high risk entry pathways such as nursery stock and the postal service.

Reference: Kil, Eui-Joon, Sunhoo Kim, Ye-Ji Lee, Hee-Seong Byun, Jungho Park, Haneul Seo, Chang-Seok Kim, et al. 2016. "Tomato Yellow Leaf Curl Virus (TYLCV-IL): A Seed-Transmissible Geminivirus in Tomatoes." Scientific Reports 6 (January). doi:10.1038/srep19013.

Milestone 2 - Evaluation of establishment potential for whitefly around post entry quarantine facilities (PEQ)

Since the commencement of this project, the federal Department of Agriculture and Water Resources consolidated the existing government Post Entry Quarantine (PEQ) facilities into a single site at 135 Donnybrook Road, Mickleham, VIC. Some operations commenced at the facility as early as 2015 and the facility will be fully operational by 2018. The facility includes a plant diagnostic laboratory.

As the facility located at Knoxfield in Victoria was still operational in 2015, an inspection was completed. This facility is no longer open and all operations were transferred to the new facility at Mickleham. This newly opened PEQ facility was inspected in March 2016. The containment level of the facility is high and the risk of insect escape from this facility is negligible. Furthermore, the likelihood of insect populations establishing within the facility is very low. Import conditions on live plants require treatment to remove live insects prior to importation or on arrival and plants are monitored regularly during grow-out periods to maintain an insect-free environment. The PEQ in NSW is also no longer in operation.

Evaluation of PEQ facilities within this project was limited to government run facilities. There are a number of private PEQ facilities in operation in various states. It is recommended that future projects on preparedness to insect-vectored viruses of cotton aim to investigate private PEQ facilities as a potential entry pathway.

Milestone 3 - Survey of cotton in Ayr and Kununurra

Surveys of commercial cotton crops in Ayr and Kununurra were not completed as no cotton was planted in either area during the project. A small number of volunteer cotton plants in the Kununurra area were inspected and sampled during a survey in April 2015. No samples showed signs of CLCuD. These plants tested negative for begomovirus by molecular indexing. Further details on these samples are captured in Milestone 4 results.

Milestone 4 - Northern Australian surveys

2014

The survey of potential begomovirus hosts from Townsville to Bowen in QLD, was completed in October 2014 (Figure 1). A total of 192 individual plants were sampled and indexed molecularly. Many samples were combined in groups of up to five individuals into composite samples (i.e. bulked) for indexing. Appendix 2 lists the plant samples collected during this survey, their symptoms, location of collection, bulk rate and indexing results.

Figure 1. Map of sites inspected as part of plant virus surveys in October 2014.

No exotic begomoviruses were detected from any of the samples. *Tomato yellow leaf curl virus* (TYLCV) was detected in two tomato samples from Gumlu. There were three distinct poleroviruses detected from the samples. None of the poleroviruses detected had high similarity to the known cotton infecting-viruses that cause cotton bunchy top or cotton blue disease.

2015 surveys

Plant health surveys were completed in three distinct northern Australian regions between March and May 2015. Surveyed areas included communities around Weipa, Mapoon and Bamaga in QLD, Kununurra, Wyndham and Kalumbaru in WA and Darwin in the NT (Figure 2). Random samples were collected of the known CLCuD malvaceous host, ornamental hibiscus. Additionally, other known CLCuD hosts were sampled if showing any virus symptoms. Whitefly was also sampled during the surveys. Details of the plant and whitefly samples collected and the localities of the survey sites are provided in Appendix 2.

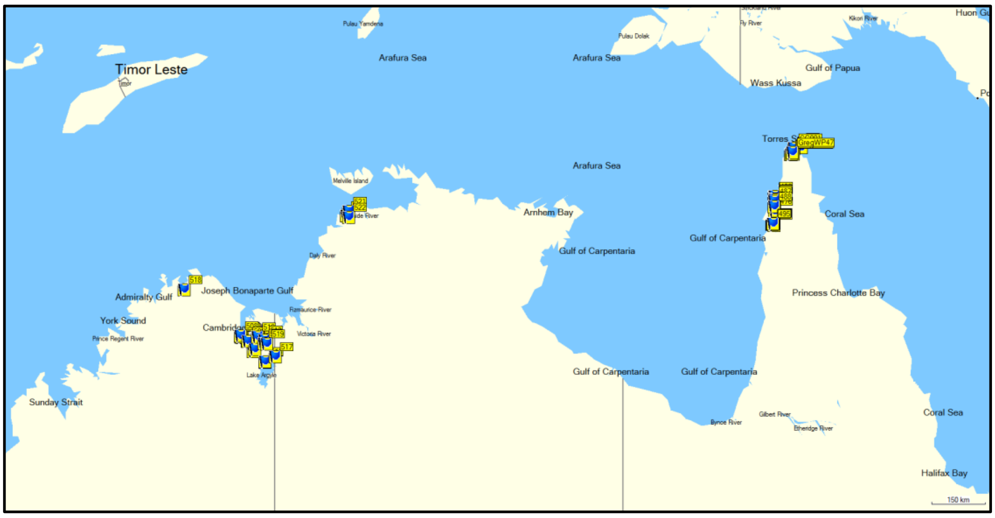

Figure 2. Map of sites inspected as part of plant health surveys from March to May 2015.

Surveys were of residential backyards and public areas. No commercial crops were inspected. The major CLCuD host detected was ornamental hibiscus and is present in most places. A selection of random samples was collected of this host and tested. In most instances, five individual plants were bulked into a single composite sample for indexing. The number of ornamental hibiscus sampled was 146 individuals from a total of 339 individuals inspected. Other malvaceous plant species were detected, and with the exception of *Abelmoschus esculentus* (okra) which is a host of CLCuD, the susceptibility of these species to CLCuD is largely unknown. A total of 739 individual plants of these potential alternative CLCuD hosts were inspected. Some samples of these hosts were collected and will be stored for future testing if required.

Additionally, cucurbits and solanaceous species were surveyed and samples were collected of plants that showed symptoms of virus infection. In total, there were five symptomatic cucurbit plants sampled from the 114 individual plants inspected. Although the symptoms in these plants are similar to begomovirus infection, they are also similar to those caused by the ubiquitously distributed cucurbit potyviruses. Three chilli plants showed signs of subtle cupping. Again this can be a symptom of begomovirus infection. Alternatively, a range of insect and physiological disorders can also cause these symptoms. In total, 145 individual solanaceous plants were inspected, of which 11 were tomato. The remaining were *Capsicum* species, mostly chilli, with capsicum only occasionally found.

Whitefly populations were detected and sampled mostly from cucurbit host plants and weed species. The details of the host plants sampled and the survey sites are provided in Appendix 2. There were no symptoms of begomovirus infections on any of these whitefly host plants.

In summary, a total of 598 individual plants of species known to be hosts of CLCuD were inspected during these surveys of northern Australia. This included 339 ornamental hibiscus, 114 cucurbit and 145 solanaceous plants. Of these, 215 plant samples were collected and molecularly indexed for begomoviruses. No begomoviruses were detected from any plant samples. Additionally, 14 whitefly samples were collected. These were molecularly identified by Dr Van Brunschot CRDC project UQ1305). The majority of whiteflies collected were identified as *B. tabaci* AUSII, Australian native whitefly. No *B. tabaci* Middle East Asia Minor 1 (MEAM; B biotype) or *B. tabaci* AUS1 (Australian native whitefly 1) were identified from the samples. The whitefly collected from cucurbit plants at a survey site in Wyndham belong to the *B. tabaci* ASIAII species grouping. Whiteflies with highly divergent mitochondrial cytochrome oxidase 1 gene (COI) sequences were detected at a second Wyndham survey site. It is likely these belong to a previously unidentified *Bemisia* spp.

The Department of Agriculture and Water Resources, Northern Australian Quarantine Service (NAQS) has collaborated to collect samples on behalf of the project. Samples of plants were collected during their surveys of northern Australia and forwarded to DAF for molecular begomovirus indexing. In total, there were 16 samples provided and all tested negative for begomovirus. Details of sample collections are provided in Appendix 2.

Visits to Indonesia as part of an Australian Centre for International Agricultural Research (ACIAR) funded project entitled the 'Sustainable productivity improvements in allium and solanaceous vegetable crops in Indonesia and sub-tropical Australia (HORT-2009-056)' commenced in 2013 and have continued until February 2016. Multiple plant and whitefly samples were collected during visits.

Experiments to identify virus species present within individual whitefly and to identify the species and/or biotype of the whitefly were completed. Indexing of the samples was either through the use of generic begomovirus PCR with subsequent cloning and sequencing of the amplified fragments. From this experiment two viruses were detected, *Ageratum yellow vein virus* (AYVV) and *Tomato leaf curl New Delhi virus* (ToLCNDV). This technique was not continued as the ratio of time input to outcomes generated for measuring virus diversity was not favourable.

Analyses of plant samples collected from Java identified a third begomovirus, *Pepper yellow leaf curl virus* (PYLCV). In total, eight symptomatic chilli plants and 20 symptomatic cucurbit plants were sampled in Indonesia. The chilli plants were infected with PYLCV and the cucurbits with ToLCNDV.

Whitefly were collected from a further six sites in Java during 2015. The samples were tested using the specific primers for *T. vaporariorum* and only those collected from the two sites at the Indonesian Vegetable Research Institute in Lembang were identified as this species. Lembang is approximately 1230 m above sea level and quite cool. This is most likely why *T. vaporariorum* was found in this location. *B. tabaci* was found at three survey sites, all at low altitude and *Aleurotrachelus trachoides* was found at the remaining site. This species of whitefly is not a known virus vector but is listed as an emerging pest risk by the European and Mediterranean Plant Protection Organization (EPPO). Begomoviruses were also amplified from the whitefly samples and identified as *Squash leaf curl virus* (SqLCV) and ToLCNDV.

In summary, through testing both whitefly and symptomatic plant samples, four begomoviruses were detected in Indonesia. Most of these species are not known to cause CLCuD. The exception is ToLCNDV which was recently reported in association with CLCuD in Pakistan (Zaidi et al 2016).

The Indonesian collaborators on the ACIAR project have identified regions in Indonesia where cotton is cultivated commercially. This includes areas around Yogyakarta on Java and in areas of Sulawesi which is located between Borneo and the Maluku Islands. Inspections of the crops was not possible during this project, however, it would be beneficial to do in future CLCuD preparedness projects.

Reference: Zaidi, Syed Shan-e-Ali, Muhammad Shafiq, Imran Amin, Brian E. Scheffler, Jodi A. Scheffler, Rob W. Briddon, and Shahid Mansoor. 2016. "Frequent Occurrence of Tomato Leaf Curl New Delhi Virus in Cotton Leaf Curl Disease Affected Cotton in Pakistan." PLoS ONE 11 (5). doi:10.1371/journal.pone.0155520.

Milestone 6 – Survey of Papua New Guinea and/or East Timor

Plant health surveys were conducted in the PNG lowland and highland provinces in collaboration with staff from the Australian Department of Agriculture and Water Resources, National Agriculture Quarantine Strategy (NAQS) and those from the PNG National Agriculture Quarantine Inspection (NAQI) group. The highland surveys were conducted from the 26th of May to the 11th of June, 2014 and those in the lowlands from the 17th to the 30th of April, 2016. This region of PNG is mostly subsistence farming, with very small village gardens. The area surveyed ranged in elevation from sea level to 1539 m above sea level. The area experiences a wet and dry season, similar to that of northern Australia. A geographical representation of the survey sites is shown in Figure 3.

(a)

(b)

Figure 3. Map showing the location of sites surveyed in Papua New Guinea during 2014 (a) and 2016 (b)

Cotton leaf curl disease (CLCuD) plant host species were observed in most areas visited. A list of plants including the number of individuals inspected and the number of inspection sites is provided in Appendix 3. This list is limited to those plants inspected for virus symptoms and doesn't represent the abundance of the plants in this region. Of these species, ornamental hibiscus was the most commonly encountered and was present in multiple locations including in most gardens surveyed and along roadside properties. Cucurbit, tomato and chilli plants were also observed in many gardens inspected, with pumpkin the most commonly grown cucurbit.

The most common *Malvaceae* species observed was *Abelmoschus manihot* (aibieka) which is commonly grown as a leaf vegetable and is vegetatively propagated. No symptoms of begomovirus infection were observed in these plant species and in general these plants appeared healthy. Occasional plants showed some symptoms of potential virus-infection but the symptoms were not consistent with begomovirus infection. In addition to these plants, the two weed species, *Ageratum conyzoides* and *Stachytarpheta australis*, showed symptoms of a potential virus infection. Both plant species were confirmed infected with either *Ageratum yellow vein virus* and/or *Tomato leaf curl Java virus*. These two viruses are not known to cause CLCuD. Infected plants were observed in many locations and samples collected for virus indexing. None of the remaining plants observed were symptomatic but representative samples were collected to test for latent virus infections.

Cotton plants were not commonly grown garden plants in the survey areas. Individual plants were, however, detected at six survey sites. The species were putatively identified as *Gossypium barbadense* and representative samples are undergoing confirmatory identification by NAQS botanists. No virus symptoms were observed on the plants. Samples were collected from each plant to monitor for asymptomatic virus infections of either CLCuD or Cotton blue disease (CBD).

Where possible, whitefly populations were also sampled during the surveys. The details of the whitefly collections are provided in Appendix 3. Samples from 2014 surveys were molecularly indexed and mostly identified as *Trialeurodes vaporariorum* (Glasshouse whitefly). Some populations were a mix with *Bemisia tabaci* AUS I (Australian Native Whitefly 1). These populations were collected from a range of host plant species grown at altitudes at least 1400 m above sea level. Of the 2016 whitefly collections tested, *T. vaporariorum* was detected only at one site of similar high altitude. The identities of the remaining collections require verification, however, given the widespread detection of a begomovirus in the survey areas it is likely *B. tabaci* MEAM 1 (Biotype B, silverleaf whitefly) will also be detected. The identifications of these collections will be completed in collaboration with the Department of Agriculture and Water Resources, Northern Australian Quarantine Service (NAQS) group and published in a peer-reviewed paper.

The NAQS staff collaborated to collect samples on behalf of the project. Samples of plants were collected during their surveys to Timor Leste and Papua New Guinea and forwarded to DAF. The samples were processed for begomovirus by PCR and all 24 samples tested negative for begomovirus. Details of sample collections are provided in Appendix 3. Samples were also tested for CBD, in collaboration with Mr Murray Sharman under project DAQ1201. The most significant result of testing these samples was the detection of a polerovirus species in a Timor Leste sample. The polerovirus has high similarity to a species causing CBD in Brazil and represents the closest detection of this virus to Australia. The virus was detected in a single sample from a *Gossypium barbadense* plant which displayed no apparent disease symptoms at the time of sampling. Further detail on this detection and implications for the cotton industry is provided in reporting through project DAQ1201.

Milestone 7 - Index ornamental imports

A review of quarantine procedures in relation to imported ‘medium risk nursery stock (MRNS)’ was completed by the then Department of Agriculture and Water Resources in August 2012 (http://www.daff.gov.au/biosecurity/import/plants-grains-hort/review_of_nursery_stock_importation_conditions). Plants categorised as “Medium Risk” are grown in post entry quarantine for a minimum of 3 months and released if showing no signs of diseases or pest infestation. The trigger for the review in quarantine procedures was increasing importation of large volume commercial consignments of nursery stock for direct retail sale. The Department reports about 75% of MRNS are in consignments in excess of 10,000 plants, 22% are in consignments of 50,000 plants and only 2% of imported nursery stock is imported in consignments of less than 1000 plants. Most of the species imported in the medium risk category are ornamentals such as orchids, bromeliads, yuccas and anthuriums.

Important outcomes of the review in relation to risk of co-importation of exotic viruses affecting cotton include:

- Amendments to import conditions to “manage insect risk by moving Full Container Load consignments (if correctly certified) to the appropriate quarantine approved premises prior to unpacking and inspection” – this will lower risk of escape of virus vectors if present in the consignment
- “A formalised Import Notification process has been developed for MRNS. This requires a Notice of Importation (NOI) form to be lodged with the relevant regional office detailing the consignments size and the Quarantine Approved Premises where plants will undergo post entry quarantine.” – knowing how much of a virus host plant was imported, where it came from and where it undergoes quarantine processing will assist in risk evaluation of potential escape and simplify post-incursion responses if escape occurs
- “A trial is currently being undertaken of a sample based inspection regime for on arrival inspections of selected MRNS genera. The following genera were selected for the trial as data

analysis has shown they have a low rate of pest and disease interceptions: *Phalaenopsis* spp. (orchid), *Dendrobium* spp. (orchid), *Sansevieria* spp. (mother-in-laws tongue), and *Gymnocalycium* spp. (cacti) grafted to *Hylocereus* spp." – the trial has potential benefit for evaluating the system, however, no detail is provided on what pests and diseases they will be inspecting for. None of the hosts listed are of relevance to the cotton industry.

Discussions were held in July, 2014 at the PEQ facility in Knoxfield, Victoria to obtain access permission for testing imported ornamental plants. The discussions were between the project leader and Dr Mark Whattam, Director of Operational Science Support (South East Region), Department of Agriculture and Water Resources. The major outcome from these discussions was a change to the import conditions for *Hibiscus* spp. entering the country as nursery stock. Previously, the import conditions consisted of a minimum period of 3 months growth in a PEQ with inspection for virus symptoms. As these species can be asymptomatic hosts of CLCuD it was important to alter these import conditions. The new conditions are stated below:

> Condition C20106
>
> **Phytosanitary certificate requirement**
>
> Goods must be accompanied by an official government Phytosanitary certificate from the country of origin of the nursery stock and/or tissue cultures, and be endorsed with the following additional declaration:
>
> "Plants were grown in [insert country of origin] which is free from Cotton leaf curl disease"
>
> OR
>
> Plants from countries known to have Cotton leaf curl disease must be endorsed with the following additional declaration:
>
> "Plants were tested negative for Cotton leaf curl disease using Polymerase Chain Reaction (PCR)."
>
> Consignments without an appropriate Phytosanitary certificate will be tested for Cotton leaf curl disease in a Government Post Entry Quarantine facility with PCR, re-exported or destroyed at the importer's expense.
>
> The following countries are known to have Cotton leaf curl disease: **Pakistan, India, China, Egypt, Sudan, Nigeria and Tanzania.**
>
> **Note:** The above condition is in addition to all other conditions for nursery stock and tissue cultures.

To obtain this change to import conditions a peer-reviewed publication reporting *Hibiscus* spp. as a host of CLCuD was required. It wasn't possible to negotiate a general permission to access and test any ornamental species suspected to be a host of the disease.

Milestone 8 - Transmission of Australian begomoviruses

A whitefly transmission experiment was conducted to determine if the endemic begomovirus *Tomato yellow leaf curl virus* (TYLCV) can infect Australian cotton plants. *Bemisia tabaci,* biotype B (Silverleaf whitefly) individuals were fed on a tomato plant infected with a known isolate of TYLCV for 48 h. The plant plus insects were then transferred to a large insect-proof cage containing virus-free test tomato and cotton plants. The whitefly colony was allowed to establish in the cage, and test plants exposed to the viruliferous insects for approximately 2 months. After this time the plants were rated for symptoms, sampled and indexed for TYLCV by a specific PCR assay (Van Brunschot et al. 2010). All 19 test tomato plants tested positive for TYLCV and varied in symptoms from none, through to severe. By contrast, no cotton plants showed symptoms of infection although 17 of the 25 test plants were confirmed infected with TYLCV by PCR. The cotton variety used in the experiment was Sicot 71. This demonstrates that cotton could support recombination between TYLCV and newly introduced begomoviruses, provided they too can infect cotton. Similarly, other host species of TYLCV could support recombination with newly introduced viruses if co-infection occurs in those hosts.

Tomato leaf curl virus (ToLCV) is also a begomovirus known to affect field crops in Australia (Stonor et al. 2003). A host range study indicated this virus infects very few plant species outside the *Solanaceae* family. Cotton (variety DP90) was included in the study and did not support ToLCV replication (Stonor et al. 2003). Although only a single variety of cotton was studied, the limited host range of this virus outside *Solanaceae* indicates cotton crops are at very low risk of infection or disease from this virus. Additionally, cotton would not provide a recombination opportunity for this virus with other begomovirus species.

These two viruses are rarely found as co-infections in tomato thus there is a very low likelihood of virus recombination between the two species. The geographic distribution of ToLCV has diminished, possibly through displacement by TYLCV and there is now a reduced overlap in the geographic distribution of the two viruses thus reducing chances of co-infection and potential recombination.

There is also a bipartite begomovirus, *Abutilon mosaic virus* (AbMV) widespread in Australia. AbMV is non-transmissible by whitefly and is used by the ornamental industry as a variegated form of Chinese Lantern. Currently, the risk of impact from endemic begomoviruses to cotton production is considered as negligible.

Outcomes

5. Describe how the project's outputs will contribute to the planned outcomes identified in the project application. Describe the planned outcomes achieved to date.

The project application listed seven expected outputs. These were:

1. *Molecular indexing of ornamental imports for potential exotic viruses.*

This output will contribute to an applied industry outcome of increased preparedness to CLCuD and contribute to science outcomes through improved knowledge on the presence and diversity of begomoviruses and DNA betasatellites in imported ornamental species. This output was not achievable due to restrictions in access to the material for testing. Instead, import conditions were changed to ensure high risk ornamental hosts such as *Hibiscus* spp. from countries known to have CLCuD were molecularly tested. This molecular indexing will be completed by the PEQ facility or sent to a recognised diagnostic facility. This change to import conditions has significantly contributed to the industry outcome of increased preparedness and will contribute to science outcomes as material is tested.

2. *Survey of areas to the north of Australia for CLCuD*

This output will contribute to an applied industry outcome of increased preparedness to CLCuD and contribute to science outcomes through improved knowledge on the diversity of begomoviruses and DNA betasatellites in these areas. Knowledge on this diversity close to Australia will increase accuracy of risk evaluations for introduction of CLCuD via northern Australia. Results from surveys of Timor Leste and Papua New Guinea have identified previously begomoviruses not known to occur in these areas. Importantly, a begomovirus associated with CLCuD in Pakistan was detected in Indonesia. Additionally, the presence of Cotton blue disease was confirmed in Timor Leste. These results provide strong evidence that there is a risk of transfer of exotic viruses.

3. *Maps showing the distribution of known and potential hosts of CLCuD in northern Australia. Survey of hosts for CLCuD.*

This output will contribute to the applied industry outcome of enhanced knowledge on potential establishment sites for CLCuD if it enters Australia from areas to the north. The subsequent improved knowledge on the distribution of potential and known alternative hosts of CLCuD in northern Australia and within horticulture production areas, predominantly in QLD is an important scientific outcome. This project has generated maps of potential CLCuD hosts detected during surveys. However, more comprehensive maps are available through NAQS and are periodically updated with new host location data generated during their routine survey activities.

4. *Report on whitefly intercepts at entry points into Australia including the virus status of the whiteflies. A map demonstrating geographical location of these intercepts relative to potential CLCuD establishment sites.*

This output was not completed as described. Instead an evaluation of this type of surveillance in relation to potential high risk pathways for CLCuD was completed. This evaluation contributed to the expected applied industry outcome which was to better evaluate the risk posed from different entry points and found the trapping and indexing whitefly at ports of entry is a low value survey option for early detection of CLCuD. Instead, surveys of northern Australia in combination with ongoing crop surveys are more cost effective. Additionally, it is recommended the Cotton Industry approaches the Department of Agriculture and Water Resources to discuss further options to mitigate the risk of co-importation of CLCuD with high risk entry pathways such as nursery stock and the postal service.

5. *Report on the diversity of whitefly species and biotypes. A map demonstrating the presence of potential CLCuD vectors in Australia and close by.*

This output contributes to the applied industry outcome of improved evaluation of the risk posed from CLCuD through enhanced knowledge on vector biology. It also has the scientific outcome of improved knowledge on the diversity of whiteflies in Australia and our northern neighbours. The report shows the presence of CLCuD vectors in northern Australia, Timor Leste and Papua New Guinea.

6. *Report on the transmissibility of endemic begomoviruses*

This output contributes to the applied industry outcome of improved knowledge on the likelihood of endemic begomoviruses impacting production. It also has the scientific outcome of improved knowledge of virus transmissibility, particularly if present in mixed infections. Cotton was shown to be an asymptomatic host of one endemic begomovirus, *Tomato yellow leaf curl virus*. Previous work showed that a second endemic begomovirus, *Tomato leaf curl virus* (ToLCV) did not infect cotton. These two viruses are rarely if ever found as mixed infections. Currently the risk of impact from endemic begomoviruses to cotton production is negligible.

7. *An updated draft of the CLCuD contingency plan.*

This output provides the expected applied industry outcome of having a contemporary contingency plan for CLCuD and a scientific outcome of improved understanding on the epidemiology of CLCuD and management/containment of whitefly-transmitted viruses. The current draft of the contingency plan is available for consideration by industry and Plant Health Australia.

6. Please describe any:-

a) technical advances achieved (eg commercially significant developments, patents applied for or granted licenses, etc.);
b) other information developed from research (eg discoveries in methodology, equipment design, etc.); and
c) required changes to the Intellectual Property register.

Improved methodologies for identification of whitefly species was developed within this project. This was the developed of a PCR assay to detect *Trialeuroides vaporariorum* (Glasshouse whitefly) which assists in differentiating whitefly species in survey samples.

Conclusion

7. Provide an assessment of the likely impact of the results and conclusions of the research project for the cotton industry. What are the take home messages?

This project has assisted in further preparedness to CLCuD and other exotic insect-transmitted viruses, such as Cotton blue disease. The main impacts of the project include:

1. *CLCuD is not known to occur in Australia*

Surveys conducted within this project in northern WA, NT and QLD have not detected causal agents of CLCuD nor any new begomoviruses. This in combination with the annual disease surveys conducted through other CRDC-funded projects and from industry crop monitors gives high confidence the disease is not present.

2. *Greater evaluation of risk for introduction of CLCuD from areas to the north of Australia.*

Begomoviruses and their whitefly vectors in Papua New Guinea, Timor Leste and Indonesia confirm there is a risk of introduction of these pests into Australia. Prior to these detections it was assumed there was an entry pathway via these countries, however, the presence of begomoviruses and whitefly vector species in those areas was largely unknown. Although CLCuD was not detected in these surveys, only a small proportion of the countries were inspected. Further surveys and sampling is needed to provide confidence in the presence or absence of the disease in this area. A begomovirus associated with CLCuD in Pakistan was detected in Indonesia.

3. *A strong relationship developed with federal government biosecurity agencies*

Collaborative surveys for exotic begomoviruses and whitefly vectors with NAQS resulted in increased awareness by this group on the importance of these pests for the cotton industry. Since the commencement of the project NAQS has actively increased surveillance for these pests, have provided input into the Cotton Industry Biosecurity Plan and assisted in evaluation of potential entry pathways. Additionally, communication with the Director of the Operational Science Program for the South East Region for the Department of Agriculture and Water Resources has resulted in improved risk mitigation for importation of high risk ornamental hosts of CLCuD such as hibiscus.

Extension Opportunities

8. Detail a plan for the activities or other steps that may be taken:
(a) to further develop or to exploit the project technology.
(b) for the future presentation and dissemination of the project outcomes.
(c) for future research.

The detection of Cotton blue disease in Timor Leste was previously reported through industry communications "Ray, J., Gambley, C, Sharman, M. and Maas, S. (April 2014). Significant virus detection in East Timor: Cotton leaf roll dwarf virus (the causal agent of Cotton blue disease). www.mybmp.com.au".

A presentation summarising preparedness to date for CLCUD was delivered at an industry forum (Fuscom) held in Toowoomba in April 2016.

A presentation outline the risks posed to Australian agriculture production by exotic whitefly transmitted viruses was delivered at a Plant Health Australia workshop held in Brisbane in August 2016. The presentation and subsequent discussions at the workshop was aimed at developing a cross-industry project for improved preparedness to exotic whitefly and whitefly-transmitted viruses.

9. A. List the publications arising from the research project and/or a publication plan. (NB: Where possible, please provide a copy of any publication/s)

First report of Cotton blue disease in Timor Leste

B. Have you developed any online resources and what is the website address?

The contingency plan for Cotton leaf curl disease will be available through the Plant Health Australia website once endorsed by them and industry.

Part 4 – Final Report Executive Summary

Cotton leaf curl disease (CLCuD) presents a major biosecurity threat to the Australian industry. The exotic Cotton blue disease (CBD) is also of concern. The industry has invested in preparedness for CLCuD and CBD through project activities commencing in 2008. These projects delivered surveillance of Australian cotton crops, a draft contingency plan for each exotic disease and a surveillance strategy for detection of CLCuD through trapping its whitefly vector. To further prepared the industry for this disease a structured surveillance system for exotic cotton viruses both pre and post border was developed and trialled. This included evaluating entry and establishment of viruliferous whitefly at ports of entry and post-entry quarantine facilities. It also included pre- and post-border surveys in northern areas to map whitefly and high risk CLCuD host distributions, in addition to looking for early detection of the disease if present.

Discussions about entry pathways with the Department of Agriculture and Water Resources (DAWR), identified ports of entry as a low risk for introduction, establishment and spread of viruliferous whitefly. Instead, there is growing evidence that wind is an active entry pathway for both insects and pathogens into northern Australia. Surveillance efforts in northern Australia in combination with the ongoing crop surveys, was considered a more effective preparedness strategy for CLCuD. Risk of virus escape from the federal government Post Entry Quarantine (PEQ) facility at Mickleham, Victoria was also evaluated and deemed negligible. However, the security of private PEQ facilities in operation in various states is unknown and it is recommended future projects on preparedness to insect-vectored viruses of cotton aim to evaluate the risk of these facilities.

Post-border surveillance included surveys of natural and urban areas of northern Australia in collaboration with the Northern Australian Quarantine Service (NAQS, DAWR). Multiple areas in northern WA, NT and QLD were completed. No evidence was obtained for the presence of CLCuD in any of these areas. All plant and whitefly samples collected during surveys tested negative for CLCuD. For the pre-border strategy, surveys were completed in areas to the north of Australia. This included Indonesia and collaborative surveys with NAQS in Papua New Guinea. Additionally, NAQS staff submitted a range of plant samples collected on other surveys in PNG, Timor Leste, Torres Strait Islands and northern Australia. No evidence for the presence of CLCuD in any of these areas was obtained. All plant samples collected during surveys tested negative for CLCuD. Evidence for the exotic CBD was obtained through indexing plant samples submitted by NAQS from Timor Leste. This is an important detection as it represents the closest detection of this disease to Australian cotton production areas. Additionally, the detection of begomoviruses in Papua New Guinea, Timor Leste and Indonesia, including at least one specific causal agents of CLCuD, highlight the importance of ongoing surveillance in these areas.

To mitigate the risk of potential introduction of exotic begomoviruses through imported ornamental material, permission was sought to access and test the material prior to its release from PEQ. Normal import requirements are limited to a very short grow out period, with visual inspection only, prior to release. Symptoms of CLCuD are transient in some hosts thus there is a very high risk infected material could go undetected. Although it was not possible to obtain permission for sampling and indexing potential CLCuD hosts grown in PEQ, it was possible to alter import conditions for a high risk host. The conditions for importation of *Hibiscus* spp. from countries known to have CLCuD now require mandatory testing for this disease, irrespective of the presence of symptoms. Finally, a new draft of CLCuD contingency plan is prepared and available for consideration by industry and Plant Health Australia.

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Appendices

Appendix 1 – Excerpt from the National Diagnostic Standard for the detection of CLCuD

Total nucleic acid extraction (TNAE) method

Cotton and other malvaceous species contain a high amount of foliage mucilage which is comprised of exopolysaccharides. This material is highly viscous and complicates the extraction of DNA for molecular indexing. The high viscosity hampers pipetting and reduces or prevents precipitation of DNA. The mucilage also binds to other metabolites such as phenolics, tannins and alkaloids and co-precipitation of these metabolites often results in PCR inhibition or low yields of precipitated DNA can cause false negative indexing results.

PCR amplifiable DNA was not reliably extracted from cotton and other malvaceous species using some commercial extraction methods such as magnetic beads (Qiagen Biosprint kit) and Concert RNA reagent (Invitrogen). By contrast, a modified version of the method published by Ghosh *et al.* (2009) reliably produced high quality PCR-amplifiable DNA from these plant species.

Procedure

Total nucleic acid extracts (TNAE) is prepared as per the method of Ghosh *et al.* (2009) with some modifications.

Preparation

a. Aliquot 10 mL of CTAB extraction buffer into separate 15 mL falcon tubes and place in a waterbath at 65°C.

b. 2 mL tubes each containing 600 µL of chloroform:isoamyl alcohol (24:1).

c. 2 mL tubes each containing 600 µL of room temperature isopropanol.

d. Record sample numbers on the TNAE record sheet.

Extraction

a. Keep the 100 mg fresh samples on ice until required for the following grinding step. If using desiccated material, weigh out 10 mg of tissue.

b. Macerate the plant tissue using a mortar and pestle with a small aliquot (200-500 µL) of the CTAB extraction buffer. As sample material is likely to be very viscous only add a small amount of buffer initially to macerate the sample and then add this to the remaining volume of CTAB extraction buffer in the falcon tube.

c. Invert the falcon tube 5-10 times to ensure the extract is well mixed and incubate for 30 min at 65°C in the waterbath. Repeat the inversion 2-3 times during the incubation period.

d. Aliquot 1 mL of extract into the 2 mL tubes containing the chloroform:IAA and mix by inversion for approximately 5 min at room temperature (prepare two tubes per extract). (Note: These 15 mL tubes can be stored frozen to repeat this section in case of pellet loss.)

e. Centrifuge tubes at about 14 000 RPM for 10 min using a microfuge.

f. Aliquot the upper aqueous phase (approximately 1 mL) into the 2 mL tubes containing the isopropanol and mix by inversion (prepare two tubes per extract).

g. Centrifuge tubes at about 14 000 RPM for 10 min using a microfuge.

h. Decant isopropanol and wash pellet with 500 µL of 70% ethanol. Take care not to dislodge or lose the pellet, caution is required at this step. Suggest pipetting off the ethanol as this can reduce the risk of dislodging the pellet.

i. Air-dry or vacuum-dry pellets.

j. Resuspend each pellet in 50 µL of Milli-Q® water. To fully resuspend the TNA pipette the pellet up and down several times both before and after incubation at 65°C for 15 mins.

k. Combine the two TNAEs per sample into a single tube. Store at -20°C.

l. Create a working dilution for use in the remainder of the protocol. Dilute neat TNAE 1:10 in water.

m. Before use in rolling circle amplification quantify DNA at A_{260} using a spectrophotometer and dilute to a concentration of 100-700 ng/µL in water.

PCR Detection

Samples require testing for DNA-A, DNA-B and DNA DNA-β genome components. The viruses associated with CLCuD are either monopartite (DNA-A only) or bipartitite (DNA-A and DNA-B) and the symptom modulator of the disease is the DNA-β thus testing for all is needed.

Table 1. PCR primers for the detection of the DNA-A genome component of begomoviruses.

Name	Sequence (5'-3')[1]	Direction	Reference
Avcore	GCCHATRTAYAGRAAGCCMAGRAT	Forward	Abdel-Salam *et al.* (2006) modified version of AV494
Accore	GGRTTDGARGCATGHGTACAIGCC	Reverse	Abdel-Salam *et al.* (2006) modified version of AC1048
SPG1	CCCCKGTGCGWRAATCCAT	Forward	Li *et al.*(2004)
SPG2	ATCCVAAYWTYCAGGGAGCTAA	Reverse	Li *et al.*(2004)
PBL1v2040	GCCTCTGCAGCARTGRTCKATCTTCATACA	Forward	Rojas *et al.* (1993)
PCRc1	CTAGCTGCAGCATATTTACRARWATGCCA	Reverse	Rojas *et al.* (1993)

[1] Where K=G or T, R=A or G, S=C or G, W=A or T, Y=C or T, H=A, T or C, B=C, G or T, V=A, C or G, and N=A, T, C or G.

Table 2. PCR primers for the detection of the DNA-β genome component associated with CLCuD and some begomoviruses.

Name	Sequence (5'-3')	Direction	Reference
β01	GGTACCACTACGCTACGCAGCAGCC	Forward	Briddon *et al.* (2002)
β02M	CCAGGGGTACACACCGCCGCG	Reverse	Gambley, unpublished

Table 3. PCR primers for internal control to confirm the presence of template and template integrity of samples which tested negative by DNA-A, DNA-B or DNA-β PCR assays.

Name	Sequence (5'-3')	Direction	Reference
AtropaNad2.1a	GGACTCCTGACGTATACGAAGGATC	Forward	Thompson *et al.* (2003)
AtrophaNad2.1b	AAACAACGCTTGTAAGGAGTCC	Reverse	Thompson *et al.* (2003)

Procedure: Detection of DNA-A molecules

a. Prepare master mix to a volume (n+2) where n = number of samples including positive and negative controls and store on ice.

b. Refer to Table 4 for components of one PCR volume

c. Aliquot 24 µL of PCR master mix per tube or well, store on ice.

d. Add 1 µL of the diluted TNAE per PCR volume

e. Centrifuge reactions at 13 000 RPM for 30-60 sec at room temperature

f. Incubate reactions in the thermocycler using the following cycling parameters:

    a. For DNA A (Avcore/Accore)
        - 95°C 1 min
        - 95°C 20 sec, 53°C 20 sec, 72°C 40 sec × 34 cycles
        - 72°C 5 min

    b. For DNA A (SPG1/SPG2)
        - 95°C 1 min
        - 95°C 20 sec, 72°C - 62°C (touchdown 1°C per cycle) 30 sec, 72°C 30 sec × 11 cycles
        - 95°C 20 sec, 60°C 30 sec, 72°C 30 sec × 24 cycles
        - 72°C 5 min

    c. For DNA B (PBL1v2040/PCRC1)
        - 95°C 30 sec min
        - 95°C 1 min, 55°C 2 min, 72°C 2 min × 34 cycles
        - 72°C 5 min

    d. For DNA-β (β01/β02(M))
        - 95°C 1 min
        - 95°C 20 sec, 50-55°C 20 sec, 72°C 45 sec × 34 cycles
        - 72°C 5 min

    e. For internal control (AtropaNad2.1a/AtrophaNad2.1b)
        - 95°C 1 min
        - 95°C 20 sec, 50°C 20 sec, 72°C 30 sec × 34 cycles
        - 72°C 5 min

Table 4. Each component of the master mix used in the begomovirus genus specific degenerate DNA-A PCR assays is listed along with its specified volume for a single reaction.

Reagent	**PCR master mix (volume for 1 reaction in µL)**
10 µM Forward primer (Avcore or SPG1)	2.0
10 µM Reverse primer (Accore or SPG2)	2.0
10 mM dNTPs	0.5
10 × PCR buffer	2.5
50 mM $MgCl_2$	0.875
Taq polymerase	0.3
Water	15.825
Total	24

Table 5. Each component of the master mix used in the begomovirus-associated DNA-B specific PCR assay is listed along with its specified volume for a single reaction.

Reagent	**PCR master mix Volume × 1 reaction (µL)**
10 µM PBL1v2040 Forward primer	0.5
10 µM PCRC1 Reverse primer	0.5
10 mM dNTPs	0.25
10 × PCR buffer	2.5
50 mM $MgCl_2$	0.75
Taq polymerase	0.2
Water	18.3
Total	23.0

Table 6. Each component of the master mix used in the begomovirus-associated DNA-β specific PCR assay is listed along with its specified volume for a single reaction.

Reagent	**PCR master mix Volume × 1 reaction (µL)**
10 µM β01 primer	0.25
10 β02(M) Reverse primer	0.25
10 mM dNTPs	0.5
10 × PCR buffer	2.5
50 mM $MgCl_2$	0.75
Taq polymerase	0.3
Water	19.55
Total	24

Table 2. Each component of the master mix used in the Internal Control PCR assay is listed along with its specified volume for a single reaction.

Reagent	**PCR master mix Volume × 1 reaction (µL)**
10 µM AtropaNad2.1a primer	0.5
10 µM AtrophaNad2.1b primer	0.5
10 mM dNTPs	0.5
10 × PCR buffer	2.5

50 mM $MgCl_2$	0.875
Taq polymerase	0.3
Water	18.825
Total	24.0

Interpretation of results

DNA-A

A positive PCR result is recorded if a DNA fragment of expected size is observed. For detection of begomovirus DNA-A a positive result with the primer set Avcore/A this is approximately 600 bp and for the primer set SPG1/SPG2 this is approximately 800 bp. An uncertain PCR result is recorded if the presence of the DNA band is uncertain, or if DNA fragments of ambiguous sizes are observed. A negative PCR result is recorded if no DNA fragment is observed and the internal control PCR demonstrated presence of amplifiable DNA.

Samples rated as uncertain by PCR where the internal control PCR demonstrated presence of amplifiable DNA require further investigation through retesting. Samples recorded as uncertain by PCR for DNA-A are further tested by a second molecular assay to establish the virus status.

DNA-B

A positive PCR result is recorded if a DNA fragment of expected size is observed. For detection of begomovirus-associated DNA-B components a positive result with the primer set PMB1v2040/PCRC1 this is approximately 500-650 bp. An uncertain PCR result is recorded if the presence of the DNA band is uncertain, or if DNA fragments of ambiguous sizes are observed. A negative PCR result is recorded if no DNA fragment is observed. Samples rated as uncertain by PCR may require further investigation through retesting.

DNA-β

A positive PCR result is recorded if a DNA fragment of expected size is observed. For detection of begomovirus-associated DNA-β components a positive result with the primer set β01/β02(M) this is approximately 700, 700 and 1400 or 1400 bp. An uncertain PCR results is recorded if the presence of the DNA band is uncertain, or if DNA fragments of ambiguous sizes are observed. A negative PCR result is recorded if no DNA fragment is observed. Samples rated as uncertain by PCR may require further investigation through retesting.

Internal control

A positive PCR result is recorded if a DNA fragment of expected size is observed. For detection of the DNA chromosome of the *ndhB* gene using the primer set Atropa2.1a/Atropa2.2b this is approximately 900 bp. The mRNA transcript of the gene is unlikely to be amplified in the PCR; however, if present it is easily distinguishable from the chromosome template as it is only approximately 200 bp in size. An uncertain PCR results is recorded if the presence of the DNA band is uncertain, or if DNA fragments of ambiguous sizes are observed. A negative PCR result is recorded if no DNA fragment is observed. **Samples rated as negative or uncertain by this PCR require further investigation through re-extraction of the sample and further indexing of freshly prepared TNAEs.**

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Appendix 2 – Tables of plant inspections and samples collected during surveys of northern Australia.

Table 1. A list of samples collected during a survey from Townsville to Bowen in October 2014. The common names of the plants sampled, their symptoms and the number of samples combined for indexing (i.e. bulk rate) are listed for each sample reference. Virus detections are highlighted in bold and the virus listed. NVD indicates no begomovirus or polerovirus was detected in that sample.

Sample Reference	Plant common name	Symptoms	Location	Bulk rate	Virus detected
NQ2014_WP389_002	Chilli	fine mosaic and cupping	Townsville	1	NVD
NQ2014_WP389_003	**Tomato**	**mottle/mosaic**	**Townsville**	**1**	**Polerovirus**
NQ_WP390_001	Tomato	necrotic rings and lines	Townsville	1	NVD
NQ_WP390_002	Tomato	necrotic rings and lines	Townsville	1	NVD
NQ_WP390_003	Chilli	necrotic rings and lines	Townsville	1	NVD
NQ_WP392_001	Capsicum	subtle cupping & bubbling	Giru	1	NVD
NQ_WP392_002	Chilli	subtle cupping & bubbling	Giru	1	NVD
NQ_WP393_001	**Chilli**	**random sample of chilli leaves**	**Giru**	**5**	**Polerovirus**
NQ_WP393_002	**Chilli**	**random sample of chilli leaves**	**Giru**	**5**	**Polerovirus**
NQ_WP393_003	**Chilli**	**random sample of chilli leaves**	**Giru**	**5**	**Polerovirus**
NQ_WP393_004	**Chilli**	**random sample of chilli leaves**	**Giru**	**5**	**Polerovirus**
NQ_WP394_005	**Capsicum**	**random sample of capsicum leaves**	**Ayr**	**5**	**Polerovirus**
NQ_WP394_006	**Capsicum**	**random sample of capsicum leaves**	**Ayr**	**5**	**Polerovirus**
NQ_WP394_007	**Capsicum**	**random sample of capsicum leaves**	**Ayr**	**5**	**Polerovirus**

NQ_WP395_003	Rockmelon	random melon sample	Ayr	1	NVD
NQ_WP395_004	Rockmelon	random melon sample	Ayr	5	NVD
NQ_WP395_005	Rockmelon	random melon sample	Ayr	5	NVD
NQ_WP395_006	Rockmelon	random melon sample	Ayr	5	NVD
NQ_WP396_002	**Tomato**	**yellowing, stunting, begomovirus-like**	**Gumlu**	**1**	**TYLCV[1]**
NQ_WP396_003	**Tomato**	**yellowing, stunting, begomovirus-like**	**Gumlu**	**1**	**TYLCV**
NQ_WP396_004	**Chilli**	**random sample of chilli leaves**	**Gumlu**	**5**	**Polerovirus**
NQ_WP396_005	**Chilli**	**random sample of chilli leaves**	**Gumlu**	**5**	**Polerovirus**
NQ_WP396_006	**Chilli**	**random sample of chilli leaves**	**Gumlu**	**5**	**Polerovirus**
NQ_WP396_007	Chilli	random sample of chilli leaves	Gumlu	5	NVD
NQ_WP397_001	Nightshade	random solanum nigrum sample	Bowen	5	NVD
NQ_WP397_002	Pumpkin	random pumpkin sample	Bowen	5	NVD
NQ_WP397_003	Unknown	random native malvaceae species sample	Bowen	5	NVD
NQ_WP397_004	Unknown	random native rosella or bladda keptmia sample	Bowen	5	NVD
NQ_WP397_005	Nightshade	random solanum nigrum sample	Bowen	5	NVD
NQ_WP397_006	Nightshade	random solanum nigrum sample	Bowen	5	NVD
NQ_WP397_007	Nightshade	random solanum nigrum sample	Bowen	5	NVD
NQ_WP397_009	Pumpkin	random pumpkin sample	Bowen	5	NVD
NQ_WP397_010	Pumpkin	random pumpkin sample	Bowen	5	NVD

NQ_WP397_011	Pumpkin	random pumpkin sample	Bowen	5	NVD
NQ_WP397_013	Unknown	random native *Malvaceae* sample	Bowen	5	NVD
NQ_WP397_014	Unknown	random native *Malvaceae* sample	Bowen	5	NVD
NQ_WP397_015	Unknown	random native *Malvaceae* sample	Bowen	5	NVD
NQ_WP397_017	Unknown	random native rosella or bladda keptmia	Bowen	5	NVD
NQ_WP397_018	Unknown	random native rosella or bladda keptmia	Bowen	5	NVD
NQ_WP397_019	Unknown	random native rosella or bladda keptmia	Bowen	5	NVD
NQ_WP400_001	Eggplant	virus infected eggplant; fine mosaic, subtle cupping/bubbling and chlorotic blotches	Bowen	1	NVD
NQ_WP400_002	Eggplant	virus infected eggplant; fine mosaic, subtle cupping/bubbling and chlorotic blotches	Bowen	1	NVD
NQ_WP400_003	Eggplant	virus infected eggplant; fine mosaic, subtle cupping/bubbling and chlorotic blotches	Bowen	1	NVD
NQ_WP401_001	Cotton	random asymptomatic cotton plants	Bowen	5	NVD
NQ_WP401_002	Cotton	random asymptomatic cotton plants	Bowen	5	NVD
NQ_WP401_003	Cotton	random asymptomatic cotton plants	Bowen	5	NVD
NQ_WP401_004	Cotton	random asymptomatic cotton plants	Bowen	5	NVD
NQ_WP401_005	Cotton	random asymptomatic cotton plants	Bowen	5	NVD
NQ_WP404_001	Tomato	upright growth, slightly stunted	Bowen	1	NVD
NQ_WP404_003	Tomato	upright growth, slightly stunted	Bowen	1	NVD
NQ_WP404_004	Tomato	upright growth, slightly stunted	Bowen	1	NVD
NQ_WP404_005	Tomato	upright growth, slightly stunted	Bowen	1	NVD

[1]TYLCV refers to *Tomato yellow leaf curl virus*

Table 2. Samples of known Cotton leaf curl disease hosts collected for molecular indexing during collaborative surveys with the Northern Australian Quarantine Strategy staff in 2015. The common names of the plants sampled, their symptoms and the number of samples combined for indexing (i.e. bulk rate) are listed for each sample reference.

Survey Site	Nearest town	Host common name	Host Species	Inspected	Sampled	Bulk rate for indexing	Symptoms
NPA_GCWP13	Injinoo	Hibiscus ornamental	*Hibiscus* sp.	16	5		asymptomatic
NPA_GCWP15	Injinoo	Hibiscus ornamental	*Hibiscus* sp.	5	5		asymptomatic
NPA_WP528	New Mapoon	Hibiscus ornamental	*Hibiscus* sp.	5	5		asymptomatic
NPA_WP529	Bamaga	Hibiscus ornamental	*Hibiscus* sp.	10	6		asymptomatic
NPA_WP530	Bamaga	Hibiscus ornamental	*Hibiscus* sp.	25	5		asymptomatic
NPA_WP533	Bamaga	Hibiscus ornamental	*Hibiscus* sp.	3	3		asymptomatic
NPA_WP534	Albany Island	Hibiscus ornamental	*Hibiscus* sp.	1	1		asymptomatic
NPA_CGWP001	Roko Island	Hibiscus ornamental	*Hibiscus* sp.	2	2		asymptomatic
NPA_CGWP002	Bamaga	Hibiscus ornamental	*Hibiscus* sp.	91	19		asymptomatic
NPA_CGWP003	Seisia	Hibiscus ornamental	*Hibiscus* sp.	22	5		asymptomatic
NPA_Seisia_General	Seisia	Hibiscus ornamental	*Hibiscus* sp.	17	5		asymptomatic
ORIA_WP499	Wyndham	Hibiscus ornamental	*Hibiscus* sp.	9	9		asymptomatic
ORIA_WP499	Wyndham	Watermelon	*Citrullus maxima*	3	3		stunting, distortion & chlorotic mottling
ORIA_Wydnum_General	Wyndham	Hibiscus ornamental	*Hibiscus* sp.	40	18		asymptomatic

ORIA_WP506	Wyndham	Hibiscus ornamental	*Hibiscus* sp.	2	2		asymptomatic
ORIA_WP508	Wyndham	Hibiscus ornamental	*Hibiscus* sp.	2	2		asymptomatic
ORIA_WP508	Wyndham	Chilli	*Capsicum* sp.	1	1		subtle cupping of leaves
ORIA_WP510	Kununurra		*Gossypium australe*	20	10		asymptomatic
ORIA_WP511	Lake Argyle		*Hibiscus aphelus*	3	3		asymptomatic
ORIA_WP512	Kununurra	Chilli	*Capsicum* sp.	1	1		subtle cupping of leaves
ORIA_WP515	Lake Argyle		*Abutilon indicum*	5	5		asymptomatic
ORIA_WP516	Lake Argyle	Hibiscus ornamental	*Hibiscus* sp.	15	10		asymptomatic
ORIA_WP517	Kununurra		*Gossypium australe*	1	1		asymptomatic
ORIA_WP518	Kalumbaru	Hibiscus ornamental	*Hibiscus* sp.	40	20		asymptomatic
ORIA_WP518	Kalumbaru	Roselle	*Hibiscus sabdariffa*	6	6		asymptomatic
ORIA_WP518	Kalumbaru	eggplant	*Solanum melongena*	1	1		leaf distortion, mild mottle
ORIA_WP519	Kununurra	Cotton	*Gossypium hirsutum*	10	10		asymptomatic
ORIA_WP521	Darwin	Cotton	*Gossypium hirsutum*	6	6		asymptomatic
Weipa_WP467	Napranum	Hibiscus ornamental	*Hibiscus* sp.	3	3		asymptomatic
Weipa_WP467	Napranum	Hibiscus ornamental	*Hibiscus* sp.	1	1		chlorotic blotches
Weipa_WP468	Napranum	Hibiscus ornamental	*Hibiscus* sp.	6	6		asymptomatic
Weipa_WP469	Napranum	Hibiscus ornamental	*Hibiscus* sp.	2	2		asymptomatic
Weipa_WP470	Napranum	Hibiscus ornamental	*Hibiscus* sp.	4	4		asymptomatic

Weipa_WP473	Weipa	Hibiscus ornamental	*Hibiscus* sp.	15	15		asymptomatic
Weipa_WP484	Mapoon	Chilli	*Capsicum* sp.	1	1		subtle cupping
Weipa_WP490	Weipa	Hibiscus ornamental	*Hibiscus* sp.	10	10		asymptomatic
Weipa_WP495	Weipa	Hibiscus ornamental	*Hibiscus* sp.	1	1		chlorotic blotches
Weipa_1	Weipa	Watermelon	*Citrullus maxima*	2	2		mosaic, distortion
Weipa_2	Weipa	Rockmelon	*Cucumis melo*	1	1		mosaic, distortion

Table 3. *Malvaceae* species detected during collaborative surveys with the Northern Australian Quarantine Strategy staff in 2015. The common names of the plants the number of plants inspected are listed. None of the plants inspected showed symptoms of begomovirus or polerovirus infections. Representative samples of species were collected and stored for future testing if required.

Species	Common name	Location	Number inspected
Abelmoschus manihot	aibika	Weipa	1
		Umagico	2
		Seisia	25
Abelmoschus esculentus	okra	Wyndham	3
		Darwin	2
Abelmoschus ficulneus	native rosella; white wild musk mallow	Kununurra	21
Abutilon indicum	Indian mallow; Indian abutilon	Kununurra	26
Abutilon spp.		Weipa	10

Gossypium australe	native cotton	Kununurra	20
		WA/NT border gate	1
Hibiscus apheles		Kununurra	3
Hibiscus meraukensis		Parry's creek	22
Hibiscus mutabilis[1]	confederate rose	Darwin	3
Hibiscus panduriformis		Kununurra	21
Hibiscus sabdariffa	rosella, roselle	Injinoo	2
		Umagico	1
		Kalumbaru	6
		Darwin	3
Hibiscus tiliaceus	beach hibiscus	Weipa	19
		Mapoon	4
Melochia corchorifolia		Wyndham	15
Melochia pyramidata		Parry's creek	10
Melochia spp.		Weipa	1
		Mapoon	10
Sida acuta		Kununurra	10
Sida cordifolia		Napranum	4
		Kununurra	115

Sida spp.		Weipa	50
		Napranum	30
		Mapoon	41
		Kununurra	5
Sida spinosa		Kununurra	20
Thespesia sp.		Mapoon	1
Urena lobata		Napranum	1
		Weipa	41
		Mapoon	4
		Bamaga	25
		Injinoo	15
		Kalumbaru	31
Waltheria indica		Wyndham	100
		Kununurra	50
Waltheria spp.		Napranum	5
		Mapoon	10

[1] Note these plants were previously inspected and sampled, although misidentified as Gossypium hirsutum (cotton). The results were reported in the CRDC travel report Smith & Gambley, 2011'Disease surveys in commercial cotton in the Ord River Irrigation Area'.

Table 4. Whitefly samples collected during collaborative surveys with the Northern Australian Quarantine Strategy staff in 2015. The whitefly populations were mostly identified as *Bemisia tabaci* AUSII (Australian native whitefly). The exceptions were, whitefly collected from cucurbit plants at survey site ORIA_WP498 which were *B. tabaci* ASIAII and those from ORIA_WP505 which belong to a putative new *Bemisia* spp.

Survey Site	Host species	Host common name	Comments	Nearest Town
NPA_WP523	*Physalis angulata*	gooseberry, balloon cherry, wild tomato etc	no begomovirus symptoms on host, whitefly collected from 3 individual host plants	Bamaga
NPA_GCWP11	*Cucurbita maxima*	Pumpkin	no begomovirus symptoms on host, single pumpkin plant	Injinoo
NPA_WP526	*Cucurbita maxima*	Pumpkin	no begomovirus symptoms on host	Injinoo
NPA_WP527	*Cucurbita maxima*	Pumpkin	no begomovirus symptoms on host, whitefly collected from 2 individual host plants	Umagico
NPA_WP528	*Calopogonium mucunoides*	Calopo	no begomovirus symptoms on host, whitefly collected from multiple host individuals	New Mapoon
NPA_WP532	*Calopogonium mucunoides*	Calopo	no begomovirus symptoms on host, whitefly collected from multiple host individuals	Bamaga
NPA_CGWP001	*Ipomoea hederifolia*	Pink morning glory	possible begomovirus symptoms on host, sample collected of host, whitefly collected from 3 individual host plants, species not a CLCuD host	Roko Island
NPA_GCWP44	*Cucurbita maxima*	Pumpkin	whitefly collected from host (4 individuals), no begomovirus symptoms on host	Seisia
NPA_GCWP47	*Synedrella nodiflora*		no begomovirus symptoms on host, whitefly collected from 3 individual host plants	Seisia
ORIA_WP498	*Citrullus lanatus* var. *lanatus* & *Cucumis sativus*	Watermelon & cucumber	no begomovirus symptoms on hosts, whitefly collected from multiple individual host plants of both species	Wyndham
ORIA_WP498	*Solanum melongena*	Eggplant	no begomovirus symptoms on host, whitefly collected from an individual host plant	Wyndham
ORIA_WP500	*Solanum melongena*	Eggplant	no begomovirus symptoms on host, whitefly collected from multiple individual host plants	Wyndham
ORIA_WP505	*Cucumis melo* subsp. *Melo*	Rockmelon	no begomovirus symptoms on host, whitefly collected from an individual host plant	Wyndham
ORIA_WP518	*Physalis angulata, Solanum melongena*	gooseberry, balloon cherry, wild tomato etc, eggplant	no begomovirus symptoms on host, whitefly collected multiple host plants	Kalumbaru

Table 5. List of plant samples submitted by Department of Agriculture and Water Resources, Northern Australian Quarantine Strategy from surveys in northern Australia and tested for exotic viruses.

Sample Reference	Collection Location	Symptoms	Plant species
3754-1	South Goulburn Island Airport	Asymptomatic	*Gossypium hirsutum*
3754-2	Kununurra, WA	Asymptomatic	*Abelmoschus ficulneus* (native rosella)
3754-3	Kununurra, WA	Asymptomatic	*Gossypium australe*
3754-4	Kununurra, WA	Asymptomatic	*Gossypium hirsutum*
3754-5	Legune Station, NT	Asymptomatic	*Hibiscus panduriformis*
3754-6	Darwin, NT	Asymptomatic	*Gossypium hirsutum*
3754-7	Darwin, NT	Asymptomatic	*Gossypium hirsutum*
3754-8	Wiligi Property, NT	Asymptomatic	*Hibiscus* sp.
3754-9	Bradshaw Military Base, NT	Some crinkle and curled leaves	*Gossypium australe*
3983-15	Hammond Island, Torres Strait	asymptomatic	*Gossypium barbadense*
3983-21	Smithfield, Queensland	yellow on green mosaic	*Abelmoschus manihot*
3983-23	Thursday Island	ringspot	*Sterculia quadrifida*
3996-1	Kununurra, WA	asymptomatic	*Gossypium hirsutum* (BRF71)
3996-3	Kununurra, WA	asymptomatic	*Hibiscus panduriformis*
3996-4	Kununurra, WA	asymptomatic	*Abelmoschus ficulneus*
3996-5	Kununurra, WA	asymptomatic	*Gossypium hirsutum*

Appendix 3 –Tables of plant inspections and samples collected during surveys of Papua New Guinea and Timor Leste.

Table 1 A list of plant species observed in Papua New Guinea 2014 and 2016 surveys. The total number of individual plants inspected is listed, in addition to the number of sites that species was detected at.

Plant Species	Common name	Number inspected 2014	Number sites 2014 (total 28)	Number inspected 2016	Number sites 2016 (total 26)
Abutilon spp.	Chinese lantern	6	2	10	1
Abelmoschus manihot	Aibeka	188	11	269	12
Capsicum annuum	Chilli	0	0	44	6
Cucurbitaceae	Cucumber/pumpkin	175	14	60	13
Gossypium spp.	Cotton	4	4	2	2
Hibiscus rosa-sinensis	Ornamental hibiscus	84	7	270	8
Hibiscus sabdariffa	Roselle	0	0	9	1
Solanum lycopersicum	Tomato	133	13	34	4
Urena lobata	Urena burr/pink flowered chinese burr	5	5	0	0

Table 2. A list of plant samples collected for virus testing during surveys of Papua New Guinea in 2014. All samples tested negative for Begomovirus.

Sample reference	Location	Plant species	Common name	Bulk rate	Symptoms
PNG001	Kainantu	*Hibiscus* sp.	Hibiscus	1	Enations
PNG003	Aiyura Valley	*Ageratum* sp.	Billy goat weed	1	subtle cupping
PNG004		*Ageratum* sp.	Billy goat weed	1	stunting, cupping,, slight yellowing
PNG005		*Ageratum* sp.	Billy goat weed	1	leaf enations, one leaf only
PNG011	Benha	*Abelmoschus manihot*	Aibika	1	mosaic, mottle
PNG012		*Abelmoschus manihot*	Aibika	1	mosaic, mottle
PNG013		*Abelmoschus manihot*	Aibika	1	mosaic, mottle, some bubbling
PNG026		*Abelmoschus manihot*	Aibika	1	mottle/mosaic
PNG015	Kamaligi	*Gossypium barbadense*	cotton	1	asymptomatic
PNG016	Goroka	*Gossypium barbadense*	cotton	1	asymptomatic
PNG017		*Hibiscus* sp.	Hibiscus	1	chlorotic mottle
PNG018		*Ageratum* sp.	Billy goat weed	1	cupping of leaves, fine enations on lower surface
PNG021		*Abelmoschus manihot*	Aibika	1	asymptomatic
PNG024	Kabiufa	*Gossypium barbadense*	cotton	1	asymptomatic
PNG027	Ku	*Abelmoschus manihot*	Aibika	1	yellowing, mottle/mosaic
PNG030	Mt Wilheim	*Ipomoea batatas*	Sweetpotato	1	cupping
PNG033		*Phaseolus* sp.	bean	1	cupping, brittle leaves
PNG034		*Gossypium barbadense*	Cotton	1	asymptomatic

PNG036		*Crassocephalum crepidioides*		1	chlorotic blotches, cupping, leafrolling
PNG037		*Urena lobata*		1	asymptomatic
PNG038		*Abelmoschus manihot*	Aibika	1	mottle/mosaic
PNG039		*Abelmoschus manihot*	Aibika	1	mottle/mosaic
PNG040		*Hibiscus* sp.	Hibiscus	10	asymptomatic
PNG041	Minz	*Abelmoschus manihot*	Aibika	1	mottle/mosaic
PNG042		*Abelmoschus manihot*	Aibika	1	mottle/mosaic
PNG043		*Abelmoschus manihot*	Aibika	1	mottle/mosaic
PNG048	Mt Hagen	*Abutilon* sp.	Chinese lantern	5	asymptomatic
PNG049		*Abelmoschus manihot*	Aibika	1	mottle/mosaic
PNG050		*Hibiscus* sp.	Hibiscus	1	asymptomatic
PNG051		*Urena lobata*		1	asymptomatic
PNG052		*Hibiscus* sp.	Hibiscus	2	asymptomatic
PNG053		*Urena lobata*		1	asymptomatic
PNG054	Sabama	*Hibiscus* sp.	Hibiscus	3	asymptomatic
PNG055		*Hibiscus* sp.	Hibiscus	3	asymptomatic
PNG056		*Hibiscus* sp.	Hibiscus	3	asymptomatic
PNG057	Rainbow	*Hibiscus* sp.	Hibiscus	3	asymptomatic
PNG058		*Hibiscus* sp.	Hibiscus	3	asymptomatic
PNG059		*Hibiscus* sp.	Hibiscus	3	asymptomatic
PNG060	East Boroko	*Hibiscus* sp.	Hibiscus	5	asymptomatic
PNG061		*Hibiscus* sp.	Hibiscus	5	asymptomatic
PNG062		*Hibiscus tiliacea*	Beach hibiscus	1	asymptomatic
PNG063		*Hibiscus* sp.	Hibiscus	1	asymptomatic

Table 4. A list of whitefly samples collected during Papua New Guinea surveys in 2014 and 2016. Details of survey location, elevation and plant hosts the insects were collected from are listed with the species detected at each site.

Survey year	Location	Host	Elevation (m)	Species detected
2014	Kainantu	hibiscus, billy goat weed, tomato, nightshade	1622	*Trialeurodes vaporariorum*
	Aiyura Valley	billy goat weed, tomato	1630	*Trialeurodes vaporariorum*
	Benha	tomato	1490	*Trialeurodes vaporariorum*
	Kamaligi	tomato	1427	*Trialeurodes vaporariorum, Bemisia tabaci AUS 1*
	Okiufa	tobacco	1639	*Bemisia tabaci AUS 1*
	Goroka	tomato, bean, billy goat weed	1644	*Trialeurodes vaporariorum, Bemisia tabaci AUS 1*
	Mt Wilheim	potato, sweet potato, pumpkin	2567	*Trialeurodes vaporariorum*
		tomato, billy goat weed	1695	*Trialeurodes vaporariorum*
	Minz	billy goat weed, tomato	1605	*Trialeurodes vaporariorum*
	Tambul	potato	2618	*Trialeurodes vaporariorum*
	Mt Hagen	billy goat weed	1632	*Trialeurodes vaporariorum*
		billy goat weed	1554	*Trialeurodes vaporariorum*
2016	Amanab DAL	sweet potato	406	TBD
	Telefomin village	tomato	1506	*Trialeurodes vaporariorum*
	Aitape high school	peanut	10	TBD
	Pasci DAL site 1	sweet potato	18	TBD
	Wusipi household	pumpkin	20	TBD
	Vanimo western tower	sweet potato	74	TBD
	Wutang village	pumpkin	15	TBD
		sweet potato	15	TBD
	Dawi Musu	cucumbers	34	TBD

	Bapa village	pumpkin	69	TBD
	Blackwater refugee camp	sweet potato	49	TBD
	Imbinis village	sweet potato	53	TBD
	Sckoptia village	sweet potato	170	TBD

Table 5. A list of plant samples submitted by the Department of Agriculture and Water Resources, Northern Australian Quarantine Strategy staff, from surveys in Timor Leste and Papua New Guinea, 2015. Samples tested negative for exotic begomoviruses.

Accession	Host	Symptoms	Location
RID6718	*Urena* sp.	Asymptomatic	Viqueque, Timor Leste
LMJ552	*Hibiscus* sp.	Asymptomatic	Alieu District, Timor Leste
LMJ586	*Gossypium* sp.	Asymptomatic	Maliana District, Timor Leste
LMJ594	*Gossypium* sp.	Marginal discolouration on a few leaves	Maliana District, Timor Leste
LMJ629	*Gomphrena* sp.	Asymptomatic	Liquica District, Timor Leste
LMJ641	Cotton relative	Asymptomatic	Dili District, Timor Leste
LMJ643	*Hibiscus* sp.	Asymptomatic	Dili District, Timor Leste
LMJ646	*Urena sinuate*	Broad darker green leaf margins	Dili District, Timor Leste
LMJ655	*Hibiscus* sp.	Asymptomatic	Boigu, Timor Leste
3755-1	*Hibiscus tiliaceus*	Asymptomatic	Timor Leste
3755-2	*Gossypium* sp.	Asymptomatic	Timor Leste
3755-3	*Gossypium barbadense*	Asymptomatic	Timor Leste
3755-4	*Hibiscus* sp.	Asymptomatic	Timor Leste
3755-6	*Hibiscus tiliaceus*	Yellow/green mottle	Timor Leste
3983-16	*Urena sinuate*	Asymptomatic	Viqueque, Timor Leste
3983-17	*Gossypium hirsutum*	Asymptomatic	Viqueque, Timor Leste
3983-18	*Gossypium barbadense*	Asymptomatic	Viqueque, Timor Leste
3983-19	*Abelmoschus ficulneus*	Asymptomatic	Timor Leste
3983-20	*Gossypium arboreum*	Asymptomatic	Dili, Timor Leste
3983-22	*Abelmoschus manihot*	leaf distortion, mosaic	Kiunga, Papua New Guinea
RID7080	*Urena lobata*	Asymptomatic	Daru, Papua New Guinea
RID7081	*Melochia corchorifolia*	Asymptomatic	Daru, Papua New Guinea
RID7082	*Hibiscus mutabilis*	Asymptomatic	Daru, Papua New Guinea
RID7084	*Gossypium barbadense*	Asymptomatic	Daru, Papua New Guinea