Final report

Project title:

Avocado industry biosecurity capacity and capability building: Phase II

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Project code:

AV21003

Date:

6 May 2026

Disclaimer:

Funding statement:

This project has been funded by Hort Innovation, using the avocado research and development levy and contributions from the Australian Government. Hort Innovation is the grower-owned, not-for-profit research and development corporation for Australian horticulture.

Publishing details:

Published and distributed by: Horticulture Innovation Australia Limited
ABN 71 602100149

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North Sydney NSW 2060

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www.horticulture.com.au

Contents

Public summary

To ensure the Australian avocado industry remains profitable and environmentally sustainable, it is critical to have a strong biosecurity system. The main purpose of this project was to develop diagnostic capacity for selected, high priority exotic pests and pathogens, namely the Persea mite (*Oligonychus perseae*), the avocado seed moth *(Stenoma catenifer*), avocado scab (*Elsinoe perseae*), and laurel wilt (*Harringtonia lauricola*). Diagnostic support was also provided to the Avocado Nursery Accreditation Scheme (ANVAS) to ensure planting material distributed to growers was free of avocado sunblotch viroid (ASBVd).

Adult female Persea mites are about 0.5 mm long, and the males even smaller. The Persea mite is a type of spider mite (family Tetranychidae), and this group of arachnids is notoriously difficult to identify, even for experts as only adult male individuals can be identified to species level using morphological features, not the females or larval stages. Molecular diagnostic assays for the Persea mite were therefore developed in this project. The first approach was to develop DNA barcoding assays, which is a family-wide method of identification based on sequencing of short stretches of DNA. The second approach was to develop a Persea mite LAMP assay, a very simple test that can be done in a farm shed if necessary. The specificity and sensitivity of these assays was demonstrated using mite specimens from Mexico.

Avocado scab is also a very difficult pathogen to identify using conventional methods, as it is very slow growing on artificial media, and does not produce the spore structures needed to make a definitive identification. A qPCR assay was developed that allowed detection of the pathogen directly in scab tissue. The assay was road-tested in Florida using fresh scab specimens, and positive identifications made within a day. The specificity of the test was examined, and no cross-reactions were observed with closely related fungal species. The robustness of the assay was also examined by demonstrating performance in a second independent laboratory, and by altering the brand of qPCR reagents and equipment.

Laurel wilt is a deadly vascular disease complex of lauraceous species including avocados, caused by an ambrosia beetle vectored fungus, *H. lauricola*. To improve disease surveillance and management, a LAMP assay was developed for rapid detection of the fungal pathogen directly from an infected plant and the beetle vector, bypassing the time-consuming laboratory diagnostic procedures. We validated this published assay and confirmed its specificity and reliability in detecting the fungus directly in infected plants and beetles imported from the USA. Improvements were also made to the assay by converting it into a colorimetric format, whereby positive results are indicated by colour change of the reaction mix from pink to yellow.

In the remaining activities of this project, a new DNA barcoding assay was developed for the avocado seed moth, and 5,770 trees certified to be free of ASBVd as part of the routine testing program for ANVAS.

Keywords

Persea americana, quarantine, diagnostics, biosecurity preparedness, exclusion benefits, mites, laurel wilt, avocado scab, avocado sunblotch viroid, avocado seed moth, exotic pests.

Introduction

Avocado is one of the most economically important fruit crops in Australia with a farmgate value of over $700 million in 2024/25 (Hort Innovation 2025). In the last decade, the industry has rapidly expanded due to growing consumption per capita, translating into stronger market prices over extended periods. Furthermore, domestically sourced fruit is now available for most of the year through expansion of the industry in southwest Western Australia. The production of avocados in Australia is expected to continue to grow as many recently planted orchards have yet to reach peak production. Superimposed upon local production are imports from New Zealand and most recently, Chile. To cope with surplus production, there is a need to develop new export markets, and biosecurity can be used as a non-tariff trade barrier. The growth of the avocado industry in Australia is mirrored in many parts of the world, with avocados now being grown in places unheard of before, enhancing the risk of spread of pests and pathogens around the world.

The most cost-effective approach to managing exotic pests and pathogens (EPPs) is to prevent introduction in the first place, but unfortunately, biosecurity breaches do occur. If a new pest or pathogen of avocado invaded Australia, then the impacts are immediate and very expensive through movement restrictions placed on the trade of fresh fruit, from destruction of orchards, and from ongoing costs associated with managing the pest or pathogen to minimize yield losses. The success of eradication or containment programs in the event of a biosecurity incursion are very dependent on the speed at which the organism can be detected and identified and on the availability of knowledge about the biology of the organism such as host range and mode of transmission. Accuracy is paramount as false identifications can be very expensive, as exemplified by erroneous reports of avocado scab (*Elsinoë perseae*) and sunblotch (avocado sunblotch viroid) from New Zealand, which cost millions of dollars to disprove. For all the above reasons, it is essential to prepare for a biosecurity incursion by developing a strong diagnostic capacity. It is also necessary to have intelligence on where major pests and pathogens are found around the world, what dispersal pathways may exist, and to have an active domestic surveillance system for these organisms.

Exotic pests and pathogens for the Australian avocado industry are prioritised in importance by an expert group based on four criteria: entry potential, establishment potential, spread potential and economic impact. This prioritisation process is valuable in focussing biosecurity research on those pests that are most likely to cause the most harm to the Australian avocado industry (high priority pests and pathogens, HPPs). HPPs that are documented on the Biosecurity Plan for the Avocado Industry (v3.0 2020) include *Elsinoe perseae*, the cause of avocado scab, *Harringtonia lauricola* and *Xyleborus glabratus*, the fungal causal agent and its vector of laurel wilt, *Oligonychus perseae* (Persea mite), and *Stenoma catenifer* (avocado seed moth).

For the avocado industry to take advantage of exclusion benefits going forward, a strong and data driven approach to biosecurity is needed. Therefore, the aims of this project were to 1) improve and rigorously validate existing diagnostic protocols to international standards for exotic pests and pathogens for the Australian avocado industry, 2) develop and validate diagnostic protocols for high priority exotic pests, the Persea mite *Oligonychus perseae* (Acari: Tetranychidae) and the avocado seed moth *Stenoma catenifer* (Lepidoptera: Depressariidae), 3) validate and update the national diagnostic protocol for laurel wilt, and 4) provide diagnostic support to ANVAS. The major outcome of this project is the establishment of an economically and environmentally sustainable Australian avocado industry through limiting the introduction or spread of new pests and pathogens and by promoting trade access utilising enhanced knowledge of the endemic pests and pathogens.

Methodology

The main research activities of this project were to develop diagnostic tools and standard operating protocols for high priority biosecurity pests and build human capacity towards enhancing the biosecurity diagnostic capability for the avocado industry. The research focused on four high priority pests listed on the Biosecurity Plan for the Avocado Industry (v3.0, 2020): Persea mite, the laurel wilt disease complex, avocado seed moth, and avocado scab. In addition, diagnostic support was provided to ANVAS for avocado sunblotch viroid (ASBVd) testing. The below methods were used.

Review of available diagnostic assays for high priority biosecurity avocado pests and pathogens

At the start of the project, a desktop review was undertaken for diagnostic assays available for the high priority exotic pests and pathogens of avocado (Biosecurity Plan v3.0, 2020) to identify the gaps in knowledge and investigate methods of identification for four target organisms that lack diagnostic preparedness and research activities suggested to address these gaps in knowledge.

Development of qPCR assays for the rapid detection of the avocado scab fungus *Elsinoe perseae*

Elsinoe isolates used in this study were either imported from CBS-KNAW (Netherlands) as live cultures under Australian Government quarantine permit 0002287435 or provided by the Brisbane Pathology Herbarium (BRIP) at Dutton Park, Queensland. Some *E. perseae* isolates used to validate the qPCR assays were used in situ at the Tropical Research and Education Centre (TREC), University of Florida (Homestead, FL 33031, United States). Non-target *Elsinoe* species used to test the specificity of the assays were: *E. mangiferae*, *E. randii*, *E. centrolobii*, *E. terminaliae*, *E. ichnocarpi*, *E. lagoa-santensis*, *E. leucospermi*, *E. piri*, *E. australis*, *E. fawcettii*, and *E. citricola*. A range of other ascomycete fungi isolated from avocados by Dr. Elizabeth Dann were also included in the testing panel for the qPCRs.

Total genomic DNA (gDNA) was extracted from approximately 50 mg of mycelia scraped from the surface of culture plates using a Wizard® Genomic DNA Purification Kit (Catalog number: A1120, Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions.

To design qPCR primers and TaqMan probes, representative ITS1 and RPB2 sequences from all species in the genus *Elsinoe* considered by Fan et al. (2017) were retrieved from GenBank and aligned using the MAFFT Alignment plugin for Geneious Prime v. 2024.0.3 (Biomatters Ltd., Auckland). Suitable sites for primers and probes were selected visually, and sequences are provided in Table 1.

Table 1. TaqMan qPCR primer and probes for *Elsinoe perseae* qPCR assay

Name	Target	Sequence (5' - 3')	5' Modification	3' Modification	Amplicon size (bp)
eITSCalFluor	ITS	TTGTAGTCGGAGTACAACCGT	CAL Fluor560	BHQ1	252
eITS-F		CGAACCAACTCTTGCACATCA	-	-	
eITS-R		GCCACACGCCCAATACCAA	-	-	
eRPB2FAM	RPB2	TGAATGGACAATGGATCGGTG	FAM	BHQ1	222
eRPB2-F		ACTAACCCAAATGCCACTAAG	-	-	
eRPB2-R		GTCACCAGGCTCGTTATTGA	-	-	

Two gBlocksTM dsDNA fragments (Integrated DNA Technologies, Coralville, IA, USA) were designed as positive controls: EpITS-gB and EpRPB2-gB. Each fragment included the target amplicon extended by an additional 20–30 nt of the fungal sequence on either end. Universal primer binding sites, M13F and M13R, were added to the 5' and 3' ends, respectively, to facilitate PCR amplification of the synthetic DNA fragment if needed. The concentrations of EpITS-gB and EpRPB2-gB in solution were quantified using a Qubit (Thermo Fisher Scientific) and then diluted to desired concentrations or copy numbers before use.

Singleplex qPCRs were run using 10 µL of Inhibitor-Tolerant qPCR Mix 2× (Catalog number: MDX013, Meridian Bioscience, Cincinnati, OH, USA), 1 µL of each primer at 8 µM, 1 µL of each probe at 4 µM, 1 µL of template, and water

to reach a total volume of 20 µL. Duplex qPCRs were run using Reactions were run using the TaqMan Fast Advanced Master Mix (Catalog number: 4444556, Thermo Fisher Scientific Waltham, MA, USA). Assays were run on a Rotor-Gene Q (Catalog number: 9002370, QIAGEN, Inc., Hilden, Germany) with one cycle at 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. Primer and probe sequences are provided in Table 1. When assays were done in singplex, all probes were labelled with a 5′ 6-FAM reporter and a 3′ BHQ1 quencher. To enable duplexing, the ITS probe was modified to include the CalFluor560 reporter, while the RPB2 probe remained unchanged.

In Florida, DNA was extracted from eight fruit and leaf samples from different cultivars, including 'Hass', 'Lula', and 'Miguel', which showed typical symptoms of avocado scab. Additionally, samples from four fruits (cultivars 'Hass', 'Reed' and 'Brook Late') that displayed other physical damage or defects that were visually like scab symptoms were tested. An asymptomatic fruit from cultivar 'Donni' served as the negative control (Fig. 1). These samples were collected from the TREC germplasm collection at Homestead, the 'Hass' fruit were purchased from a local supermarket.

DNA barcoding of spider mites (Tetranychidae)

Complete or near-complete sequences of the COI gene and rRNA operon were extracted from the nucleotide and genome databases of GenBank for the Tetranychidae, as well as more distant relatives from the suborder Acariformes. Sequence alignments were created using the MUSCLE algorithm, and PIR format alignment files exported. These PIR files were then used as input data for GPRIME, a program that advances a sliding window of user defined width across the sequence alignment one column at a time and then calculates the degeneracy value for each window of sequence. PCR primers were then designed to the most conserved regions of sequence. Finally, parameters such as melting temperature (Tm), secondary structure, primer-dimer formation and self-annealing were checked using OligoEvaluator™ (Sigma-Aldrich). Primer sequences are provided in Table 2.

Table 2. Cytochrome c oxidase subunit I (COI) DNA barcoding primers, and PCR parameters

PCR	Primer name	Sequence (5′-3′)	Final conc. (µM)	Annealing temp.	Extension time	Amplicon length (bp)
1	TetraCOI364-F	GGDTGAACHATRTAYCCYCC	3.33	60°C	30 s	353
	TetraCOI716-R	TCNGGRTGHCCAAARAAYCA	6.67			
2	TetraCOI364-F	GGDTGAACHATRTAYCCYCC	3.33	55°C	30 s	569
	TetraCOI932-R	TCNGGRTGHCCAAARAAYCA	1.66			
3	TetraCOI16-F	TCHACNAAYCATAARRATATTGG	3.33	50°C	30 s	917
	TetraCOI932-R	ATVGCAATAATTATWGTAGC	0.66			

A range of spider mite species including *Oligonychus perseae* and *O. punicae* (Table 3) were used for validating the DNA barcoding primers. DNA of these specimens were extracted from individual spider mites using the Quick-DNA Tissue/Insect Microprep Kit (Catalogue Number: D6015 Zymo Research, Orange, CA) according to the manufacturer's instructions. PCR assays were then done using Phusion™ High-Fidelity DNA Polymerase (Catalogue Number: M0530 New England Biolabs, Beverly, MA) as per the manufacturer's instructions. Phusion™ High-Fidelity DNA Polymerase (Catalogue Number: M0530 New England Biolabs, Beverly, MA) was used for all PCR assays as per the manufacturer's instructions. Reaction mixes contained 1 × Phusion DNA polymerase buffer, 200 µM of each dNTP, 0.5–1 µL of DNA template, and forward and reverse primers to the final concentrations listed in Table 1. Phusion HF buffer was used for the ITS2 and 18S-V4 reaction mixes, and DMSO added to a final concentration of 3%, whereas Phusion GC buffer was used for all COI reaction mixes and no DMSO added. Water was added to give final reaction volumes of 15 µL in all cases. Thermocycling conditions comprised an initial denaturation at 98°C for 30 s, 35 cycles of 98°C for 30 s, primer pair dependent annealing temperatures and extension times at 72°C (see Table 1) and a final elongation step at 72°C for 5 min.

Amplicons were electrophoresed on a 1–1.5% agarose gel in 0.5 × Tris-Borate-EDTA buffer and visualised by ethidium bromide staining. Primer annealing temperatures were optimised using the temperature gradient function of the SuperCycler, choosing increments of c. 1–2°C on either side of the predicted Tm. Amplicons were electrophoresed on a 1–1.5% agarose gel in 0.5 × Tris-Borate-EDTA buffer and visualised by ethidium bromide staining. Direct Sanger sequencing of the PCR amplicons was done at Macrogen, South Korea, using the amplification primers for the sequencing reactions. Where direct sequencing did not provide high quality sequence reads, the PCR amplicons were cloned using a NEB® PCR Cloning Kit (Catalogue Number: E1202S New England Biolabs, Beverly, MA) and colony PCRs done using T7-forward and

M13-reverse universal primers and Phusion™ High-Fidelity DNA Polymerase according to the manufacturer's instructions. The resulting amplicons were directly sequenced as described above. Sequenced data was assembled using Geneious Prime Version 2025 1.2 (Biomatters Ltd) and identified using BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 3. Mite specimens used for DNA barcoding experiments and LAMP assays

Species	Specimen ID	Host	Collection date	Collector	Nearest town	Country
Tetranychus ludeni	LSP21	*Phaseolus vulgaris*	18/04/24	L. Pretorius	Dutton Park, QLD	Australia
T. urticae	LSP11[a]	*Prunus amygdalu*	18/4/24	L. Pretorius	Dutton Park, QLD	Australia
	LSP46	*Citrus* sp.	4/6/24	L. Mannes	Dutton Park, QLD	Australia
	NT6	*Rosa* sp.	6/2/24	T. Santillan-Galicia	Montecillo	Mexico
*T. lambi**		*Phaseolus vulgaris*	14/6/25	L. Pretorius	Dutton Park, QLD	Australia
T. neocaledonicus	LSP55	*Passiflora edulis*	15/12/23	L. Pretorius	Fairfield, QLD	Australia
Oligonychus coffeae	LSP43	*Persea americana*	23/1/24	C. Russel	QLD	Australia
	LSP44	*P. americana*	6/2/24	L. Pretorius	QLD	Australia
	LSP52[a]	*P. americana*	18/5/24	E. Dann	QLD	Australia
O. perseae	NT2 Oper	*P. americana*	6/2/24	H. G. Hernández	Tequexquinahuac	Mexico
	NT4	*P. americana*	6/2/24	H. G. Hernández	Tequexquinahuac	Mexico
	NT5	*P. americana*	6/2/24	H. G. Hernández	Tequexquinahuac	Mexico
	NT8	*P. americana*	6/2/24	E. Estrada	Montecillo	Mexico
O. punicae	NT1	*P. americana*	6/2/24	H. G. Hernández	Tequexquinahuac	Mexico
	LSP31	*P. americana*	6/2/24	H. G. Hernández	Tequexquinahuac	Mexico
	LSP70	*P. americana*	6/2/24	H. G. Hernández	Tequexquinahuac	Mexico
	NT3	*Alnus acuminata*	6/2/24	H. G. Hernández	Tequexquinahuac	Mexico
Eutetranychus africanus	NT16	*Plumeria acutifolia*	25/4/24	J. Beard	QLD	Australia
Eotetranychus sexmaculatus	NT184[a]	*P. americana*	7/10/25	J. Beard	Manjimup, WA	Australia
E. lomandrae	NT194[a]	*Lomandra lomgifolia*	18/8/25	n/a	QLD	Australia
Eotetranychus sp.	NT7	*Salix bonplandiana*	6/2/24	T. Santillan-Galicia	Montecillo	Mexico
E. africanus ex. *murraya*	NT165	*Murraya panniculata*	25/4/24	J. Beard	QLD	Australia
Petrobia harti	NT166a	*Oxalis corniculata*	25/4/24	J. Beard	Anstead, QLD	Australia
Tyrophagus putrescentiae	NT167[a]	Infested agar plate	1/7/24	C. O'Dwyer	Dutton Park, QLD	Australia

[a]not tested by LAMP assays

Development of LAMP assays for diagnosis of the Persea mite and avocado brown mite

To develop species-specific LAMP primers, COI, 28S, ITS1, and ITS2 sequences from *O. perseae*, *O. punicae* and related species in the family Tetranychidae were retrieved from GenBank, and alignments made. Two to three sets of primers were designed for each species using PrimerExplorer v4 software (https://primerexplorer.jp/e/). To allow simultaneous detection (i.e., duplexing) of the two target species in one reaction, a species-specific fluorescent assimilating probe (Kubota et al. 2011; Hamilton et al. 2020) was also designed for each species. The primers and probes were checked for secondary structure, dimerization and self-annealing using Oligonucleotides Property Calculator (https://www.biosyn.com/gizmo/tools/oligo/oligonucleotide%20properties%20calculator.htm). *In silico* validation was done on the sequence alignments in Geneious Prime v. 2021.0.1 (Biomatters Ltd., Auckland), narrowing the selection to one set of primers targeting the 28S rDNA gene of *O. perseae* and one set targeting the ITS1 region for *O. punicae* (Table 4).

For each LAMP assay, a gBlocks™ gene fragment was synthesized for use as a positive control (Table 4). To assist with recognition of false positive results due to DNA contamination of the assay, the gBlocks gene fragments were designed to include (i) the primer binding sites and randomly generated sequences to approximately the length of the LAMP amplicons, (ii) a restriction site (e.g. EcoRV), that is not present on the mite target sequence fragment, and (iii) a higher GC content, achieved by adding runs of 'ccc' and 'ggg' between the primers, to provide clear differentiation of the positive control from the target in melting point analyses.

DNA of spider mite specimens (Table 3) were extracted from a single mite using the Quick-DNA Tissue/Insect Microprep Kit as described previously. A simple, rapid, and non-destructive DNA extraction method was also developed for use in the field, and situations where the mite cadaver needs to be retained for confirmatory tests. The method was based on the TE boiling method of Aoyama et al. (2015) with modifications. Briefly, individual mites were submerged in 20 µL of 1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0 within a 0.6 ml plastic microfuge tube and incubated for 5 min at 40°C. The tubes were then briefly centrifuged, and 15 µL of the supernatant transferred to a fresh tube for use in LAMP.

Three formats of the LAMP assays were validated: i) intercalating fluorescent dye-based assay in singleplex, hereafter referred to as dye-based LAMP, ii) assimilating probe-based LAMP in singleplex and duplex, hereafter referred to as probe-based LAMP, and iii) colorimetric LAMP. While the probe assay is often more specific as the probe is designed to be specific to the target sequence, and therefore less likely to produce late non-specific amplification, it is not colorimetric compatible, does not allow melt curve analyses, and synthesizing probe can add costs. The dye-based LAMP assays can sometimes result in late non-specific amplification, but it allows melt curve analyses to be performed to confirm positive results, it allows the conversion of the LAMP to colorimetric format.

LAMP assay optimization and validation were done using a WarmStart LAMP kit (Catalog number: E1700, New England BioLabs) following the manufacturer's instructions except reaction volumes reduced to 10 µL. Assays were run on a QuantStudio 5 (ThermoFisher Scientific) using standard PCR tubes. The assays were subjected to a vigorous validation process following the validation and verification of quantitative and qualitative test methods (National Association of Testing Authorities (NATA) Australia 2018), and specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity (EPPO 2021). The validation parameters include analytical specificity, sensitivity (limit of detection), accuracy (repeatability and reproducibility) and ruggedness (reproducibility under minor changes of test conditions). The assays were tested using an Isothermal Master Mix (catalog number: ISO-004, OptiGene, UK) and the LAMP device Genie II (OptiGene) to fulfil the assay's ruggedness validation. In addition, colourimetric detection method was performed using a WarmStart Colorimetric LAMP kit (Catalog number: M1800, New England BioLabs) as per manufacturer's instructions.

An undergraduate student was involved in validation of the avocado brown mite LAMP assay.

Table 4. Sequences of LAMP assay oligonucleotides and gBlocks™ gene fragments

Target species	Oligo names	Oligo types	Sequence (5'-3')
O. perseae	Ope28S-46-F3	Primer	CATCATWYGTAACTATGACCACA
	Ope28S-46-B3		TCACAGKCCAAGTCTCAA
	Ope28S-46-FIP		GAATAAATTCACAACTCCTCACATCAGGAGTGCATTTTCTCGTA
	Ope28S-46-BIP		TTCTCTGGTGATATCAGCAGGGTCTTGCAAGAAGAATGAGTGG
	Ope28S-46-LF		CGCGGTTTGAGCACGAGAG
	Ope28S-46-LB		GTACTAACCATACACCCAATCAACT
	Ope28S-46-FAM*	Assimilating probe	6FAM-ACGCTGAGGACCCGGATGCGAATGCGGATGCGGATGCCGAGTACTAACCATACACCCAATCAACT
	OpeQ-BHQ1	Quencher	TCGGCATCCGCATCCGCATTCGCATCCGGGTCCTCAGCGT-BHQ1
	Op28S-46gB	Synthetic positive control	GCCGCTACACGATTAGTTCTATCGCGGGCATCATTTGTAACTATGACCACACCCAGGAGTGCATTTTCTCGTAGGGCTCTCGTGCTCAAACCGCGCCCGATGTGAGGAGTTGTGAATTTATTCGGGGATATCTTCTCTGGTGATATCAGCAGGGTCCCGTACTAACCATACACCCAATCAACTGGGCCACTCATTCTTCTTGCAAGCCCTTGAGACTTGGCCTGTGAGGGGAAATGGCATGAGTCGAAATACCAGTC
O. punicae	OpuITS1-155-F3	Primer	WTAGTACYCTTTGCTYMCTT
	OpuITS1-155-B3		TCATGTTAATCTACATCAYAGTG
	OpuITS1-155-FIP		YCTCCTAACTGATTCCTTTATTGCTAGTATAAGGTTGCAAGGTTCA
	OpuITS1-155-BIP		AAGATCCGCCAGGTCTAGGGTATTCAATCTAGAAATAGGACCGTC
	OpuITS1-155-LF		CCAGCGGAGGGYAAYATCTCTT
	OpuITS1-155-LB		GGCATCATTACTTCGGTAATGGTGT
	OpuITS1-155-Cy5*	Assimilating probe	Cy5-ACGCTGAGGACCCGGATGCGAATGCGGATGCGGATGCCGAGGCATCATTACTTCGGTAATGGTGT
	OpuQ-BHQ3	Quencher	TCGGCATCCGCATCCGCATTCGCATCCGGGTCCTCAGCGT-BHQ3
	OpuITS1-155gB	Synthetic positive control	TTACCACGAAATAAATGCCGAAAGTGTTTACCCTACCTTATAGTACCCTTTGCTTCCTTAACTGGGAGTATAAGGTTGCAAGGTTCAGGGAAGAGATGTTACCCTCCGCTGGTCCCACTGGGTTAGCAATAAAGGAATCAGTTAGGAGGTCCCTGATATCAAGATCCGCCAGGTCTAGGGTCCCAGCGGCATCATTACTTCGGTAATGGTGTCCCGACGGTCCTATTTCTAGATTGAATAGGGCACTGTGATGTAGATTAACATGAGGGCATGCATGGGAGAGGTAGTATTCGGCCGAAATG

*Underlined parts in probe sequences are loop backward (LB) primers.

DNA barcoding of the avocado seed moth

Specimens of *S. catenifer* used in this study were imported from Mexico (Table 5). DNA was extracted using two different methods: a crude extraction method originally developed for whitefly and a commercial Quick-DNA Tissue/Insect Miniprep Kit (Zymo Research, Irvine, California) following the manufacturer's instructions.

The whitefly extraction method was based on that of De Barro and Driver (1997) with modifications by Sharon van Brunschot (unpublished). A small piece of the larvae or adult moth was placed in a microfuge tube containing 50 µL of lysis buffer (10 mM Tris-HCl, pH 8.0, 50 mM KCl, 0.45% (v/v) Tween 20, 0.45% (v/v) Triton X-100, and 60 µg/mL proteinase (New England Biolabs, MA, USA)) and homogenized using a plastic pipette tip. The tube was then incubated at 65 °C for 30 min, followed by 95 °C for 10 min to inactivate the proteinase K, and finally 50 µL of sterile distilled water was added before storage at -20°C.

Table 5. Details of *Stenoma catenifer* specimens imported from Mexico

Specimen ID	Stage	Host	Collection date	Collector	Nearest town	Origin
2/1/F	Larvae	*Persea americana*	8/03/23	N. S. Peñaranda	State of Colima	Mexico
2/2/F	Larvae	*P. americana*	8/03/23	N. S. Peñaranda	State of Colima	Mexico
2/5/H	Larvae	*P. americana*	8/03/23	N. S. Peñaranda	State of Colima	Mexico
GEO3	Adults	*P. americana*	8/03/23	N. S. Peñaranda	State of Colima	Mexico

We tested new primers targeting the cytochrome oxidase *c* subunit I (COI) of species in Lepidoptera developed from this project and published primers targeting the ITS regions (Table 6).

COI PCR amplifications using primers LepCOI-F1 and LepCOI-R2 were performed in a 20 µL reaction using Phusion High-Fidelity DNA Polymerase (Catalogue number: M0530L, New England Biolabs). Each reaction contained 4 µL of 5× Phusion HF Buffer, 0.4 µL of 10mM dNTP, 3.2 µL of LepCOI-F1 (10mM) primer and 2.4 µL of LepCOI-R2 (10mM) primer, 0.6 µL of DMSO, 0.2 µL of Phusion DNA polymerase, 1 µL of gDNA template (or water as no-template control), and water to the volume of 20 µL. The following thermocycling conditions were used: at 98°C for 20 s, 35 cycles of 98°C for 10 s, 50°C for 20 s, and 72°C for 20 s and a final elongation step at 72°C for 5 min. PCR products were size fractionated on 1.5% agarose gels prior to being directly sequenced by Macrogen Incorporated using an AB 3730xl DNA Analyzer (Applied Biosystems) to identify the species.

ITS PCR was performed using Phusion High-Fidelity DNA Polymerase. A 3× master mix of the reaction components was created and stored and -20°C: 540 µL of 5× HF Buffer, 54 µL of 10mM dNTP, 281 µL of milli-Q water and 25 µL of Phusion DNA polymerase (Phusion 3× MM). PCR amplification using the ITS1 and ITS2 primers (Table 6) used the same reaction, consisting of 5 µL of the Phusion 3× MM, 1 µL of each primer at 10 µM except primers 5.8SFc/28SB1d used at final concentration of 0.6 µM and 1.3 µM, respectively due to degeneracy, 1 µL of diluted (1 in 10) DNA as template and milli-Q water to a final reaction volume of 15 µL. Thermocycling conditions were as follows: 98 °C for 30 s; 35 cycles, each of 98 °C for 30s, 50 °C for primers 18SF1/5.8SB1d, 65 °C for primers 5.8SFc/28SB1d, and 69 °C for ILYC2F/ ILYC2RB for 30s, and 72 °C for 30s; and a finally 72 °C for 5 mins.

Table 6: Primers used to sequence for PCR and sequencing of *Stenoma catenifer*

Gene region	Primer name	Sequence (5' - 3')	Reference
COI	LepCOI-F1	TCWACWAAYCATAARGATATTGG	This paper
	LepCOI-R2	TCTATNCCDACNGTRAATATRTG	
ITS1	18SF1	TACAC ACCGC CCGTC GCTAC TA	Dai et al. (2012)
	5.8SB1d	ATGTG CGTTC RAAAT GTCGA TGTTCA	
ITS2	5.8SFc	TGAAC ATCGA CATTT YGAAC GCACAT	
	28SB1d	TTCTT TTCCT CCSCT TAYTR ATATG CTTAA	
	ILYC2F	GAGAAACATCCAGGACCACT	Shapoval and Lukhtanov (2015)
	ILYC2RB	CTGATCTGAGGCCA ACG	

Validation of a LAMP assay for detection of the laurel wilt pathogen

A Loop-Mediated Isothermal Amplification (LAMP) assay has been developed for rapid detection of the laurel wilt fungal pathogen (*Harringtonia lauricola*) from infected plant tissues and the beetle vector (*Xyleborus glabratus*) by Hamilton et al. (2020). In this project, we validated this published probe-based LAMP assay using DNA samples imported from the USA (Table 7). In addition, the following modifications were made to improve the assay: i) a gBlocks dsDNA fragment was designed, as described previously for mite LAMP assays, and synthesized by IDT to use as positive control, ii) intercalating dye-based format was validated as it is compatible with the colorimetric LAMP.

Probe-based LAMP reaction set up was as per Hamilton et al. (2020) except a WarmStart LAMP kit (Catalog number: E1700, New England BioLabs) was used and reaction volumes reduced to 10 µL. Dye-based LAMP reactions used the WarmStart LAMP kit following manufacturer's instructions except reaction volumes reduced to 10 µL. Colourimetric

LAMP was performed using a WarmStart Colorimetric LAMP kit (Catalog number: M1800, New England BioLabs) as per manufacturer's instructions.

In total, 77 DNA samples (Appendix 3) were imported from the University of Georgia (USA) with representative samples (Table 7) used for validation of the LAMP assay.

Specimens and sequence data

Exotic insect and plant pathogen specimens obtained during this project have been lodged in the most appropriate public collection, including the Queensland Museum Entomology Collection and the Queensland Department of Plant Pathology Herbarium and Insect Collections. Nucleotide sequence data corresponding to these specimens will be uploaded to GenBank upon publication of the research.

Creation of standard operating procedures

National Diagnostic Protocols (NDP) have been prepared for the target pests and pathogens: avocado sunblotch viroid (ASBVd), avocado scab (*Elsinoe perseae*), laurel wilt (*Harringtonia lauricola*), and Persea mite (*Oligonychus perseae*) following standard format provided by the Subcommittee for Plant Health Diagnostics (SPHD). NDP procedures were coordinated and administered by the National Plant Biosecurity Diagnostic Network (NPBDN) through Plant Health Australia.

Ongoing diagnostic support for ANVAS and other biosecurity surveillance activities

The project team continued to support ANVAS by providing testing services for ASBVd throughout the course of the project. Testings for ASBVd were done using the qPCR assay we developed as per NDP created from this project.

Results and discussion

Review on diagnosis of the high priority exotic pests and pathogens for the Australian avocado industry

The literature review is provided as Appendix 1. This review, although not yet published, was used to guide research done in this project.

Enhancing biosecurity diagnostic capacity

The avocado industry in Australia is dispersed across all six states. To transfer knowledge generated in this and previous projects to other diagnostic laboratories, a joint avocado and citrus biosecurity diagnostic workshop was held on 10-11 October 2023 in Brisbane. The workshop was jointly funded by projects AV21003 (UQ) and CT21005 (NSW DPIRD) as there is overlap in the teams of these projects, and the biosecurity challenges for avocado and citrus are similar. Fourteen delegates attended the workshop, representing UQ, NSW DPIRD, WA DPIRD, DAFF Mickleham, Biosecurity Queensland, Auscitrus.

The workshop was organized over one and a half days with main activities presentations on exotic pests and pathogens of avocado and citrus given by Andrew Geering and Nerida Donovan, group discussions on challenges and the way forward for biosecurity diagnostics of avocado and citrus EPPs, and finally demonstration by the two project teams on diagnostic techniques.

Minutes and recommendations from this workshop are presented in Appendix 2.

Development of a qPCR assay for the rapid detection of the avocado scab fungus *Elsinoe perseae*

Elsinoe perseae is a slow growing ascomycete fungus that rarely if ever produces a sexual stage in the field or in culture. Spore and hyphal morphologies of the asexual stage are non-diagnostic, and the scab symptoms on the fruit are also easily confused with symptoms caused by wind rub or thrips feeding damage. In this project, research was done to develop and validate qPCR assays to allow the fungus to be directly detected in symptomatic tissue, bypassing axenic

culturing steps, which may take months to complete. Two genetic loci were targeted, the internal transcribed spacer 1 region (ITS1) region of the rDNA, and the DNA-directed RNA polymerase subunit II (RPB2) gene. TaqMan™ probes were used, each labelled with a different fluorophore to allow duplexing of the two assays.

Using chemically synthesized DNA fragments as the target, the lowest copy number of DNA that was consistently and reliably detected by both the ITS and RPB2 primer and probe sets was 10^3 copies/µL (equivalent to 1.0 fg/µL DNA), corresponding to Ct values of 29.4 for the ITS and 26.4 for RPB2. The lowest concentration of *E. perseae* DNA from pure culture (gDNA) that was consistently detected using both the ITS and RPB2 assays was 0.1 ng/µL, with Ct values of 23.4 and 28.5, respectively. At a gDNA concentration of 0.01 ng/µL, *E. perseae* was also detected using the ITS primers and probe, with a Ct value of 27.1; however, the Ct value for RPB2 at this concentration was 32.4, exceeding the cutoff point of 30. Repeatability tests of the duplex qPCR assay demonstrated consistent amplification of the target *E. perseae* isolates, with no amplification of non-target isolates, negative controls, or no-template controls across various occasions under constant conditions, including the same experimentalist. Changes in the brand of DNA polymerase mix did not affect the specificity of the duplex qPCR assay, as all six *E. perseae* isolates and synthetic positive controls (gBlocks) were detected.

Elsinoe perseae was detected in fruit and leaves exhibiting typical avocado scab symptoms (Fig. 1) using ITS primers and probe (Ct values = 23.7–29.1). However, it was not found in asymptomatic fruit or in fruit with skin defects (Ct values >30), which were determined to be caused by other factors. The infection status of each sample was verified using the conventional ITS PCR assay of Everett et al. (2011), which produced amplicons of approximately 250 bp for positive samples (data not shown). The infected samples included different cultivars such as 'Lula', 'Miguel', and 'Hass', indicating that host genotype did not adversely affect assay performance.

Detection efficiency using the RPB2 primers and probe was poor, as only one of the previously confirmed positive samples tested borderline positive (Ct = 30) using this assay, while the other seven samples had Ct values above the detection threshold.

Fig. 1. Avocado plant samples collected in Florida (USA) used to validate the duplex TaqMan qPCR assay for *Elsinoe perseae* detection in plant tissues. Marked areas indicate the regions sampled for DNA extraction. Samples TREC-NT1 to TREC-NT6 exhibited avocado scab symptoms and tested positive by the qPCR assay, whereas TREC-NT7 to TREC-NT10, which showed other defects visually similar to scab, tested negative. TREC-11 was asymptomatic and also tested negative.

DNA barcoding methods for spider mites (Tetranychidae)

The DNA barcoding loci that have been adopted for spider mites are the cytochrome oxidase *c* subunit I (COI) gene, and the internal transcribed spacer region 2 (ITS2) of the rDNA. Existing PCR primers for these two DNA barcoding loci were designed 20 or more years ago using small sequence datasets, often without representative sequences from the Tetranychidae. There are no COI universal PCR primers that cover all species in the Tetranychidae, and while the existing ITS2 PCR primers have much greater species coverage than the COI PCR primers, they are not located in the most conserved positions. In this project, new DNA barcoding assays were designed, utilizing the much larger set of sequences for spider mites that have now been deposited in GenBank to identify optimal sites for PCR primer design.

Sixty-three entire COI gene sequences of spider mites were aligned, representing 19 species and six genera from subfamily Tetranychinae. From the alignment, two forward and two reverse primers were designed (Table 1). To investigate the theoretical coverage of the TetraCOI364-F and TetraCOI716-R primers, BLASTN searches of GenBank were done to identify matches with the much larger set of partial COI gene sequences that have been generated for the Tetranychidae. Both primer sequences were strongly conserved among 16 different genera from the Tetranychidae (Table 8), and even more broadly across subclass Acari.

The TetranychidaeCOI364-F and TetranychidaeCOI716-R primers were utilized in PCR, and amplicons of the expected size obtained from a taxonomically diverse panel of spider mites, including representatives of both subfamilies of the Tetranychidae, five different genera and 11 different species (Fig. 2). Positive PCR results were also obtained with the more distantly related mould mite *Tyrophaghus putrescentiae* (Acari: Acaridae), which was included in the testing panel as an outgroup.

Table 8. *In silico* validation of the TetraCOI$_{364}$-F and TetraCOI$_{716}$-R primers

Subfamily	Genus	TetraCOI$_{364}$-F		TetraCOI$_{716}$-R	
		M[1]	MM	M	MM
Tetranychinae	*Acanthonychus*	1	0	1	0
	Amphitetranychus	1	0	2	0
	Aplonobia	N/A[2]	N/A	1	0
	Eotetranychus	7	0	24	2
	Eurytetranychoides	N/A	N/A	1	0
	Eutetetranychus	N/A	N/A	2	0
	Mononychellus	3	0	1	0
	Oligonychus	15	0	24	0
	Panonychus	3	0	8	0
	Paraponychus	1	0	1	0
	Schizotetranychus	6	0	8	1
	Stigmaeopsis	6	0	7	0
	Tetranychus	70	1	29	2
	Yezonychus	N/A	N/A	0	1
Bryobiinae	*Bryobia*	44	4	3	0
	Petrobia	N/A	N/A	2	0
	Total	**157**	**6**	**114**	**6**

[1]M = perfect match, MM = mismatch at one position in the alignment

[2]N/A = not applicable, sequences not available spanning the primer binding site

Success was had generating a longer fragment of the COI gene from the panel of mite species under consideration using

the TetranychidaeCOI364-F primer in combination with a new reverse primer, TetraCOI932-R (Fig. 2). In a BLASTN search of GenBank, 144 sequences were retrieved covering the TetraCOI932-R primer binding site, of which 86 had exact matches. Finally, an even longer fragment of the COI gene (917 bp) was amplified when a new forward primer, TetraCOI16-F, was used in combination with the TetraCOI932-R primer. The TetraCOI16-F primer binding site is identical to that of the original LCO1490 primer of Folmer et al. (1994), although the TetraCOI16-F primer is two nucleotides shorter at the 5' end, and contains sequence degeneracy to account for variation observed within Tetranychidae sequences.

To design new ITS2 primers for the Tetranychidae, 5.8S and 28S rDNA gene sequences were extracted from GenBank and aligned. More strictly conserved sequence motifs were identified 124-nt upstream and 22-nt downstream of the forward and reverse primer-binding sites of Ben-David et al. (2007), to where new ITS2 primers were positioned. The newly designed Acari5.8S-F1 and Acari23S-R1 primers successfully amplified the ITS2 region of all spider mite species used in this study (Fig. 2). The broader applicability of these PCR primers for amplifying the ITS2 region of more taxonomically divergent species was demonstrated using the eriophyid mite *Aceria cynodoniensis* (data not shown) and the mould mite *Tyrophaghus putrescentiae* (Fig. 3).

To add extra confidence to identifications of spider mites made by sequence analysis, a third DNA barcoding locus was chosen, the variable 4 (V4) domain of the 18S rDNA. The V4 domain is widely used for metabarcoding of eukaryotic communities but has yet to gain traction with the Tetranychidae. Using the 18S rRNA structural model for *Demodex folliculorum* as a guide, the V4 region was defined as nucleotide positions 707–1093 of Tetranychus urticae GenBank sequence XR_003083727.1. PCR primers (AcariSSU_V4-F1/R1) were designed to conserved sequences on either side of the V4 domain, which are predicted to be universal across the Acari. Success was had using the AcariSSU_V4-F1/R1 primer pair in PCR, with amplicons of the expected size obtained from all ten spider mite species tested in our experiments (Fig. 3). Benefit was obtained from including DMSO in the PCR mixture, which reduced background non-specific amplification and increased the intensity of the target amplicons.

All PCR amplicons shown in Figs 2 and 3 were sequenced to demonstrate specificity of amplification, and significant matches (>99 %nt identity) obtained to reference sequences of each species in GenBank, when they existed.

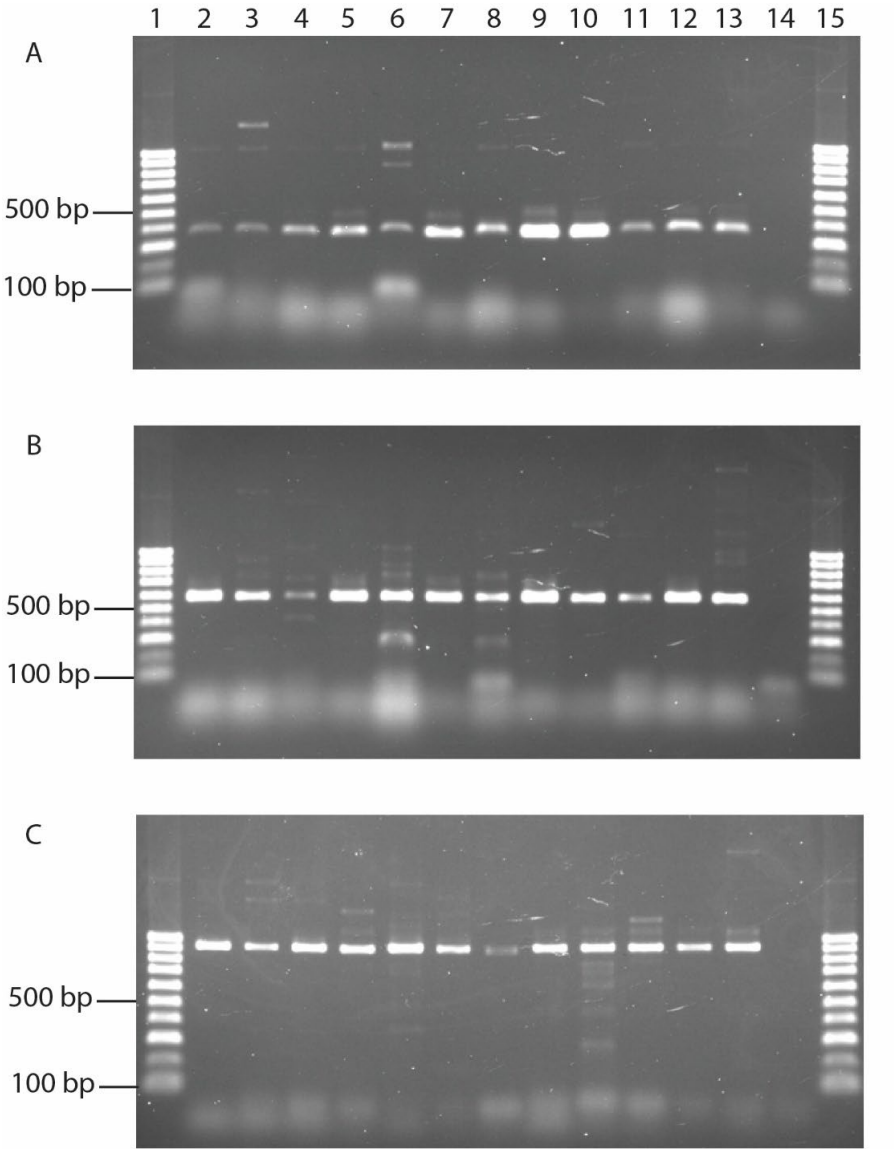

Fig. 2. Amplification of a fragment of the cytochrome c oxidase subunit I gene from mite taxa using (A) the TetraCOI364-F and TetraCOI716-R primer pair; (B) the TetraCOI364-F and TetraCOI932-R primer pair; and (C) the TetraCOI16-F and TetraCOI932-R primer pair. Lanes 1 and 15 are a 100 bp DNA ladder and lane 14 is a no-template negative control. In loading order, lanes 2–13 are: *Eotetranychus sexmaculatus*, *E. lomandrae*, *Eutetranychus africanus*, *Oligonychus coffeae sensu Mexico, O. perseae*, *O. punicae*, *Petrobia harti*, *Tetranychus ludeni, T. urticae*, *T. neocaledonicus*, *T. lambi* and *Tyrophaghus putrescentiae* (Acari: Acaridae). Two µL of PCR product was loaded in each lane.

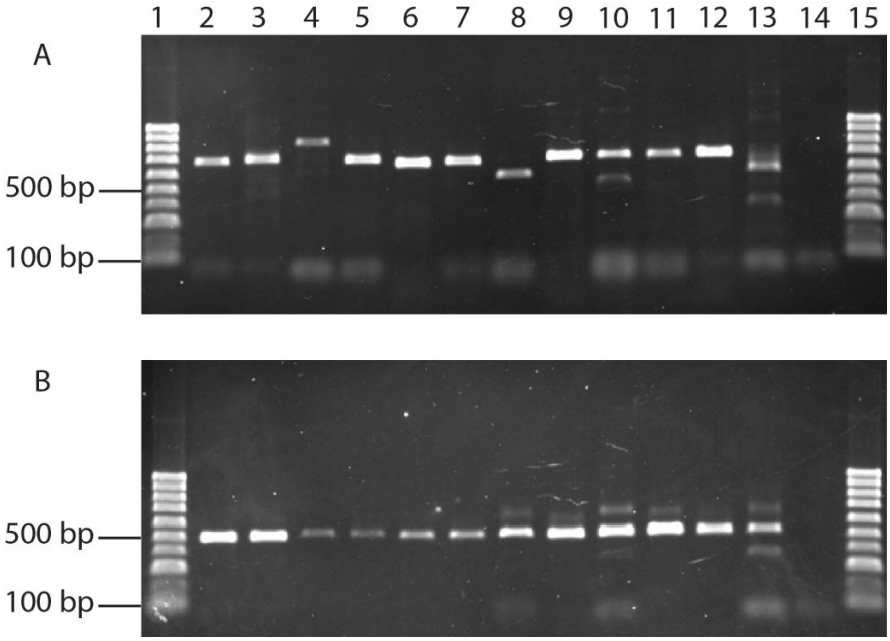

Fig. 3. Amplification of fragments of the 18S rDNA from mite taxa using (A) the Acari5.8S-F and Acari28S-R primer pair; (B) the Acari18SV4-F and Acari18SV4-R primer pair. Lanes 1 and 15 are a 100 bp DNA ladder and lane 14 is a no-template negative control. In loading order, lanes 2–13 are: *Eotetranychus sexmaculatus*, *E. lomandrae*, *Eutetranychus africanus*, *Oligonychus coffeae sensu Mexico*, *O. perseae*, *O. punicae*, *Petrobia harti*, *Tetranychus ludeni*, *T. urticae*, *T. neocaledonicus*, *T. lambi* and *Tyrophaghus putrescentiae* (Acari: Acaridae). Two µL of PCR product was loaded in each lane.

Development of LAMP assays for diagnoses of the Persea mite and avocado brown mite

The Persea mite (*Oligonychus perseae*) has spread to many parts of the world and is ranked as one of the highest priority biosecurity threats to Australia. The avocado brown mite (*O. punicae sensu lato*) is recorded as present in Australia (Beard 2018), but the type found in Mexico is genetically distinct from that found throughout the Old World and will likely be reclassified as a new species. Quarantine is necessarily conservative in nature, and following the precautionary principle, it is safest to assume that *Oligonychus punicae sensu* Mexico is also exotic to Australia. *O. perseae* and *O. punicae* are often found on the same but alternative sides of the leaf, and in the event of an interception at international ports of entry, it is necessary to be able to diagnose the specimens with speed and accuracy. However, morphological identification of species in the genus *Oligonychus* can be difficult even for experts due to the limited number of diagnostic characters, presence of intraspecific variation, minute differences in male aedeagus morphology and interspecific similarities in females (Mushtaq et al. 2021). *Oligonychus coffeae* is a common pest of avocado on the eastern seaboard of Australia (Rand and Schicha 1981), and there is potential to confuse this species with exotic *Oligonychus* species.

In this project, Loop-Mediated Isothermal Amplification (LAMP) assays were developed for the rapid diagnosis of *O. perseae* and *O. punicae sensu Mexico*. The LAMP assay format is advantageous over conventional PCR as it is isothermal, faster to complete, and has simpler reaction conditions, making it ideal for point-of-care and resource-limited settings. Most importantly, LAMP can be done by a person with limited technical skills. Each assay was rigorously validated following the requirements of the National Association of Testing Authorities (NATA) Australia 2018, and specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity (EPPO 2021). Validation was done for both singleplex and duplex formats with the latter allowing detection of both target species simultaneously.

Using the dye-based LAMP assay for *O. perseae*, the limit of detection was 10^2 copies of DNA/µL of the gBlocks synthetic gene fragment in 15 min (Fig. 4A), while for the *O. punicae* assay, the limit of detection was 10^3 copies of DNA/µL in 17.5 min (Fig. 4B). Distinct melt curve profiles were observed for native DNA and gBlocks amplicons for each species, with melt temperatures of 83.6 ± 0.09 °C and 84.6 ± 0.10 °C for *O. perseae,* respectively (Fig. 4C) and 83.6 ± 0.37 °C and 85.37 ± 0.80 °C for *O. punicae* (Fig. 4D), respectively.

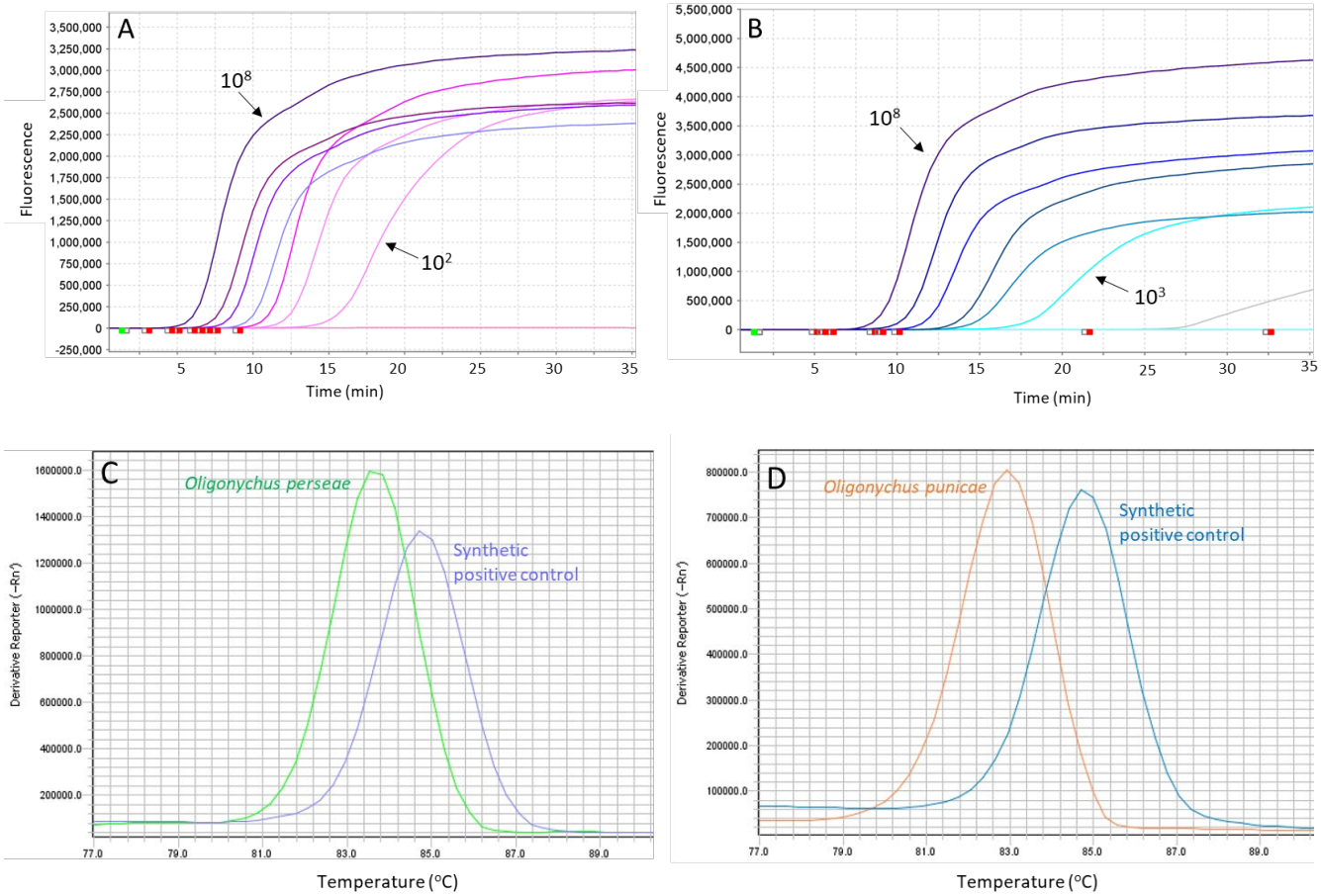

Fig. 4. Sensitivity of dye-based, singleplex LAMP assays, for detection of (A) *Oligonychus perseae* and (B) *O. punicae.* Detection of each species was evaluated using synthetic positive controls (gBlocks) at ten-fold serial dilutions, ranging from 10^8 to 10 copies/µL. Differences in melting temperatures of the gBlocks compared to native DNA amplicons are shown for (C) *O. perseae* and (D) *O. punicae*.

The probe-based LAMP assays worked well in both singleplex and duplex formats (Fig. 5). Sensitivity of the *O. perseae* LAMP assay was similar between singleplex and duplex, with a limit of detection at 10 copies/µL (Fig. 5A) in under 18 min. The lowest concentrations detected for both singleplex and duplex formats of the *O. punicae* LAMP assays were 10^3 copies/µL, at which a delayed detection was found for the duplex format (25 min) compared to the singleplex format (17 minutes) (Fig. 5B). Further optimization of the primer ratios, probe and quencher rations may improve performance of the *O. punicae* multiplexed assay.

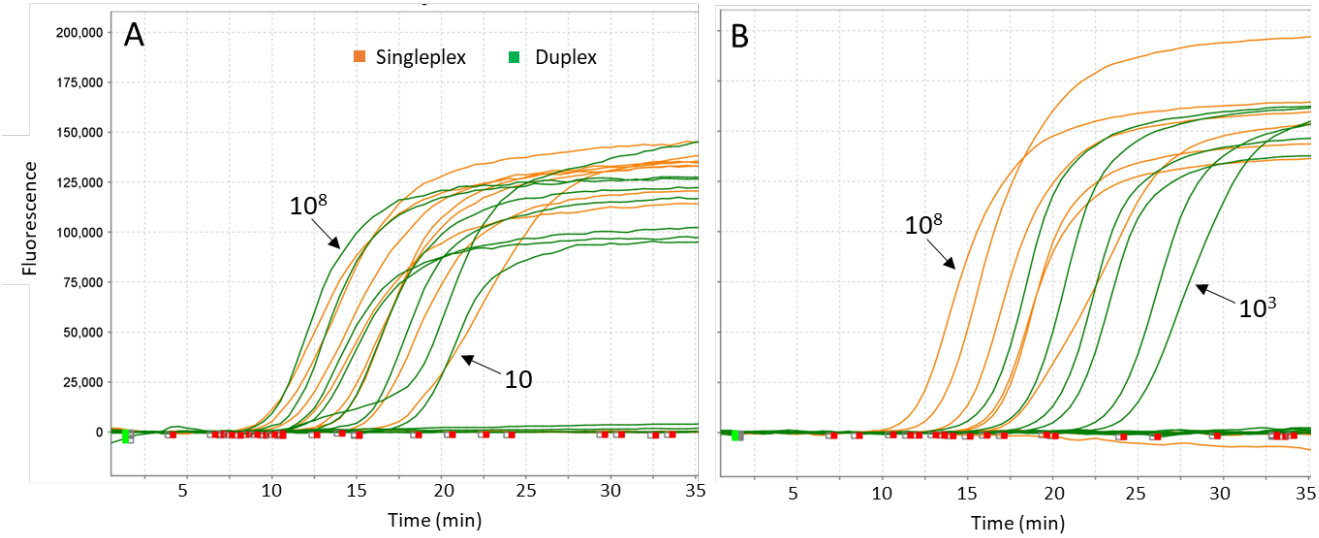

Fig. 5. Sensitivity of probe-based LAMP assays for detection of (A) *Oligonychus perseae* and (B) *O. punicae* in singleplex (orange) and duplex (green). Detection of each species was evaluated using synthetic positive controls (gBlocks) at ten-fold serial dilutions, ranging from 10^8 to 10 copies/µL.

The specificity of the LAMP assays was validated using target species specimens imported from Mexico and a range of non-target taxa. All *Oligonychus perseae* and *O. punicae* specimens were detected by the corresponding LAMP assays with no cross-reactivity between these two species, and no off-target amplification to other taxa observed.

The dye-based LAMP assays were generally faster to reach completion than the probe-based LAMP assays: all O*. perseae* specimens gave positive reactions within 10 min and all *O. punicae* specimens within 20 min using the dye-based LAMP assays, whereas the equivalent probe-based LAMP assays took 20 min and 25 min, respectively. However, the dye-based assays produced late non-specific amplification for some non-target species (e.g. *Tetranychus ludeni*) when reactions were run longer than 20 min. Hence, the recommended cut-off time for positive amplification of the dye-based LAMP assays is 20 min. It is also recommended that amplicon melting temperatures be analysed to confirm true positive reactions. The probe-based LAMPs showed no late non-target amplification and thus a cut-off time for a positive reaction is not necessary.

The LAMP assays were shown to be robust, as they performed well using other LAMP kit (i.e. OptiGen) on the portable LAMP device Genie II, and using DNA extracted by our simple, rapid, and non-destructive DNA extraction method (data not shown).

Colorimetric LAMP assays were accessed by the color change in the reaction tubes from pink to yellow for positive reactions while negative reactions remained pink (Figs. 6 and 7). We showed that the *O. perseae* and *O. punicae* colorimetric LAMP assays were specific to detect only the target species within 30 minutes of incubation at 65°C. No cross-reactivity with non-target species was observed. A time series assessment was done for the *O. perseae* assay from which color was found started to change at 15 minutes after incubation (data not shown) and developed to a stronger yellow color at 30 min. Further works are still required to test the assays on a wider range of specimens to confirm the assay's specificity. In addition, comparing the sensitivity and time to positivity of colorimetric with the standard LAMP formats (probe-based and intercalating dye-based) to better understand the assay performance to allow fit for purpose application.

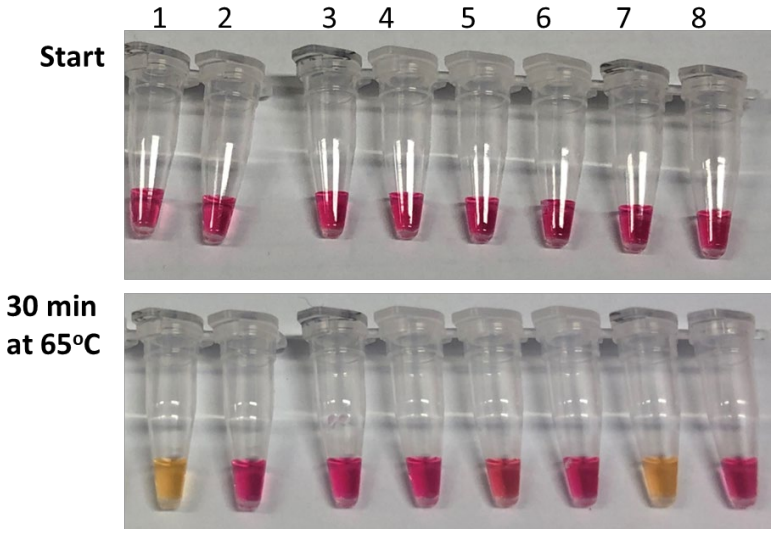

Fig. 6. Detection of *Oligonychus perseae* using species-specific colorimetric LAMP. Tube 1 = *O. perseae* (NT4), tube 2 = *O. punicae* (NT2 Opun), tube 3 = *Tetranychus ludeni*, tube 4 = = *O. punicae* (NT1), tube 5 = *O. coffeae* (LSP44.1), tube 6 = *T. urticae* (LSP46.1), tube 7 = Ope28S-46gB at 10^5 copies per µL (positive control), tube 8 = water (no-template negative control).

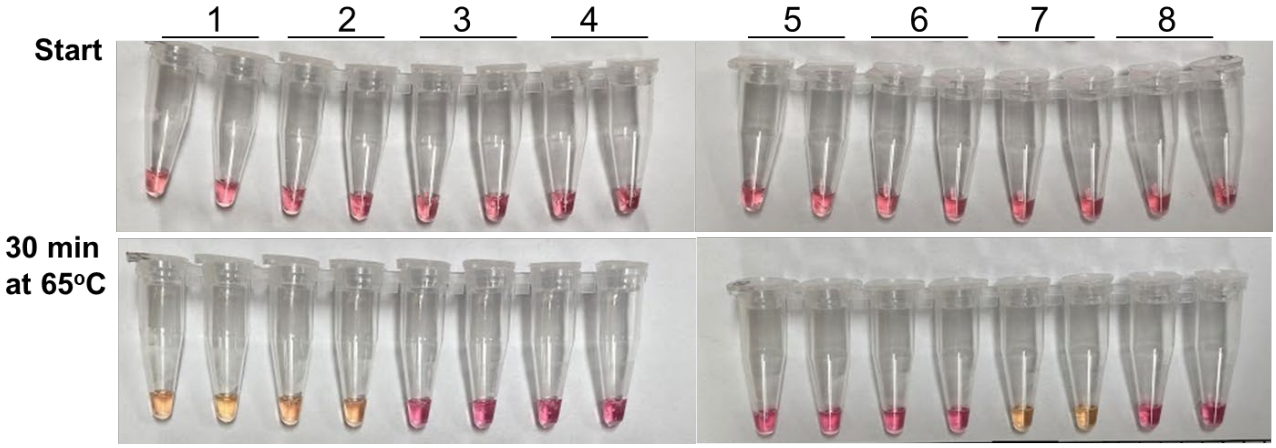

Fig. 7. Detection of *Oligonychus punicae* using species-specific colorimetric LAMP. Positive reactions showed by colour changed from pink to yellow while negative reaction tubes remained pink. Samples were tested in duplicate. Tubes 1 = *O. punicae* (NT1), tubes 2 = *O. punicae* (NT2 Opun), tubes 3 = *Tetranychus urticae* (LSP46.1), tubes 4 = *T. ludeni* (LSP21), tubes 5 = *perseae* (NT5), tubes 6 = *O. coffeae* (LSP44.1), tubes 7 = OpuITS1-155gB at 10^4 copies per µL (positive control), tubes 8 = water (no-template negative control).

Development of DNA barcoding for identification of avocado seed moth

The genus *Stenoma* is exclusively a neotropical genus of moths, with a primary centre of diversity in Central America. The detection of any *Stenoma* species in Australia would be of biosecurity significance as none currently occur here. The population genetics of the avocado seed moth *S. catenifer* is poorly understood. We therefore designed new primers targeting the COI of the family Lepidoptera as well as validated ITS primers published for other moth species (Dai et al. 2012; Shapoval and Lukhtanov 2015). The PCR assays were validated using *S. catenifer* specimens imported from Mexico.

Our new COI primers worked well, amplifying an approximately 870 bp product for avocado seed moth specimens (Fig. 8). Sequencing of the PCR products provided a positive identification of the species, and the four specimens that were sequenced had >99.8% nucleotide identity to each other. All sequences of *S. catenifer* available on GenBank correspond to the COI gene and were generated using published primers LCO1490/HCO2198 (Folmer et al. 1994). Our forward primer

binds to similar position as the LCO1490 primer but our reverse primer binding site is further upstream of HCO2198, resulting in approximately 165 nucleotide (nt) longer sequence.

An additional 21 sequences of *S. catenifer* specimens, collected from different locations in Mexico, generated using the published primers were provided to us by N. Sanhueza-Peñaranda Colegio de Postgraduados. We aligned the *S. catenifer* sequences with all *Stenoma* spp. sequences retrieved from GenBank, resulting in a 636 nt long sequence alignment which showed small variation of 93.9–100% nt identity within *S. catenifer* and 84.6–89.7% nt identity between *Stenoma* spp. This lack of a large DNA barcode gap between *Stenoma* spp. Poses challenges for designing species-specific assays in the future, and further research is required to understand the population genetics of this genus of moths.

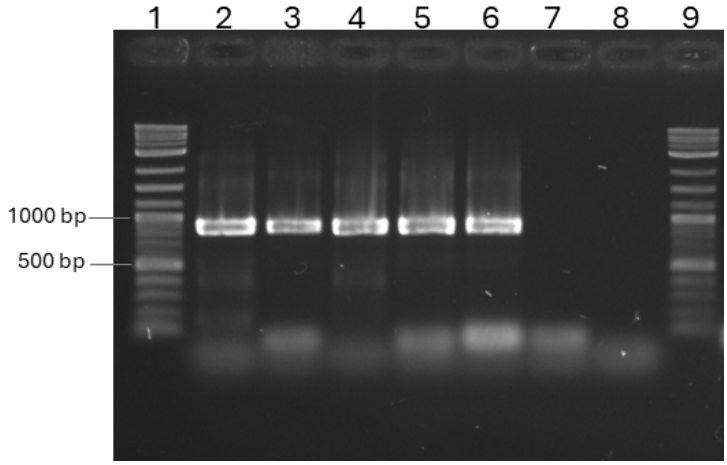

Fig. 8. PCR amplification of the COI region of the genus *Stenoma* using new primers LepCOI-F1 and LepCOI-R2 developed from this study. Lanes 1 and 9 are a 100-bp DNA ladder, lane 2 is specimen 2/1/F, lanes 3 and 6 are 2/2/F, lane 4 is 2/5/F, lane 5 is Geo3 and lanes 7 and 8 are no-template negative control. Predicted amplicon size is 870 bp.

Development of a LAMP assay for detection of the laurel wilt pathogen *Harringtonia lauricola*

Both probe- and dye-based LAMP assays specifically detected *H. lauricola* within 15 min using DNA extracted from fungal cultures and 25 minutes of DNA extracted from infected plant tissues or vector beetles (*Xyleborus glabratus*). No non-specific detection of non-target species were observed (Table 7). Similar to the mite LAMP assays, the dye-based detection method was found to produce late non-specific amplifications when reactions were run past 25 min, but these amplifications were confirmed to be true negatives by melt curve analysis. The probe-based LAMP was shown to be specific with no non-specific amplifications.

The newly designed gBlocks was successful amplified by both probe- and dye-based assays (Fig. 9). A clear difference in melting point for *H. lauricola* DNA (89.2±0.07°C) and for the gBlocks (90.1 ±0.12°C) (Fig. 9), which helps to differentiate the detection of the target species to the positive control.

Table 7. Specificity of LAMP assay for detection of *Harringtonia lauricola* from infected plant tissues and vector beetles.

Isolate code	Species	Host/Source	Origin	LAMP results[1]
CV-2017-004	*Harringtonia lauricola*	*Xyleborus glabratus*	South Carolina, United States	+
CV-2017-005	*Harringtonia lauricola*	*Xyleborus glabratus*	South Carolina, United States	+
CV-2017-006	*Harringtonia lauricola*	*Xyleborus glabratus*	South Carolina, United States	+
CV-2017-010	*Harringtonia lauricola*	*Persea borbonia*	South Carolina, United States	+
CV-2017-014	*Harringtonia lauricola*	*Sassafras* sp.	Early County, Georgia	+
CV-2017-021	*Harringtonia lauricola*	*Persea borbonia*	Georgia, United States	+
CV-2017-067	*Harringtonia lauricola*	*Sassafras* sp.; *Xyleborus glabratus*	Alabama, United States	+
CV-2017-101	*Raffaelea ambrosiae*	*Quercus* sp.; *Platypus cylindrus*	Southern England	-
CV-2017-040	*Raffaelea arxii*	*Xyleborus volvulus*	Florida, United States	-
CV-2018-026	*Raffaelea brunnea*	n/a	Florida, United States	-
CV-2017-102	*Raffaelea canadensis*	*Pseudotsuga menziesii; Platypus wilsonii*	Canada	-
CV-2017-104	*Raffaelea ellipticospora*	*Xyleborus glabratus*	South Carolina, United States	-
CV-2017-029	*Raffaelea fusca*	*Xyleborus volvulus*	Florida, United States	-
CV-2017-030	*Raffaelea fusca*	*Xyleborus volvulus*	Florida, United States	-
CV-2017-097	*Raffaelea gnathotrichi*	*Picea engelmannii*; *Gnathotrichus retusus*	Colorado, United States	-
CV-2017-064	*Raffaelea* PL1004 *aguacate*			-
CV-2017-042	*Raffaelea* PL6099 *xyleborina*	*Xyleborus glabratus*	Florida, United States	-
CV-2017-033	*Raffaelea* sp. PL1001	*Xyleborus volvulus*	Florida, United States	-
CV-2017-024	*Raffaelea subalba*	*Xyleborus bispinatus*	Florida, United States	-
CV-2017-032	*Raffaelea subalba*	*Xyleborus volvulus*	Florida, United States	-
CV-2017-027	*Raffaelea subfusca*	*Xyleborus volvulus*	Florida, United States	-
CV-2017-028	*Raffaelea subfusca*	*Xyleborus volvulus*	Florida, United States	-
Beetle-01	*Xyleborus glabratus*	n/a	n/a	+
Beetle-02	*Xyleborus glabratus*	n/a	n/a	-
Beetle-13	*Xyleborus glabratus*	n/a	n/a	-
RL4	*Harringtonia lauricola*	*Persea americana*	United States	+
TREC-NT12	*Harringtonia lauricola*	*Persea americana*	United States	+
TREC-NT13	*Harringtonia lauricola*	*Persea americana*	United States	+
TREC-NT14	*Euplatypus parallelus*	n/a	United States	-
LWP665	*Raffaelea sulphurea*	n/a	United States	-

[1]"+" indicates positive results and "-" indicates negative results.

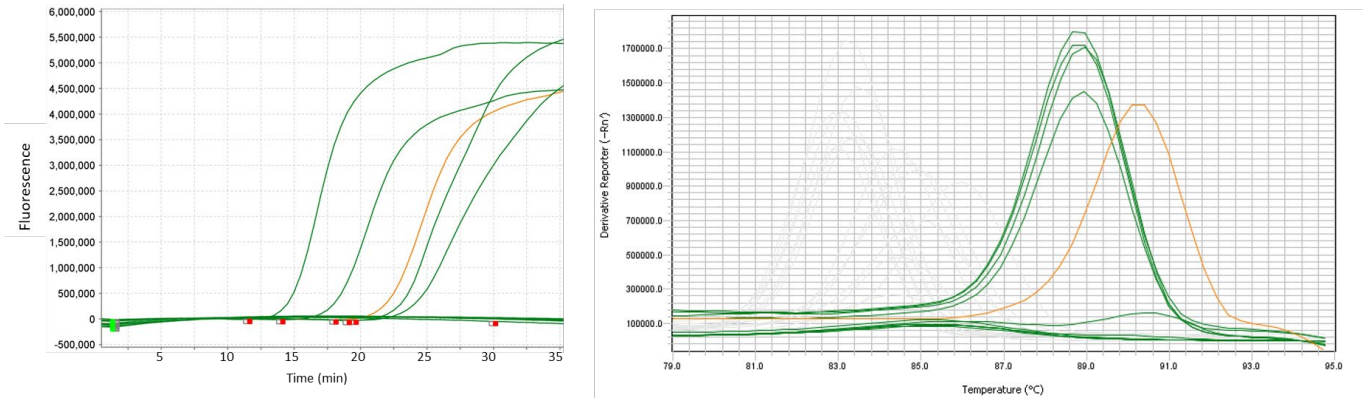

Fig. 9. Detection of *Harringtonia lauricola* (green) and synthetic positive control (orange) using dye-based LAMP assay showing (left) amplification plot and (right) melt curve plot.

The target fungal species *H. lauricola* and positive control were successful detected by colorimetric LAMP (Fig. 10) while non-target samples and negative controls were negative. However, we noted that sample TREC-NT13 (tube 3), which is a DNA sample extracted from infected plant tissues, was negative after 30 minutes incubation while it was tested positive by the probe- and dye-base LAMP assays. It is possible that quantity of the target fungal DNA was low in this sample and that the standard LAMP formats are more sensitive than colorimetric format. Further work to compare the sensitivities of different LAMP formats would provide insights into the assay performance and to investigate if longer incubation time would reduce false negatives by colorimetric LAMP in samples with low concentration of the target species.

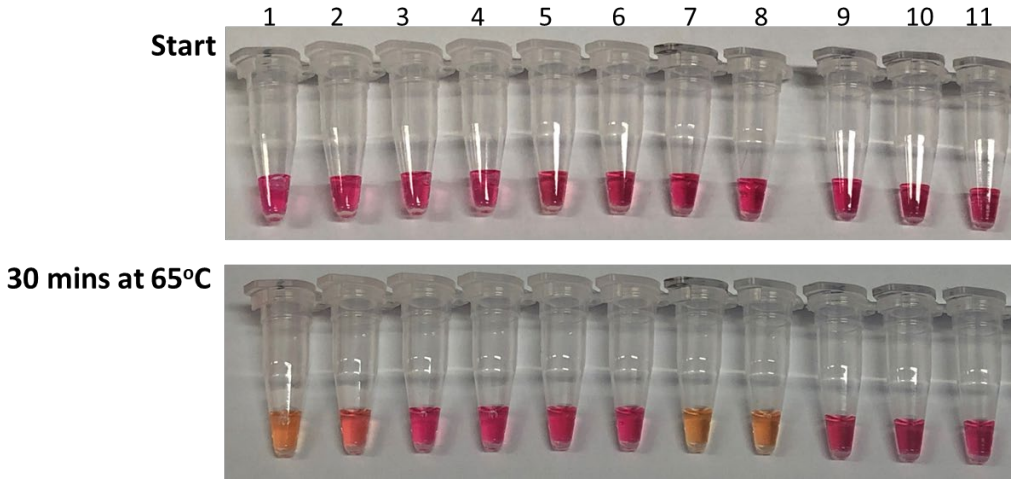

Fig. 10. Detection of *Harringtonia lauricola* using colorimetric LAMP assay. Positive reactions showed by colour changed from pink to yellow while negative reaction tubes remained pink. Tube 1 = *H. lauricola* (CV-2017-006), tube 2 = *H. lauricola* (TREC-NT12), tube 3 = *H. lauricola* (TREC-NT13), tube 4 = *Euplatypus parallelus*, tubes 5 = *Raffaelea* PL1004 *aguacate*, tube 6 = *Raffaelea sulphurea*, tube 7 = *H. lauricola* (RL4), tube 8 = gBlocks RT-BTgB 10^5 copies per µL (positive control), tube 9 = healthy avocado cv, Lula (negative control), tube 10 = healthy avocado cv. Hass, and tube 11 = water (no-template negative control).

Specimens and sequence data

List of specimens obtained from overseas during this project are provided as Appendix 3. Submission of nucleotide sequence data generated from this project to GenBank is in progress and sequences will make available upon request.

Development of national diagnostic protocols

National Diagnostic Protocols (NDP) have been prepared for the target pests and pathogens: avocado sunblotch viroid (ASBVd), avocado scab (*Elsinoe perseae*), laurel wilt (*Harringtonia lauricola*), and Persea mite (*Oligonychus perseae*) following standard format provided by the Subcommittee for Plant Health Diagnostics (SPHD).

NDP procedures were coordinated and administered by the National Plant Biosecurity Diagnostic Network (NPBDN) through Plant Health Australia. However, during this project, the coordination of the NPBDN and administration of NDP has been transitioned to the Australian Government (Department of Agriculture, Fisheries and Forestry) leading to some delay in the NDP submission process.

Diagnostic support for ANVAS

The project team continued to support ANVAS by providing testing services for avocado sunblotch viroid (ASBVd) throughout the course of the project. In total, 5,770 samples were submitted to the laboratory from different nurseries/orchards to test for ASBVd. Testings for ASBVd were done using the qPCR assay we developed as per NDP created from this project. All were tested negative (Table 9).

The economic impact of ASBVd can be significant, as yield reduction of up to 80% can be seen in severely infected trees

and the disease downgrades fruit quality (Geering 2018). The presence of the viroid in Australian orchards is very rare and therefore the more damaging impact the viroid poses to the industry is trade impediment due to quarantine conditions imposed on the movement of fresh fruit due to the risk of transmitting (Geering 2018). The diagnostic support provided by this project has allowed Australian avocados to meet the export market requirements and thus facilitating export opportunities (Briggs 2024). The continuation of this service also allows infected trees to be removed towards a pest-freedom status of ASBVd in Australia.

Table 9. Details of avocado samples tested for ASBVd from 2022-2026

Date	Nursery	Number of samples	Type of testing	Results
24-Feb-22	Anderson Horticulture	758	Multiplication	Negative
4-Mar-22	Anderson Horticulture	193	Multiplication	Negative
29-Aug-22	Anderson Horticulture	13	Multiplication	Negative
29-Nov-22	Anderson Horticulture	9	Multiplication	Negative
6-Feb-23	Anderson Horticulture	45	Multiplication	Negative
29-Sep-24	Anderson Horticulture	550	Multiplication	Negative
31-Aug-22	Chislett Farms and Nursery	19	Multiplication	Negative
27-Sep-22	Costa Group Renmark	480	Multiplication	Negative
5-Jul-22	Cottrell Farms	300	Multiplication	Negative
11-Jan-22	Howe Farming	2	Orchard trees	Negative
29-May-24	Sunshine Horticultural Services	19	Budwood testing	Negative
25-Mar-22	Fleming's Nurseries	166	In-line	Negative
22-Jun-22	Fleming's Nurseries	67	In-line	Negative
21-Apr-23	Fleming's Nurseries	418	In-line	Negative
15-Sep-25	Fleming's Nurseries	81	In-line	Negative
25-Mar-22	Turkinje Nursery	239	In-line	Negative
20-Jun-22	Turkinje Nursery	164	In-line	Negative
11-Oct-22	Turkinje Nursery	282	In-line	Negative
16-Jan-23	Turkinje Nursery	311	In-line	Negative
7-Jun-23	Turkinje Nursery	67	In-line	Negative
3-Sep-24	Turkinje Nursery	89	In-line	Negative
5-Jan-25	Turkinje Nursery	372	In-line	Negative
17-Jun-25	Turkinje Nursery	287	In-line	Negative
8-Dec-25	Turkinje Nursery	379	In-line	Negative
14-Apr-26	Turkinje Nursery	460	In-line	Negative
	Total	**5,770**		

Outputs

Table 10. Output summary

Output	Description	Detail
Validated diagnostic assays and standard operating procedures (SOPs) for high priority avocado pests: avocado sunblotch viroid (ASBVd), avocado scab, Persea mite, laurel wilt and the avocado seed moth.	National diagnostic protocol (NDP) for avocado sunblotch viroid (ASBVd) contains methods to detect and identify the viroid, and other information to support surveillance activities. It has been reviewed by SPHD and the final revised version is ready for resubmission for publication through the National Plant Biosecurity Diagnostics Networks (NPBDN) for access by NPDBN members.	The ASBVd diagnostic protocol has been published (https://doi.org/10.1016/j.jviromet.2022.114455 and a National Diagnostic Protocol (NDP) submitted to SPHD. The NDP has also been shared with colleagues at the NSW DPIRD, WA DPIRD, NASQ, DAFF Mickleham, and Biosecurity Queensland. The ASBVd NDP was provided as Appendix 3 with milestone 107 report submission. The revised version will be provided upon request.
	A novel qPCR assay has been developed and vigorously validated following NATA and EPPO standards for specific, accurate and rapid diagnosis of the avocado scab pathogen *Elsinoe perseae;* NDP describing the methods for diagnosis of this this pathogen has been developed.	The qPCR assay targeting the ITS region successfully detected *E. perseae* using DNA extracts from pure cultures as well as infected plants tissues without the need to culture the pathogen. Additionally, the assay can be used in duplex format to simultaneously target the ITS and RPB2 to increase confidence of the test results. An expression of interest (EOI) to develop a NDP for this pathogen has been approved by SPHDS; the NDP has been developed and ready for submission. NDP was provided as Appendix 4 with milestone 107 report submission. The updated version will be provided upon request. Due to transition of the coordination of the NPBDN and administration of NDP from Plant Health Australia to the Australian Government, Department of Agriculture, Fisheries and Forestry during this project, the NDP submission process has been slow. The submission process will continue in project AV25012.
	A published LAMP assay for diagnosis of *Harringtonia lauricola* has been validated and NDP detailing the diagnostic methods has been created.	The published LAMP assay (Hamilton et al. 2020) has been validated in our laboratory using DNA of *H. lauricola* extracted from infected host plants including redbay (*Persea borbonia*), avocado (*Persea americana*), the beetle vectors (*Xyleborus glabratus*), related plants species and beetles imported from the USA. Improvements were made to the published assay, including: (i) intercalating dye-based assay was developed in addition to validating the originally published assimilating probe-based assay. Intercalating dye often comes with LAMP kit and thus it is a more cost-effective detection method than synthesizing a probe. (ii) a colorimetric LAMP was developed from which a positive reaction changes color from pink to yellow for visible detection.

		This is a simple method, requiring no expertise, field and low resource settings compatibility. (iii) a synthetic DNA (gBlocks dsDNA fragment) designed to be used positive control. This is particularly beneficial for diagnosis of exotic pests and pathogens where positive DNA sources are often limited and it provides consistent control across test runs/laboratories etc. for improved test's reliability. The assays were shown to be accurate, simple and rapid with detection of *H. lauricola* from both infected plants and vector beetles in under 25 min for the probe- and dye-based assays and 30 minutes for the colorimetric assay. An expression of interest (EOI) to develop a NDP for laurel wilt has been approved by SPHDS; the NDP has been developed and ready for submission which will continue on to AV25012. The last draft of this NDP was provided as Appendix 3 with milestone 104 report submission. The updated version is available upon request.
	NDP for Persea mite (*Oligonychus perseae*) has been created that describes the novel LAMP assay we have developed and validated for this exotic pest. PCR assay utilising the new DNA barcoding primers developed from the present study is also included as a confirmatory test.	The LAMP assay was designed to be used either as singleplex or duplex formats detecting both persea mite and avocado brown mite (*O. punicae sensu Mexico*) simultaneously. Either format was shown to be specific, with no cross-reactivity, in detecting the target species. Detection was achieved within 25 minutes from mite DNA samples. New DNA barcoding PCR assays targeting the cytochrome oxidase *c* subunit I (COI) gene and internal transcribed spacer 2 (ITS2) region, and the variable region 4 (V4) of the 18S rDNA developed that allows identification of members of the family Tetranychidae. Expression of interest (EOI) to develop NDP for persea mite has been approved by SPHD; the NDP has been developed and ready for submission which will continue on to AV25012. The NDP was provided as Appendix 2 with milestone 107 report submission - updated version is available upon request.
	DNA barcoding PCR assays have been developed for avocado seed moth (*Stenoma catenifer*).	There were only three sequences, all of the COI, publicly available for *S. catenifer* at the start of this project. This hindered the development of a molecular diagnostic assay. We therefore evaluated published primers of genomic regions including the ITS and new COI primers for Lepidoptera. Validation of the PCR assays was done using specimens imported from Mexico. Details of the PCR assays and sequence information are provided under 'Results and Discussion' section. The new sequence information will improve our understanding of the population genetics prior to developing diagnostic assays which will be continued in project AV25012.
Data on surveys and identification of high priority pests and pathogens to underpin pest free status	A collection of specimens and/or DNA of high priority pests and pathogens (HPPs) were imported overseas. In addition, specimens of endemic species that are related to the target HPPs	A list of HPPs species collected and identified from the present study is included as Appendix 3. Endemic samples are provided under 'Results and Discussion' section.

	were collected in Australia for validation of diagnostic assays and for identification to underpin pest free status.	
Testing services for ASBVd provided to the industry to support for ANVAS	During this project 5,770 samples were tested for ASBVd, all negative, with 26 diagnostic reports generated for sample submitters (e.g. growers/extensionists).	Diagnostic reports will be available upon request.
Secondary testing centre established in Perth and WA DPIRD & NSW DPIRD staff trained through diagnostic workshops and sharing of diagnostic protocols	A joint avocado and citrus biosecurity diagnostic workshop was held in Brisbane in 2023 to share knowledge and diagnostic techniques generated in this and previous projects to other diagnostic laboratories.	The workshop was jointly funded by projects AV21003 (UQ) and CT21005 (NSW DPIRD) as there is overlap in the teams of these projects, and the biosecurity challenges for avocado and citrus are similar. Fourteen delegates attended the workshop, representing UQ, NSW DPIRD, WA DPIRD, DAFF Mickleham, Biosecurity Queensland, Auscitrus. The workshop was organized over one and a half days with main activities presentations on exotic pests and pathogens of avocado and citrus given by Andrew Geering and Nerida Donovan, group discussions on challenges and the way forward for biosecurity diagnostics of avocado and citrus EPPs, and finally demonstration by the two project teams on diagnostic techniques. Regular communications are ongoing between the project team and WA and NSW DPIRD regarding biosecurity diagnostics. Minutes and recommendations from this workshop are presented in Appendix 2.
Manuscripts/reports on literature review, development & validation of diagnostic assays for high priority pests & pathogens, and survey results. Articles published in Talking Avocados	Several reports and manuscripts have been generated from the present project.	The below reports are provided as Appendices. Appendix 1. Review report: Geering ADW, and Tran NT, 2023. Diagnosis of the high priority exotic pests and pathogens for the Australian avocado industry. Appendix 2. Diagnostic workshop minutes and recommendations. Appendix 3. List of specimens obtained during this project. Appendix 4. Article: Geering A, Tran N, 2024. Mexico and Florida biosecurity study tour. *Talking Avocados*. Spring 2024 issue. Appendix 5. Presentation: Tran N, Pretorius L, Parkinson L, Geering A, 2024. Innovations in plant biosecurity research for the Australian avocado industry. Colegio de Postgraduados, Mexico, 9 February 2024. Appendix 6. Presentation – Tran N, 2024. Towards finalizing NDPs for avocado scab and laurel wilt. Annual Diagnostic Workshop 19-20 March 2024. Tweed Heads NSW. Appendix 7. Poster: Tran N, Pretorius L, Parkinson L, Geering A, 2025. Rapid detection of avocado biosecurity threats. Prepared for Avocado Australia to present as various industry meetings and events.

		Appendix 8. Poster: Garty S, Tran N, Pretorius L, Geering A, 2025. Shining a light on the avocado mite: A novel LAMP assay for rapid detection of biosecurity pest *Oligonychus punicae*. Research project I (SCIE3121) final presentation, The University of Queensland. Professor Geering contributed to the biosecurity section for "The avocado problem solver field guide (2nd edition)", accessible via Avocados Australia: https://avocado.org.au/public-articles/problem-solver/ The below manuscripts have been prepared: 1. Manuscript: Tran NT, Parkinson LE, Pretorius LS, Gazis R, Ganan-Betancur L, Garty S, and Geering ADW, 2026. Development of a qPCR assay for the rapid detection of the avocado scab fungus *Elsinoe perseae*. *Australasian Plant Pathology*. *Under review*. This manuscript was provided as Appendix 5 with milestone 107 report submission. 2. Manuscript: Pretorius LS, Tran NT, Garty S, Beard JJ, and Geering ADW, 2026. Updated DNA barcoding methods for spider mites (Tetranychidae). *Ready for submission*. 3. Manuscript: Tran NT, Garty S, Pretorius LS, Hernández HG, Galici TS, and Geering ADW, 2026. Development of a LAMP assays for the rapid diagnosis of two spider mites (Trombidiformes: Tetranychidae) that infest avocados. *In preparation.* All manuscripts will be available upon request. They will also be sent to Hort Innovation when seeking permission for submission. Hort Innovation will be notified once papers are published.

Outcomes

Table 11. Outcome summary

Outcome	Alignment to fund outcome, strategy and KPI	Description	Evidence
Intermediate outcomes: Improved diagnostic capacity and capability for high priority pests and pathogens for the avocado industry through updated and/or development of novel diagnostic protocols subjected to a rigorous validation process. Improved ability of the avocado industry to respond to exotic pests and pathogens in a timely manner through effective and fit for purpose disease diagnostics under Australian conditions. Improved human capability and enhanced understanding of pest and pathogen invasion pathway, biology & epidemiology	Relevant SIP outcome: The Australian avocado industry has improved profitability, efficiency and sustainability through globally competitive production systems, orchard management, varieties, innovative R&D and sustainable BMPs.	Rigorously validated diagnostic protocols in the forms of national diagnostic protocols and/or scientific papers communicated with relevant stakeholders including diagnosticians, researchers and growers via various forms of communications such as the NPBDN networks, conferences, industry events etc. The avocado industry is prepared with regards to the availability of fit for purpose diagnostic tools validated under Australian laboratory conditions, if an incursion of the four high priority pests and pathogens that were the focus of this project: avocado scab (*Elsinoe perseae*), Persea mite (*Oligonychus perseae*), the laurel wilt complex (*Harringtonia lauricola* and *Xyleborus glabratus*) and the avocado seed moth (*Stenoma catenifer*), was to occur. The project has resulted in a mid-career researcher trained and mentored in avocado pathology, biosecurity and diagnostic, and an improved insight into the epidemiology and management of a range of endemic and exotic pests. The researcher has also established networks with relevant national and international avocado stakeholders. This important and has allowed the industry to build and maintain the necessary expertise and skills across various generations of avocado plant protection research. An undergraduate student (Samuel Garty) has been trained in avocado biosecurity diagnostics from which they has developed interest in pathology and diagnostics, equipped with the knowledge and skills to	A joint avocado and citrus diagnostic workshop was held including delegates representing research institutes, government departments and industry representatives (i.e. UQ, NSW DPIRD, WA DPIRD, DAFF Mickleham, Biosecurity Queensland, Auscitrus) who rated it as very useful, especially as the biosecurity challenges for avocado and citrus are similar. The diagnostic protocols developed from this project including ASBVd and avocado scab have been adopted/used by other national and overseas laboratories (e.g. TREC, Florida). The presentations and shed posters on the project achievements delivered at scientific conferences and industry meetings by the project have been well received.

		work in the area. The student has made significant contributions to the mite studies that made them an authors on the mite DNA barcoding and LAMP assay papers. The delegates attended the diagnostic workshop trained in biosecurity threats of both avocado and citrus industries and the diagnostic techniques developed for the exotic pests and pathogens.	
End of project outcome: The establishment of an economically and environmentally sustainable Australian avocado industry through limiting the introduction or spread of new pests and pathogens and by promoting trade access utilising enhanced knowledge of the endemic pests and pathogens		The most cost-effective approach to managing exotic pests and pathogens (EPPs) is to prevent introduction in the first place. The improved knowledge, collaboration and networks achieved from this project allowed the monitoring of the movement of EPPs overseas which contributed to the prevent of EPPs introduction and keeping Australia free of the economically important EPPs. The improved diagnostic capability achieved this project has provided the necessary tools to support surveillance activities to achieve evidence for pest freedom status. In addition, it has prepared the industry for an incursion event and the success of eradication in case an incursion event takes place. Moreover, it allows safe import of avocado germplasm and fruit during months of short domestic fruit supplies. The pest freedom status resulted from this project, particularly the ASBVd indexing program facilitated export opportunities for Australian avocados (e.g. to New Zealand).	Australia remains free of the four high priority pests and pathogens (HPPs) that were the focus of the current project: avocado scab (*Elsinoe perseae*), Persea mite (*Oligonychus perseae*), the laurel wilt complex (*Harringtonia lauricola* and *Xyleborus glabratus*) and the avocado seed moth (*Stenoma catenifer*). The Biosecurity Plan for the Avocado Industry (v3.0, February 2020) is being reviewed to update, with Prof Geering and Dr Tran being members of the Avocado Technical Review Panel. The last panel meeting was held on 28 November 2025 in which the EPPs were accessed and the four HPPs confirmed to exotic and are of the species that may have the most significant impact if introduced to Australia. An impact assessment of the investment on project: Avocado sunblotch viroid survey (AV18007) was independently carried out by Ag Econ in project MT2105 which highlights that the support of meeting export market requirements was the clearest impact for AV18007.

Monitoring and evaluation

Table 12. Key Evaluation Questions

Key Evaluation Question		Project performance	Continuous improvement opportunities
Effectiveness			
1. To what extent has the project achieved its expected outcomes?	Has literature research identified gaps in R&D for avocado exotic pests and diseases?	Cryptic speciation has been highlighted as a major problem for diagnosis of many groups of organisms.	Review the literature for new knowledge on taxonomy of target species. National and international collaborations on species of interest to access information before publication.
	To what extent has the project enhanced diagnostic capability for priority pathogens for the avocado industry through updated and/or novel diagnostic protocols?	World leading diagnostic technologies have been developed for all target organisms studied in this project.	Pests and pathogens evolve which may result in new strains and/or species. In addition, technology is developing fast and new diagnostic technologies may become available. Therefore, continuous review of the target pest and pathogen population genetics, movement as well as staying up today with the development of diagnostic technology will allow timely revision and/or update of the current diagnostic assay to maintain accuracy and reliability of test results. There are high priority pests and pathogens (as highlighted in the literature review attached) listed on the Biosecurity Plan that lack of diagnostic capability and require research.
	To what extent has the project increased the ability of the avocado industry to respond to exotic pests and diseases?		
	Are major avocado nurseries in Australia distributing viroid-free planting material?	Diagnostic testing done in this project has affirmed pest freedom from ASBVd in the major avocado production nurseries	The testing protocol for ASBVd is rapid, sensitive and reliable, and there is no reason to change it for the foreseeable future. However, testing technologies do improve, and minor or major adjustments to the protocol may be desirable in coming years.
	To what extend has the project improved understanding of the disease cycles, epidemiology, and invasion pathways of high priority avocado pests and diseases?	Disease epidemiology and pest ecology were not specifically addressed in this project, but tools have been developed to differentiate populations of insects, mites and fungi, which will help elucidate invasion pathways.	Research will need to be done in countries of origin to investigate disease epidemiology or pest ecology.
	To what extent has the project improved the diagnostic capacity for the	A training workshop was held in the first year of the project for scientists from state and	Incentives are needed such as scholarships for domestic students to enter the avocado

	avocado industry through training and development of existing and new researchers?	federal departments of agriculture. A third-year undergraduate student was engaged to assist with development of LAMP assays for mites. A succession plan has been implemented to allow Dr Nga Tran to take over leadership of the research area from Professor Andrew Geering.	research community. Science is no longer a popular career choice because of a lack of job opportunities.
Relevance			
2. How relevant was the project to the needs of intended beneficiaries?	To what extent has the project met the needs of industry levy payers?	The project contributed to keeping Australia free of high priority pests and pathogens which has greatly benefitted the levy payers through (i) preventing losses caused by pests and pathogens, ii) limiting the input costs such as pesticide and fungicides, iii) improved fruit quality and v) improved market access facilitating export opportunities.	Continue to monitor patterns of spread of exotic pests and pathogens and remain alert to the emergence of new threats.
Process appropriateness			
3. How well have intended beneficiaries been engaged in the project?	Have regular project updates been provided through publication of articles in the grower magazine 'Talking Avocados'?	The project team published an article in *Talking Avocados*.	Project outputs have been and will continue to be published in *Talking Avocados* as well as other channels including shed posters, presentation at meetings/events, and scientific articles.
	Have project team members been able to directly liaise with avocado growers to provide advice on diagnostics and pest & disease management?	The project team members have directly liaised with: - avocado nurseries as part of support provided to ANVAS. - to avocado fruit export companies by providing declarations of pest freedom.	Continue to directly liaise with growers to provide advice and support on disease and pest diagnostics and management.
4. To what extent were engagement processes appropriate to the target audience/s of the project?	Did the project engage with industry levy payers through their preferred learning style?	Various means were employed to engage the project with levy payers including communicating the project via the grower magazine *Talking Avocados* and industry events. Prof Andrew Geering provided content for *The Avocado Problem Solver Field Guide*, 2nd edition.	Regular publications of project achievements in *Talking Avocados* and attendance of project teams at industry meetings.
	How accessible were extension events to industry levy payers?	The project team attended avocado R&D days but were not invited to present by Avocados Australia Limited.	

Efficiency			
5. What efforts did the project make to improve efficiency?	What efforts did the project make to improve efficiency?	Improved efficiency was achieved by engaging university students in the project. The project over-delivered by developing a LAMP assay for avocado brown mite (*Oligonychus punicae*), which was done by a student. This not only increased the project outputs but also trained new generations of researchers. International collaborations are paramount to the success of research on exotic pests and pathogens and enabled us to meet project milestones	Funding/support to study disease cycles, epidemiology, and invasion pathways of high priority avocado pests and diseases overseas? Industry could consider providing scholarships to help recruiting good students as good students (and also staff) can be hard to secure. Greater awareness and linkages across projects and industries, with international industries and research organisations.

Recommendations

1. There is an ongoing need for plant biosecurity research, as well as a requirement to maintain existing diagnostic capabilities for the Australian avocado industry. Desirably, funding should be continuous to employ a full- or part time scientist to service the diagnostic needs of ANVAS, to monitor the emergence of new pests and pathogens of the avocado industry both overseas and here in Australia, and to continue to build the biosecurity capacity of the nation.
2. Spider mites are the most intercepted group of arthropods at ports of entry into Australia, and there are many species that are considered major biosecurity threats. The skills base for identification of spider mites in Australia has always been small and continues to diminish. DNA barcoding provides an attractive alternative to traditional morphological identification, particularly as the techniques can be applied by people with only general training in molecular biology and are not dependent on the life stage or gender of the mite specimen. The DNA sequence data that can be utilized to develop LAMP or PCR diagnostic assays. However, the effectiveness of DNA barcoding for mite identification relies upon the availability of an accurate DNA barcode library, a resource that is not yet available for Australian spider mite species and should be a priority for future cross-industry funding.
3. The success of a biosecurity system for Australian avocados is strongly dependent on international partnerships, particularly with research organizations in Latin America as this is where many avocado pests and pathogens originate from, and countries like Mexico and Peru are dominant players in the fresh fruit trade. It is important that these international partnerships are cultivated in future avocado biosecurity projects through exchange of technical knowledge and country visits.
4. Several areas of concern about the Australian biosecurity system were highlighted in a workshop held during this project. Although already listed in Appendix 2, these concerns are reiterated here to bring them to the fore.

Response-related

- Lack of basic biological information on many Emergency Plant Pests (EPPs) makes it difficult to predict establishment and spread potential in Australia.
- Lack of dialogue between biosecurity agencies and the scientific community. Failure of biosecurity agencies to obtain proper advice due to a perceived risk of loss of confidentiality through consulting the wider plant pathology community.
- When technical experts do report a detection or provide advice, the biosecurity agencies do not inform the people of the outcome unless it is an ongoing response.
- Investment in regular maintenance (e.g. annual) of diagnostic protocols – reagents refreshed, methods updated, diagnostic assays validated against new target organisms. This would also enhance capability and workforce stability.

Are we ready if an incursion was to occur?

- Upscaling diagnostics remains an issue, particularly given delimiting surveillance can generate hundreds or even thousands of samples. High throughput diagnostic methods need to be developed, for example simplified nucleic acid extraction protocols.

- ‘Off the shelf’ diagnostic assays cannot be used for many plant diseases – often need a research approach to determine cause of a disease. Diagnostics should be integrated into larger research projects, to include research on endemic pests, as some endemic pathogens and pests are similar to exotic threats, confounding surveillance exercises.

Capability

- Diagnostic laboratories exist but there is a lack of specialist expertise to advise industry and government during responses, without appropriate succession planning for those who can provide that expertise.
- There are not enough people studying plant pathology and entomology to maintain succession plans. There is little incentive for students to take up plant pathology or entomology due to the lack of employment opportunities, short-term contracts, and lower pay relative to other professional roles.
- There is continual decline in subjects offered at universities relating to plant pathology and entomology. Only very general education is now provided at undergraduate level, and it is necessary for students to undertake postgraduate education to receive specialist training in any of the plant protection disciplines.

Refereed scientific publications

Journal article

Tran NT, Parkinson LE, Pretorius LS, Gazis R, Ganan-Betancur L, Garty S, and Geering ADW, 2026. Development of a qPCR assay for the rapid detection of the avocado scab fungus *Elsinoe perseae*. *Australasian Plant Pathology*. *Under review*.

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Intellectual property

No project IP or commercialisation to report.

Acknowledgements

We were able to deliver this project as we received enormous support and contributions from many people in Australia and overseas. We gratefully thank Dr Elizabeth Dann (Hort Health) for her support, helpful discussions and generously providing access to her pathogen collection for use in our diagnostic assay validation. We thank Dr Jenny Beard (Queensland Museum) for helping with mite sample collection and collaboration on the spider mite study. Dr Don Loch (UQ) is thanked for providing endemic mite specimens. We acknowledge the support and collaboration with Dr Nerida Donovan (NSW DPIRD) and Dr Monica Kehoe (WA DPIRD). The significant contribution from Samuel Garty is acknowledged. We thank Dr Mark Jackson for helping with DNA cloning experiments.

Research on exotic pests and pathogens would not have been delivered without international collaborations. Thank you to Dr Caterina Villari and Mr Colton Meinecke (University of Georgia, USA) for providing us the laurel wilt DNA samples. We are grateful for the support and collaboration with Dr Romina Gazis and the team at Tropical Research and Education Centre (TREC), University of Florida who provided us the laurel wilt and avocado scab DNA samples and validated our diagnostic assays, for hosting Nga Tran's visit to their lab. We thank Dr Hector González Hernández, Dr Teresa Santillan Galicia, Ms. Valentina Diaz Grisales, Dr Edith Estrada and Dr Armando Estrada and the team at the Colegio de Postgraduados, Mexico for hosting Nga Tran and Andrew Geering's visit, for their hospitality, collaboration and support. We acknowledge the contribution of Dr Thuy Duong (Hue University, Vietnam) for the work on ASBVd diagnostics.

The support from Plant Health Australia through the Residential Diagnostics program that allowed Nga Tran to visit the TREC is gratefully acknowledged.

Appendices

Appendix 1. Review report: Geering ADW, and Tran NT, 2023. Diagnosis of the high priority exotic pests and pathogens for the Australian avocado industry.

Appendix 2. Diagnostic workshop minutes and recommendations.

Appendix 3. List of specimens obtained during this project.

Appendix 4. Article: Geering A, Tran N, 2024. Mexico and Florida biosecurity study tour. *Talking Avocados*. Spring 2024 issue.

Appendix 5. Presentation: Tran N, Pretorius L, Parkinson L, Geering A, 2024. Innovations in plant biosecurity research for the Australian avocado industry. Colegio de Postgraduados, Mexico, 9 February 2024.

Appendix 6. Presentation – Tran N, 2024. Towards finalizing NDPs for avocado scab and laurel wilt. Annual Diagnostic Workshop 19-20 March 2024. Tweed Heads NSW.

Appendix 7. Poster: Tran N, Pretorius L, Parkinson L, Geering A, 2025. Rapid detection of avocado biosecurity threats. Prepared for Avocado Australia to present as various industry meetings and events.

Appendix 8. Poster: Garty S, Tran N, Pretorius L, Geering A, 2025. Shining a light on the avocado mite: A novel LAMP assay for rapid detection of biosecurity pest *Oligonychus punicae*. Research project I (SCIE3121) final presentation, The University of Queensland.

Diagnosis of the high priority exotic pests and pathogens for the Australian avocado industry

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1. Introduction

The Australian avocado industry had a farmgate value of AUD$364 million in the 2021/22 financial year, derived from the sale of 122,197 tonnes of fruit. About 80% of this production is based on just a single cultivar, 'Hass', which makes the industry quite vulnerable to a pest or pathogen incursion due to the narrow genetic base of the industry. The industry is divided into several discrete production regions, the largest being the Atherton Tableland in north Queensland (33% of production), closely followed by south-west Western Australia (30% of production) and the Bundaberg/Childers region in central Queensland (19% of production). Each production region has a distinct environment, some favouring the establishment of specific exotic pests or pathogens more than others. Most avocados consumed in Australia are produced domestically, although fresh fruit is imported from New Zealand over summer to fill a gap in local production. In 2020, import permits were also granted to Chilean growers, although the volumes of fruit from this country are still very low.

Australia remains free of many of the major pests and pathogens of avocado, a reflection of its strong and long running quarantine system, as well as its geographic isolation, particularly from Central America, the centre of origin for avocado and its cohort of coevolved pests and pathogens. To minimise the risk of any future incursions of exotic pests and pathogens (EPP), a 'Biosecurity Plan for the Avocado Industry' has been developed, which lists and then prioritises the major EPPs based on four criteria: entry potential, establishment potential, spread potential and economic impact. The rankings are done by an expert technical panel and the plan is regularly updated, with version 3.1 being the current working document (Anonymous 2020). Nineteen insect and five pathogen species have been identified as high priority EPPs for the Australian avocado industry and just over half of the insects are fruit flies. Other insects in the EPP list that form a natural biological group are the three seed weevils,

Conotrachelus aquacatae, *Conotrachelus perseae* and *Heilipus lauri*, and the two leafroller moths from New Zealand, *Ctenopseustis herana* and *Ctenopseustis obliquana*.

The diagnosis of any agricultural pest or pathogen is a multistep process, beginning with an assessment of pest damage or disease symptoms observed on the plant. The identification of an insect or mite can be quickly narrowed down by morphological examination of a specimen, whereas plant pathogens are more cryptic in nature due to their microscopic size and a preliminary diagnosis may be dependent on the observation of fungal reproductive structures or bacterial ooze, followed by culturing of the microorganism on artificial media. For the better characterized pests or pathogens, accurate molecular detection tests may be available that bypass the need to mount or culture specimens for traditional morphological examination. Finally, any diagnosis is dependent on a robust classification scheme for the target organism, and confusion over the taxonomy of a specimen may alter or delay a biosecurity response. For example, the guava rust *Austropuccinia psidii* was mistakenly diagnosed as *Uredo rangelii* when first detected in Australia, leading to a downplaying of the significance of the incursion and a cessation of the biosecurity response after only nine days (Carnegie et al. 2010; McTaggart et al. 2016).

In this review, the diagnosis of each high priority EPP in the 'Biosecurity Plan for the Avocado Industry' is discussed, with the exception of the fruit flies, which have been the subject of a dedicated investment strategy (NationalFruitFlyCouncil 2020) and relevant diagnostic resources are provided at Fruit Fly ID Australia https://www.fruitflyidentification.org.au/. Comment is also provided on the taxonomy of each organism and whether there is potential to confuse the pest or pathogen of concern with a closely related endemic or exotic species.

2. Exotic insects and mites

2.1 *Scirtothrips perseae* (avocado thrips)

Scirtothrips perseae Nakahara (Thysanoptera: Thripidae) is endemic to the mountainous regions (>1500 m altitude) of Central America from southern Mexico to Guatemala (Hoddle et al. 2002; Rugman-Jones et al. 2007) and has naturalised in California and Hawaii (Rugman-Jones et al. 2007; Mound et al. 2017). This thrips species was not known to science when first recorded as a pest of avocados in California in June 1996. A scientific name and accompanying formal description of the species was provided by Nakahara (1997), with a female specimen collected from an avocado tree

growing at Oxnard, Ventura County, California, designated the holotype. Five additional *Scirtothrips* species from avocados in Mexico have been described, namely *Scirtothrips aguacatae*, *S. kupandae*, *S. manihotifloris*, *S. tacambarensis*, and *S. uruapaniens*, but these taxa have been reduced to synonyms of *S. perseae* based on morphological comparisons (Hoddle et al. 2008).

Cryptic speciation is observed within *S. perseae*, with three distinct mitochondrial haplotype lineages (1, 2 and 3) differentiated by cytochrome c oxidase subunit I (COI) gene sequencing (Rugman-Jones et al. 2007; Mound and Hoddle 2016). The invasive population of *S. perseae* in California belongs to haplotype lineage 1, and was likely initiated by a small number of individuals arriving from Coatepec Harinas in Mexico (Rugman-Jones et al. 2007). *Scirtothrips perseae* has not been split to reflect this population structure but it is reasonable to assume that haplotype lineage 1 would represent *S. perseae* in the strictest sense. It is not known whether the three haplotype lineages of *S. perseae* vary in field ecology but all infest avocado and it should be assumed that they have similar pest potential until proven otherwise.

Avocado is the only known breeding host of *S. perseae*, although individuals have been collected from other plant species when pest densities are high, presumably because they are resting on these plants (Hoddle et al. 2002). Immature leaves and fruit are the preferred feeding and oviposition sites, hence populations of this insect peak in late spring and early summer coinciding with the leaf growth flushes (Yee et al. 2001; Hoddle 2002b). Feeding damage by the thrips causes leaf distortion, and brown scarring along the midrib and veins on the leaf underside becomes increasingly visible as leaves mature (Hoddle 2002a). When the new leaves harden and become unsuitable for feeding, the thrips move onto the developing fruit. The fruit are most susceptible to feeding damage in the 2-week period during and shortly after fruit set, when the fruit is less than 1.5 cm long (Yee et al. 2001). The amount of fruit scarring is directly proportional to the mean count of thrips on each leaf prior to fruit set (Yee et al. 2001). The patterns of scarring on the fruit are not diagnostic and may be caused by different species of thrips, as well as abiotic factors such as wind rub. *Scirtothrips perseae* has a haplodiploid breeding system (females are diploid and males are haploid) and therefore most specimens that are collected are females (Rugman-Jones et al. 2007).

Assignment of specimens to the genus *Scirtothrips* is relatively straightforward using traditional taxonomic methods. However, classification to species level using morphological features can only be

done by people with expert training in thrips taxonomy (Rugman-Jones et al. 2006). Members of the genus *Scirtothrips* are pale and often less than 1 mm in length. Their small size and oily body contents make preparation of undamaged but fully cleared specimens technically difficult but nevertheless essential for accurate examination of the surface sculpture of the insect such as the distribution patterns of microtrichia and small setae (Mound and Palmer 1981). Most specimens can be fully cleared by treatment in 5% sodium hydroxide prior to mounting in canada balsam (Mound & Pitkin, 1972) but a few unmacerated specimens should also be mounted as colour is a useful ancillary character for identification (Mound and Palmer 1981). Female specimens of *S. perseae* are generally yellow in colouration with either distinct or coalesced brown spots on the pronotum (a plate-like structure that covers the prothorax), whereas these spots are absent in the males (Nakahara 1997).

Twenty-one *Scirtothrips* species are recorded from Australia, and the majority of indigenous species have narrow host ranges restricted to a single native plant genus (Hoddle and Mound 2003). In Australia, the only other *Scirtothrips* species likely to be encountered on avocado is the polyphagous pest species, *Scirtothrips dorsalis*. Rugman-Jones et al. (2006) have developed a PCR method to distinguish the different pest species Scirtothrips, including *S. perseae* and *S. dorsalis*. This method depends on relative size differences of the ITS1 and ITS2 regions of the rRDNA between the different species, which can be resolved by simple agarose gel electrophoresis. However, this method still depends on a diagnostician sorting the thrips to genus level by morphological examination, as the PCR primers are universal for all thrips taxa.

2.2 *Ctenopseustis obliquana* and *Ctenopseustis herana* (brown-headed leafroller moth)

Ctenopseustis (Lepidoptera: Tortricidae: Tortricinae) is a genus of moths comprising five species, *C. obliquana*, *C. herana*, *C. fraterna*, *C. filicis*, and *C. servana*, all of which are endemic to New Zealand. One of these, *C. obliquana*, may have been introduced into Hawaii (Langhoff et al. 2009; Dombroskie and Sperling 2013; Dugdale 1990). The *Ctenopseustis* species are differentiated by the sex pheromones emitted by the females and adult morphological characteristics have been identified that corroborate these pheromone groupings (Dugdale 1990). *C. obliquana* males are attracted to a 90:10 blend of (Z)-8-tetradecenyl acetate (Z8-14:OAc) and (Z)-5-tetradecenyl acetate (Z5-14:OAc) (Foster et al. 1986; Young et al. 1985), whereas *C. herana* males are attracted to Z5-14:OAc alone (Foster and Roelofs 1987).

A putative sixth species, referred to as *Ctenopseustis obliquana* "Type II North Island", is morphologically indistinguishable from *C. obliquana* but has a distinct pheromone profile, thereby creating a reproductive barrier between these populations of insects (Dugdale 1990; Newcomb and Gleeson 1998). However, there is a hesitancy to formally recognize this taxonomic entity until more rigorous comparative studies are done, particularly with *C. herana*, which produces the same female pheromone (Dugdale 1990).

The different *Ctenopseustis* species vary in habitat requirements. *Ctenopseustis filicis* and *C. fraterna* are specialist feeders, living on native ferns such as *Cyathea* and *Dicksonia* spp. (Dugdale 1990). *Ctenopseustis servana* feeds on coastal woody angiosperms and has not been found more than 2 km inland of the coast on the North Island (Dugdale 1990). *Ctenopseustis obliquana* and *C. herana* are polyphagous and have become major pest species as they feed on a range of horticultural and forestry species in New Zealand such as avocado, apple, stonefruit, kiwi fruit, pip fruit and persimmon. *Ctenopseustis obliquana* is found on both the North and South Island, whereas *C. herana* is restricted to the South Island (Langhoff et al. 2009; Dugdale 1990). Hence, it is reasonable assume that *Ctenopseustis obliquana* would the main species affecting commercial avocado orchards in New Zealand given that the avocado industry is primarily centred around the Bay of Plenty and Northland on the North Island.

The larvae of at least 37 members of the Tortricidae have been recorded feeding on either the leaves or fruit of avocado, as reviewed by Gilligan et al. (2011). Three of these species (*Cryptoptila immersana*, *Homona spargotis* and *Isotenes miserana*) are recorded from Australia and could conceivably be confused with *Ctenopseutis* as they belong to the same subfamily, Tortricinae. A binary key to identify adult specimens of the *Ctenopseustis* - *Planotortrix* subgroup of genera is provided by Dugdale (1990) but differentiation of the species is not easy. LUCID keys covering 98 tortricid moth taxa of agricultural importance (TortAI) have also been developed and published by the Identification Technology Program in the USA (Gilligan and Epstein 2012) and can be accessed at https://idtools.org/id/tortai/index.html. Two keys are provided, one for adult specimens and the other for larval specimens, and both include *C. obliquana*.

DNA barcoding by cytochrome oxidase subunit 1 (COI) gene sequencing allows assignment of specimens to the genus *Ctenopseustis* but this analysis must be combined with a morphological

examination for species-level diagnosis (Langhoff et al. 2009; Newcomb and Gleeson 1998). In phylogenetic trees constructed using COI gene sequence alignments, *Ctenopseustis* and its sister genus, *Planotortrix*, are reciprocally monophyletic but the species-trees for each genus are polyphyletic (Langhoff et al. 2009). There are examples of the DNA barcodes of specimens of *C. obliquana* more closely matching those of *C. herana* (>99.5% nt identity) than those of different individuals of the same species (*c*. 96% nt identity). The observation that intraspecies species divergence is higher than interspecies divergence means that there is no DNA barcode gap to enable one species to be distinguished from another using DNA barcode sequence data alone. The reasons for the polyphyly of the species-trees is unclear but may reflect incomplete lineage sorting, unrecognized hybridisation between the species, horizontal gene transfer or human errors in specimen identification (Langhoff et al. 2009).

2.3 *Heilipus lauri* (large seed weevil), *Conotrachelus perseae* (small seed weevil) and *Conotrachelus aguacatae* ()

In this review, *Heilipus lauri*, *Conotrachelus aguacatae* and *Conotrachelus perseae* are treated together as they all belong to the weevil family, Curculionidae, and consequently share many biological features. The adult females of these insects bore a hole in the flesh of the developing avocado fruit, into which they deposit their eggs. The first signs of an infestation are small perforations in the fruit skin that are surrounded by a white powdery substance, perseitol. Upon hatching, the larvae burrow into the seed of the fruit.

The genus *Heilipus* (Coleoptera: Curculionidae) comprises 91 species, all originating from the New World and predominantly from the tropical regions of Central and South America (Castañeda-Vildózola et al. 2013). Eight of these species affect avocado, three by boring into the fruit, namely *H. lauri*, *H. pittieri* and *H. trifasciatus* (Castañeda-Vildózola et al. 2007), and the remaining five by boring into the stems or trunk of the tree, namely *Heilipus albopictus* Champion (Castañeda-Vildozola et al. 2010) , *H. squamosus* (LeConte, 1824) (Anzaldo and Díaz-Grisales 2022), H. elegans Guérin and *H. catagraphus* Germar and H. rufipes Perty.

Currently the only way to identify the *Heilipus* species that infest avocado is by morphological means. The biology, geographic distribution, life cycle and morphological descriptions for each developmental stage of these eight species have recently been reviewed by Castañeda-Vildózola et al. (2013) and

high resolution images provided for the diagnostically-informative body parts of *H. lauri*. Nevertheless, identification of the larval stages of these insects still poses great challenges (Garcia Arellano 1975), particularly when not routinely encountered, and larvae should be raised to adulthood to confirm a diagnosis.

The molecular systematics of *Heilipus* species have not been investigated. A search of the GenBank in January 2023

2.4 *Paracoccus marginatus* (papaya mealybug)

Paracoccus marginatus Williams and Granara de Willink (Hemiptera: Coccoidea: Pseudococcidae) was first described in 1992 from specimens collected in Mexico, Belize, Costa Rica, and Guatemala. This mealybug is highly polyphagous, with a host range including over 200 plant species, with papaya, hibiscus and annona species being the worst affected (García Morales et al. 2016; Watson 2022). From its native range in Mexico and/or Central America, this mealybug has invaded the islands of the Caribbean and the USA (Florida), Southern and South-Eastern Asia, China and Taiwan, Central Africa and the Middle East (Israel) (Finch et al. 2021). CLIMEX modelling suggests that in Australia, it is most likely to establish along the east coast of Queensland, from Cape York to Bundaberg (Finch et al. 2021).

In the original taxonomic treatment of *P. marginatus* by Williams and Granara de Wilink (1992), only the adult female was described and it was left to Miller and Miller (2002) to provide complete descriptions of all instars including both sexes of the adult. Adult females remain the standard for taxonomic and phylogenetic studies of mealybugs in the Pseudococcidae because adult males are mobile and difficult to capture and some species (not *P. marginatus*) are entirely parthenocarpic (Bahder et al. 2015). Good quality slide mounts of adult females must be made for morphological discrimination of the different *Paracoccus* species using the key of Williams and Granara de Wilink (1992). Given the increasing reliance on DNA sequence data for identifying and classifying mealybugs (Rung et al. 2008; Wang et al. 2016), it is recommended that specimens are mounted using a method that does not destroy the DNA (Bahder et al. 2015). *Paracoccus marginatus* adult females can be distinguished from all other *Paracoccus* species by the presence of oral-rim tubular ducts dorsally restricted to marginal areas of the body, and the absence of pores on the hind tibiae. Adult males may be distinguished from other related species by the presence of stout fleshy setae on the antennae and the absence of fleshy setae on the legs (Walker et al. 2021). Comprehensive data sheets on *P. marginatus* are provided by Walker

et al. (2021) and Watson (2022). A Threat Specific Contingency Plan for *P. marginatus* has been developed for the Australian papaya industry but not a National Diagnostic Protocol (Anonymous 2011).

As with many other groups of insects, DNA barcoding is growing in popularity for identifying mealybugs. The two most common genetic loci used for DNA barcoding are the mitochondrial COI gene and the D2 region of the 28S rDNA, and methods are provided by Wang et al. (2016) and Heya et al. (2020), among others. The population structure of *P. marginatus* across mainland China and South-east Asia is highly uniform, suggesting a recent introduction of a small number of individuals (Wang et al. 2016; Ahmed et al. 2015), whereas in Kenya, 14 specimens had 99-93% nucleotide identity across the D2 region of the 28S rDNA with a reference sequence from China, and no two specimens were identical (Heya et al. 2020). Virtually nothing is known about the population structure of *P. marginatus* within its native geographic range in Central America, where it is not regarded to be a serious pest, presumably because there are natural biocontrol agents.

2.5 *Oligonychus perseae* (persea mite)

Oligonychus perseae Tuttle, Baker and Abbatiello (Acari, Prostigmata, Tetranychidae) was described from specimens collected by quarantine services at El Paso, Texas on a *Persea* sp. imported from Matehuala in the state of San Luis Potosí, Mexico (Tuttle et al. 1976). The genus Oligonychus is further divided in species subgroups based on consistent morphological characters, and *Oligonychus peruvianus* (McGregor, 1917), *Oligonychus sumatranus* Ehara, 2004 and *O. perseae* are classified in the peruvianus species subgroup (sensu Pritchard & Baker, 1955) based on the presence of more than seven (eight or nine) tactile setae on tibia I of the females (Mushtaq et al. 2021). *Oligonychus peruvianus* also infests avocados in Central America (Aponte and McMurtry 1997) and is easily confused with *O. perseae*, especially as some morphological features (e.g., length and width of idiosoma) may vary according to the host plant species the mite is feeding upon (Sandoval et al. 2011).

Morphological identification of *Oligonychus* is challenging due to the limited number of potential diagnostic characters, cryptic speciation, interspecific similarity in females and the inadequacy of some of the original species descriptions (Mushtaq et al. 2021)(J. Beard, pers. comm.). There are at least 16 species of *Oligonychus* previously recorded from Australia. Of these, three polyphagous species have been recorded from avocado either in Australia or elsewhere in the world, viz. *O. coffeae*, O. *mangiferus*, *O. punicae*. Additionally, the six-spotted mite, *Eotetranychus sexmaculatus*, has also been

recorded on avocado in Australia but the identity of this mite is currently under scrutiny and the Australian population was recently synonymised with *E. queenslandicus* (Seeman et al. 2017).

The population structure of *O. perseae* in Mexico has been studied in reasonable detail, resulting in strong molecular evidence for the occurrence of three potential cryptic species within the taxon *O. perseae* (Lara et al. 2017). Sequences of ITS2, 28S and COI independently grouped *O. perseae* specimens into three congruent divergent genetic clusters, which can be interpreted as strong evidence for the existence of cryptic species (Lara et al. 2017). However, it is not known which COI haplotype cluster represents *O. perseae* in the strictest sense as determined by morphological comparisons with the species holotype. COI haplotype cluster 1 appears to be the most invasive type, as it is the type found in California and Israel (Lara et al. 2017).

Some avocado cultivars are more resistant to *O. perseae* than others, and cv. Hass is renowned has been highly susceptible to this pest. Damage from this pest mainly occurs on the underside of the leaf, especially along the midrib, main veins and leaf depressions, as comprehensively described by Aponte and McMurtry (1997). Colonies of the mite are protected by a canopy of webbing, which helps repel predatory mites. The six-spotted mite *Eutetranychus sexmaculatus* (Riley) also mainly affects the underside of the leaf and produces webbing along the midrib and veins but the damage from *O. perseae* is subtly different as it produces circular necrotic spot patterns from feeding. The necrotic spot patterns caused by the six-spotted mite are more irregular, and the webbing less dense. The avocado brown mite *O. punicae* feeds on upper leaf surfaces and its feeding damage results in bronzing of the leaf surface (Hoddle 1988).

3. Exotic pathogens

3.1 *Harringtonia lauricola* (laurel wilt)

Laurel wilt disease of avocado and other lauraceous plants is caused by the fungus *Harringtonia lauricola* (T.C. Harr., Fraedrich & Aghayeva) Z.W. de Beer & M. Procter, formerly known as *Raffaelea lauricola* (de Beer et al. 2022; Harrington et al. 2008). *Harringtonia lauricola* is a nutritional symbiont of the scolytid beetle *Xyleborus glabratus* Eichoff (Coleoptera: Curculionidae: Scolytinae), which is native to southern and eastern Asia from India to Vietnam, China, Taiwan and Japan and is naturalised in the south-eastern states of the USA (Cognato et al. 2019). Blastospores of the fungus are carried in paired sac-like structures, called mycangia, which are located beneath each mandible on the head of the beetle

(illustrated in Fraedrich et al. (2008)). Winged females of *X. glabratus* tunnel into the sapwood of host trees and create brood galleries. During excavation of these galleries, the beetle inoculates the fungus, which infects the wood surrounding the gallery, and the beetle larvae then feed on the fungal hyphae that is produced. Other fungi are also variably found in the mycangial communities of *X. glabratus*, depending on the beetle population sampled, including six different *Raffaelea* spp., *Trichosporon dermatis*, *Verticillium leptobactrum*, *Cladosporium sphaerospermum* and *Candida* spp. (Harrington and Fraedrich 2010; Harrington et al. 2010; Campbell et al. 2016). However, *H. lauricola* is always the dominant fungus present and is most consistently associated with *X. glabratus* (Campbell et al. 2016).

ITS sequences are difficult to amplify in many ophiostomatoid fungi

Populations of both *H. lauricola* and *X. glabratus* are genetically uniform in the USA, suggesting a founder effect associated with a single introduction event involving a small number of individuals (Wuest et al. 2017; Cognato et al. 2019). However, in Asia, both the beetle and fungus are genetically diverse.

Harringtonia lauricola can either be isolated from either *X. glabratus* or the sapwood of the tree.

MYEA (2% Difco malt extract, 0.2% Difco yeast extract, and 1.5% agar) for 4–10 days at room temperature (Harrington et al. 2010)

Highest captures of X. glabratus were obtained with α-copaene alone, and the combination of α-copaene + ethanol resulted in a significant decrease in captures (Cloonan et al. 2022).

Lindgren traps baited with manuka oil or cubeb oil (Wuest et al. 2017)

3.2 *Elsinoë perseae* (avocado scab)

Avocado scab is caused by the fungus *Elsinoë perseae* (Jenkins) Rossman & W.C. Allen, comb. nov.), which in the older literature is referred to by its basionynym, *Sphaceloma perseae* Jenkins (Jenkins 1934). *Elsinoë* and *Sphaceloma* were names given to the sexual and asexual morphs, respectively, for fungi causing scab and spot anthracnose diseases on a variety of plant species. However, following the adoption of the ‘one fungus = one name’ concept, the two genera were synonymised and *Elsinoë* had naming precedence (Rossman et al. 2016). Evolutionary relationships within the genus *Elsinoë* have been inferred using a concatenated alignment of ITS, LSU, rpb2 and TEF1-α sequence data and *E. perseae* forms a subclade with *Elsinoë terminaliae* from *Terminalia catappa*, *Elisinoë lagoa-santensis* from *Byrsonima coccolobifolia*, and *Elisinoë mangiferae* from *Mangifera indica* (Fan et al. 2017). No

single locus is adequate for distinguishing all 75 recognized species in the genus but the *rpb2* and TEF1-α loci perform best with a success rate of 91.5% and 87.7% at species resolution, respectively, compared to the ITS locus, the standard DNA barcode for fungi, which only resolves 82.4% of the species (Fan et al. 2017).

Scab symptoms on avocado fruit are very obvious but not diagnostic as they can be confused with damage caused by thrips or wind rub (Newett et al. 2013; Everett et al. 2011). There is a correlation between the severity of thrips damage and scab disease on the fruit, suggesting that the feeding action of the thrips facilitates entry of the fungus (Ávila-Quezada et al. 2003). The first signs of infection are oval to irregular, slightly raised brown to purplish-brown spots on the skin of the fruit (Menge and Ploetz 2003). As the disease progresses and secondary infections develop, the spots coalesce to form large, rough, corky areas on the surface of the fruit. The fruit is only susceptible to infection from emergence until about half grown (Ávila-Quezada et al. 2003). The fungus will also infect the leaves to cause small lesions (<3.5 mm in diameter) that become necrotic with age.

Elsinoë perseae can be cultured on artificial media such as potato dextrose agar (PDA) but it is very slow-growing and prone to be overgrown by other fungi and yeasts when isolated directly from plant tissue. For fresh plant tissue isolations, a semi-selective medium should be used containing antibiotics such as streptomycin and tetracycline and a fungicide such as dodine, which is tolerated by *Elsinoë* (Whiteside 1986; Everett et al. 2011). The sexual morph of *Elsinoë* is rarely observed in nature, and the asexual morph is morphologically conserved, providing few characters to distinguish different species (Fan et al. 2017).

Any *Elsinoë* isolated from avocado is most likely to be *E. perseae* as members of this fungal genus are very host-specific (Fan et al. 2017), although a fungus purportedly isolated from avocado in Mexico and deposited in the American Type Collection as accession ATCC 11190 was identified as *Elsinoë araliae* by ITS sequencing (Everett et al. 2011). For a definitive identification, a molecular diagnostic assay must be run. A conventional PCR for *E. perseae* was developed by Everett et al. (2011) using primers (SpF5/R6) targeting the ITS region of the rDNA. Although these primers were designed using sequence data from only six different *Elsinoë* species that were available at the time, they still are at least conceptually specific when compared against a much larger sequence alignment representing 75

Elsinoë species (ADW Geering, unpublished). The PCR assay of Everett et al. (2011) can be used to detect the fungus in DNA extracts from scabs on the fruit.

3.3 *Phytophthora mengei*

Phytophthora mengei causes trunk canker and crown rot of avocado in the USA (Hong et al. 2009). This oomycete has undergone several name changes since its discovery in California in 1914 (Fawcett 1916), and was previously classified as an avocado strain of *Phytopthora citricola*, hence the alternative name for the disease, 'citricola canker' (El-Hamalawi and Menge 1994; Zentmyer et al. 1974).

Phytophthora mengei is placed in the homothallic-paragynous-semipapillate group of the morphological key of Gallegly and Hong (2008), and can be distinguished from other members of this group (*P. citricola*, *P. syringae*, *P. pseudosyringae*, *P. primulae*, *P. porri* and *P. siskiyouensis*) by its smaller oogonia, lack of hyphal swellings, and asymmetric capitate antheridia on many of the oogonia. The sporangia are also distinctive because they form "many large, bizarre shapes" (Hong et al. 2009). *Phytophthora cinnamomi*, the most common *Phytophthora* species in avocado orchards, is heterothallic, and therefore requires two compatible mating types to produce oospores, whereas *P. mengei* is homothallic and will produce oospores in single cultures (Zentmyer 1984). These two *Phytophthora* species also have very different growth patterns on artificial media such as V8 (Zentmyer 1984).

As one of the most economically important groups of plant pathogens in the world, there are many good diagnostic resources for *Phytophthora*.

3.4 Bacterial canker complex (*Pseudomonas syringae* pv. *syringae*, *Pantoea agglomerans*, *Xanthomonas campestris*)

Bacterial cankers are observed on the trunks of avocado trees in all major avocado-growing areas of South Africa and in southern California in the USA (Korsten and Towsen 1997). In the USA, these cankers are attributed to infection by *Xanthomonas campestris*, whereas in South Africa, *Pseudomonas syringae* is held responsible. These cankers appear as slightly sunken and darker areas on the bark, hiding a necrotic and watery pocket underneath. As the canker ages, the bark splits and ooze seeps out, creating a powdery white residue at the periphery as it dries out. The cankers appear first at the base of the tree and then spread upwards, mostly in a straight line (Korsten and Towsen 1997).

Bacterial-like cankers have also been observed in avocado orchards on Tamborine Mountain in South-east Queensland, and *P. syringae* isolated from these cankers, but the bacterial isolates were not pathogenic when experimentally back-inoculated onto healthy avocado seedlings (Pegg et al. 2002). In this instance, it was concluded that boron deficiency was the primary cause of the cankers, and that *P. syringae* was only present as a secondary infection.

the last two decades, there has been a revolution in bacterial taxonomy, with whole genome sequencing data now required for proposals of novel bacterial taxa, as mandated by the *International Journal of Systematic and Evolutionary Microbiology*, journal of the International Union of Microbiological Societies. Phylogenomic analyses are recommended for classification at generic or suprageneric ranks, whereas measures of overall genome relatedness such as average nucleotide identity (ANI) are used for differentiation of closely related species (Chun et al. 2018). *Pseudomonas syringae* and *Xanthomonos campestris* in the broadest sense are now considered species complexes (Potnis et al. 2011; Harrison et al. 2023; Parisi et al. 2019; Marcelletti and Scortichini 2019). There is an urgent need to revisit the taxonomy of the bacterial isolates associated with avocado canker disease, as all identifications were done using traditional diagnostic techniques such as gram staining, biochemical testing and morphology, which are no longer considered adequate for species identification. Until molecular genetic studies are done, preferably by whole genome sequencing, there are no easy wasy to discriminate exotic bacterial isolates from the background of isolates within the same species complexes that already occur in the Australian environment.

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Joint Avocado and Citrus Diagnostic Workshop

10-11th October 2023

List of attendees:

QAAFI	Andrew Geering, Nga Tran, Lara Pretorius, Zali Mahony
NSW DPI	Nerida Donovan, Grant Chambers, Anna Englezou
DAFF	Richard Davis, Stephanie Rosch, Jennifer Morrison, Akila Prabhakaran
Qld DAF	Tuan Nguyen
WA DPIRD	Monica Kehoe, Brenda Coutts
Citrus Australia	Jessica Lye

Group discussion: Diagnostic challenges for avocado and citrus EPPs

Capability

- Diagnostic laboratories exist but there is a lack of specialist expertise to advise industry and government during responses, without appropriate succession planning for those who can provide that expertise.
- There are not enough people studying plant pathology and entomology to maintain succession plans. There is little incentive for students to take up plant pathology or entomology due to the lack of employment opportunities, short-term contracts, and lower pay relative to other professional roles.
- There is continual decline in subjects offered at universities relating to plant pathology and entomology. Only very general education is now provided at undergraduate level, and it is necessary for students to undertake postgraduate education to receive specialist training in any of the plant protection disciplines.

Sampling

- Difficulties of sampling large trees.
- Ensuring adequate sample information is collected, even when standard templates are provided there can be constraints to obtaining the data.
- Large geographic spread of both industries.

Diagnostics

- 'Off the shelf' diagnostic assays cannot be used for many plant diseases – often need a research approach to determine cause of a disease. Diagnostics should be integrated into larger research projects.
- Lack of baseline knowledge on diversity of endemic pests and pathogens.
  - citrus e.g., citrus tristeza virus – endemic and exotic strains
  - avocado e.g., thrips, mites
- Some endemic pathogens and pests are similar to exotic threats, confounding surveillance exercises.

e.g., *Cryptosporiopsis citri* induces leaf spots often mistaken for citrus canker caused by the exotic pathogen *Xanthomonas citri* subsp. *citri*.

- Non-specific amplification with 16S rRNA – moving away from the use of this in diagnostic assays.

<u>Response-related</u>

- Lack of basic biological information on many Emergency Plant Pests (EPPs) makes it difficult to predict establishment and spread potential in Australia.
- Lack of dialogue between biosecurity agencies and the scientific community.
  Failure of biosecurity agencies to obtain proper advice due to a perceived risk of loss of confidentiality through consulting the wider plant pathology community.
- When technical experts do report a detection or provide advice, the biosecurity agencies do not inform the people of the outcome unless it is an ongoing response.

Citrus specific

- Cross reaction leading to false positives for '*Candidatus* Liberibacter africanus' when testing samples that are positive for '*Ca*. L. asiaticus'.
- Non-specific amplification when testing Murraya samples for '*Ca*. L. asiaticus' – potential solution provided by MinION sequencing of amplicons.
- Non-specific amplification when testing for phytoplasmas. Looking more closely at phytoplasma diagnostics in citrus in Hort Innovation funded project CT21005.

Avocado specific

- Most high priority EPPs for the avocado industry originate from Latin America, where resources for research are limited. Molecular techniques such as DNA barcoding have not been widely applied to study the EPPs, and thus there is still a strong reliance on traditional morphological analyses for identification. Morphological identification is particularly challenging for the larval stages of the insects, even for experts.
- The demographic of insect taxonomists is ageing.

Group discussion: The way forward

<u>What improvements can be made?</u>

- Succession planning to ensure current capability is maintained.
- Remove some of the regulatory barriers to working with and exchanging infected plant material provided the biosecurity risk is managed. For example, dried leaf tissue containing '*Ca*. L. asiaticus' is no longer infectious.
- Maintain strong links with plant pathologists and entomologists in countries of origin of EPPs.
- Investment in regular maintenance (annual?) of diagnostic protocols – reagents refreshed, methods updated, diagnostic assays validated against new target organisms. This would also enhance capability and workforce stability.
- Innovative use of new technologies e.g., potential use of LAMP for efficient triage of diagnostic samples

Are we ready if an incursion was to occur?

- It depends on the commodity and the situation.
  For example, a new pathogen with a wide host range and a mobile vector in an urban environment would have little likelihood of eradication – a potential scenario for *Xylella fastidiosa*.
- Surveillance and early detection remain the weak link.
  Progress has been made for citrus in recent years with the evolution of the surveillance structure from being all government based, to industry joining citrus-focused government surveys, and now we have industry driven surveillance (Citrus Watch) with government partners to complement government surveillance. However, there needs to be clear communication between all the surveillance programs to value add rather than duplicate activities.
- Upscaling diagnostics remains an issue, particularly given delimiting surveillance can generate hundreds or even thousands of samples. High throughput diagnostic methods need to be developed, for example simplified nucleic acid extraction protocols.

Avocado insect pest specimens imported from Mexico February 2024

Provider Colegio de Postgraduados, Mexico

Biosecurity E NA24015394

Biosecurity Entry Number: **Q20309948**
Released from Biosecurity: 06-Oct-23

			Pest/Pathogen common name	Pest/Pathogen species	Nearest_town	State	Countr	Plant p	Collection_Date	Primary_Colle	Other_Collectors	Import permit	Entry number	Date released	Hand carried to Australia by
VD3	95% Ethanol		Large seed weevil	*Heilipus lauri*	Ixtapan de la Sal	Estado de México	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD4	95% Ethanol		Large seed weevil	*Heilipus lauri*	Ixtapan de la Sal	Estado de México	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD5	95% Ethanol		Large seed weevil	*Heilipus lauri*	Ixtapan de la Sal	Estado de México	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD6	95% Ethanol		Large seed weevil	*Heilipus lauri*	Ixtapan de la Sal	Estado de México	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD7	95% Ethanol		Large seed weevil	*Heilipus lauri*	Ixtapan de la Sal	Estado de México	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD8	95% Ethanol		Large seed weevil	*Heilipus lauri*	Ixtapan de la Sal	Estado de México	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD9	95% Ethanol		Large seed weevil	*Heilipus lauri*	Ixtapan de la Sal	Estado de México	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD10	95% Ethanol		Large seed weevil	*Heilipus lauri*	Ixtapan de la Sal	Estado de México	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD11	95% Ethanol		Large seed weevil	*Heilipus lauri*	Ixtapan de la Sal	Estado de México	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD12	95% Ethanol		Large seed weevil	*Heilipus lauri*	Ixtapan de la Sal	Estado de México	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD13	95% Ethanol		Large seed weevil	*Heilipus lauri*	Ixtapan de la Sal	Estado de México	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD14	95% Ethanol		Large seed weevil	*Heilipus lauri*	Ixtapan de la Sal	Estado de México	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD15	95% Ethanol		Large seed weevil	*Heilipus lauri*	Ixtapan de la Sal	Estado de México	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD16	95% Ethanol		Large seed weevil	*Heilipus lauri*	Ixtapan de la Sal	Estado de México	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD17	95% Ethanol		Large seed weevil	*Heilipus lauri*	Ixtapan de la Sal	Estado de México	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD18	95% Ethanol		Large seed weevil	*Heilipus lauri*	Ixtapan de la Sal	Estado de México	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD19	95% Ethanol		Large seed weevil	*Heilipus lauri*	Ixtapan de la Sal	Estado de México	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD20	95% Ethanol		Large seed weevil	*Heilipus lauri*	Ixtapan de la Sal	Estado de México	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD21	95% Ethanol		Large seed weevil	*H. lauri*	Malinalco	Estado de México	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD22	95% Ethanol		Large seed weevil	*H. lauri*	Malinalco	Estado de México	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD23	95% Ethanol		Large seed weevil	*H. lauri*	Malinalco	Estado de México	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD24	95% Ethanol		Large seed weevil	*H. lauri*	Malinalco	Estado de México	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD25	95% Ethanol		Large seed weevil	*H. lauri*	Malinalco	Estado de México	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD26	95% Ethanol		Large seed weevil	*H. lauri*	Coatepec Harinas	Estado de México	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD27	95% Ethanol		Large seed weevil	*H. lauri*	Coatepec Harinas	Estado de México	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD28	95% Ethanol		Large seed weevil	*H. lauri*	Tetela del Volcán	Morelos	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD29	95% Ethanol		Large seed weevil	*H. lauri*	Tlacuilotepec	Puebla	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD30	95% Ethanol		Large seed weevil	*H. lauri*	Ixtapan de la Sal	Estado de México	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD31	95% Ethanol		Large seed weevil	*H. lauri*	Ixtapan de la Sal	Estado de México	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD32	95% Ethanol		Large seed weevil	*H. lauri*	Ixtapan de la Sal	Estado de México	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD33	95% Ethanol		Large seed weevil	*H. lauri*	Ixtapan de la Sal	Estado de México	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD34	95% Ethanol			*H. pittieri*			Costa Rica				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD35	95% Ethanol			*H. pittieri*	Carrizal	Alajuela	Costa Rica				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD36	95% Ethanol			*H. pittieri*	San Rafael	Cartago	Costa Rica				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD37	95% Ethanol			*H. pittieri*	Bajo Caliente, Miramar	Puntarenas	Costa Rica				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD38	95% Ethanol			*H. pittieri*	Bajo Caliente, Miramar	Puntarenas	Costa Rica				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD39	95% Ethanol			*H. pittieri*	Bajo Caliente	Puntarenas	Costa Rica				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD40	95% Ethanol			*H. pittieri*	Birrí	Heredia	Costa Rica				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD41	95% Ethanol			*H. trifasciatus*	Bajo Caliente, Miramar	Puntarenas	Costa Rica				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD42	95% Ethanol			*H. trifasciatus*	Coyol	Alajuela	Costa Rica				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD43	95% Ethanol			*H. trifasciatus*	Solania	Guanacaste	Costa Rica				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD44	95% Ethanol			*H. trifasciatus*	Solania, Tilarán	Guanacaste	Costa Rica				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD45	95% Ethanol			*H. trifasciatus*	Solania, Tilarán	Guanacaste	Costa Rica				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD46	95% Ethanol			*H. albopictus*	Ixtapan de la Sal	Estado de México	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD47	95% Ethanol			*H. albopictus*	Ixtapan de la Sal	Estado de México	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD48	95% Ethanol			*H. albopictus*	Ixtapan de la Sal	Estado de México	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD49	95% Ethanol		Large seed weevil	*H. lauri*	Almoloya de Alquisiras	Estado de México	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD50	95% Ethanol		Large seed weevil	*H. lauri*	Almoloya de Alquisiras	Estado de México	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD51	95% Ethanol		Large seed weevil	*H. lauri*	Almoloya de Alquisiras	Estado de México	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD52	95% Ethanol		Large seed weevil	*H. lauri*	Almoloya de Alquisiras	Estado de México	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD53	95% Ethanol		Large seed weevil	*H. lauri*	Almoloya de Alquisiras	Estado de México	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD54	95% Ethanol		Large seed weevil	*H. lauri*	Huatusco	Veracruz	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD55	95% Ethanol		Large seed weevil	*H. lauri*	Sola de Vega	Oaxaca	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD56	95% Ethanol		Large seed weevil	*H. lauri*	Sola de Vega	Oaxaca	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
VD57	95% Ethanol		Large seed weevil	*H. lauri*	Sola de Vega	Oaxaca	Mexico				Valentina Diaz Grisales	not required	NA24015394	12/02/2024	Andrew Geering
S1T1	95% Ethanol		Large seed weevil	*H. lauri*	Cuauhtenco, Almoloya de Aqu	Estado de México	Mexico		8/02/2024	Hector Gonzál	Valentina Diaz Grisales, Nicolas S	not required	NA24015394	12/02/2024	Andrew Geering
S1T2	95% Ethanol		Large seed weevil	*H. lauri*	Cuauhtenco, Almoloya de Aqu	Estado de México	Mexico		8/02/2024	Hector Gonzál	Valentina Diaz Grisales, Nicolas S	not required	NA24015394	12/02/2024	Andrew Geering
S2T1	95% Ethanol		Large seed weevil	*H. lauri*	Manzana, Almoloya de Aquisi	Estado de México	Mexico		8/02/2024	Hector Gonzál	Valentina Diaz Grisales, Nicolas S	not required	NA24015394	12/02/2024	Andrew Geering
S2T2	95% Ethanol		Large seed weevil	*H. lauri*	Manzana, Almoloya de Aquisi	Estado de México	Mexico		8/02/2024	Hector Gonzál	Valentina Diaz Grisales, Nicolas S	not required	NA24015394	12/02/2024	Andrew Geering
2/1/F	95% Ethanol			*Stenoma catenifer*		State of Colima, Mu	Mexico		3/08/2023	Nicolas Sanhueza Peñaranda		not required	NA24015394	12/02/2024	Andrew Geering
2/2/F	95% Ethanol			*Stenoma catenifer*		State of Colima, Mu	Mexico		3/08/2023	Nicolas Sanhueza Peñaranda		not required	NA24015394	12/02/2024	Andrew Geering
2/5/H	95% Ethanol			*Stenoma catenifer*		State of Colima, Mu	Mexico		3/08/2023	Nicolas Sanhueza Peñaranda		not required	NA24015394	12/02/2024	Andrew Geering
GEO3	95% Ethanol			*Stenoma catenifer*		State of Colima, Mu	Mexico		3/08/2023	Nicolas Sanhueza Peñaranda		not required	NA24015394	12/02/2024	Andrew Geering
NT1 Opun	95% Ethanol and DNAlater			*Oligonychus punicae*			Mexico		1/2//2024	Hector González Hernández		not required	NA24015394	12/02/2024	Andrew Geering
NT2	95% Ethanol and DNAlater			*Oligonychus perseae*	Tequexquinahuac	Texcoco Edo. Mexic	Mexico		6/02/2024	Hector González Hernández		not required	NA24015394	12/02/2024	Andrew Geering
NT2 Opun	95% Ethanol and DNAlater			*Oligonychus punicae*	Tequexquinahuac	Texcoco Edo. Mexic	Mexico		6/02/2024	Hector González Hernández		not required	NA24015394	12/02/2024	Andrew Geering
NT3 Oper		DNA later		*Oligonychus perseae*	Michoacan		Mexico		1/02/2024	Hector González Hernández		not required	NA24015394	12/02/2024	Andrew Geering
NT3 Opun		DNA later		*Oligonychus punicae*	Michoacan		Mexico		1/02/2024	Hector González Hernández		not required	NA24015394	12/02/2024	Andrew Geering
NT4	95% Ethanol			*Oligonychus perseae*	Tequexquinahuac	Texcoco Edo. Mexic	Mexico		6/02/2024	Hector González Hernández		not required	NA24015394	12/02/2024	Andrew Geering
NT5	95% Ethanol and DNAlater			*Oligonychus perseae*	Tequexquinahuac	Texcoco Edo. Mexic	Mexico		6/02/2024	Hector González Hernández		not required	NA24015394	12/02/2024	Andrew Geering
NT6	95% Ethanol and DNAlater			*Tetranychus urticae*		Texcoco	Mexico		6/02/2024	Teresa SANTILLAN-GALICIA		not required	NA24015394	12/02/2024	Andrew Geering
NT7		DNA later		*Oligonychus perseae*	Montecillo	Mexico state	Mexico		6/02/2024	Teresa SANTILLAN-GALICIA		not required	NA24015394	12/02/2024	Andrew Geering
NT8	95% Ethanol and DNAlater			*Oligonychus* sp.		Texcoco	Mexico		5/02/2024	Estrada and Equihua		not required	NA24015394	12/02/2024	Andrew Geering
NT9	95% Ethanol and DNAlater			*Heilipus lauri*		Huatusco, Veracruz	Mexico		TBC	Estrada and Equihua		not required	NA24015394	12/02/2024	Andrew Geering

Avocado insect pest and pathogens imported from Florida, USA February 2024

Provider The University of Florida
Import permit 0007068286
Biosecurity Ent NA24015394

identifier	Preservation_Methods	Host_Symptoms	Pest/Pathogen common name	Pest/Pathogen species	Nearest_town	State	Countr	Plant p	Collection_Date	Primary_Colle	Other_Collectors	Import permit	Entry number	Date released	Hand carried to Australia by
TREC-NT1	DNA	avocado scab			Homestead	Florida	USA		11/02/2024	Dr Romina Gaz	Nga Tran	0007068286	NA24015394	19/02/2024	Nga Tran
TREC-NT2	DNA	avocado scab			Homestead	Florida	USA		11/02/2024	Dr Romina Gaz	Nga Tran	0007068286	NA24015394	19/02/2024	Nga Tran
TREC-NT3	DNA	avocado scab					Produc	of Dom	11/02/2024	Dr Romina Gaz	Nga Tran	0007068286	NA24015394	19/02/2024	Nga Tran
TREC-NT4	DNA	avocado scab					Produc	of Dom	11/02/2024	Dr Romina Gaz	Nga Tran	0007068286	NA24015394	19/02/2024	Nga Tran
TREC-NT5	DNA	maybe avocado scab?					Produc	of Dom	11/02/2024	Dr Romina Gaz	Nga Tran	0007068286	NA24015394	19/02/2024	Nga Tran
TREC-NT6	DNA	maybe avocado scab?					Produc	of Dom	11/02/2024	Dr Romina Gaz	Nga Tran	0007068286	NA24015394	19/02/2024	Nga Tran
TREC-NT7	DNA	maybe avocado scab?					Produc	of Dom	11/02/2024	Dr Romina Gaz	Nga Tran	0007068286	NA24015394	19/02/2024	Nga Tran
TREC-NT8	DNA	maybe avocado scab?			Homestead	Florida	USA		11/02/2024	Dr Romina Gaz	Nga Tran	0007068286	NA24015394	19/02/2024	Nga Tran
TREC-NT9	DNA	maybe avocado scab?			Homestead	Florida	USA		11/02/2024	Dr Romina Gaz	Nga Tran	0007068286	NA24015394	19/02/2024	Nga Tran
TREC-NT10	DNA	maybe avocado scab?			Homestead	Florida	USA		11/02/2024	Dr Romina Gaz	Nga Tran	0007068286	NA24015394	19/02/2024	Nga Tran
TREC-NT11	DNA	none			Homestead	Florida	USA		12/02/2024	Dr Romina Gaz	Nga Tran	0007068286	NA24015394	19/02/2024	Nga Tran
TREC-NT12	DNA	laurel wilt, half dead tree, striking on lim, several sugar volcanos observed			Homestead	Florida	USA		12/02/2024	Dr Romina Gaz	Monica Navia-Urrutia	0007068286	NA24015394	19/02/2024	Nga Tran
TREC-NT13	DNA	laurel wilt, completely dead tree, striking on lim, a few sugar volcanos and toothpicks observed			Homestead	Florida	USA		12/02/2024	Dr Romina Gaz	Monica Navia-Urrutia	0007068286	NA24015394	19/02/2024	Nga Tran
TREC-NT14	DNA	laurel wilt, completely dead tree, beetles collected from holes made by the beetles in sample TREC-NT13			Homestead	Florida	USA		12/02/2024	Dr Romina Gaz	Monica Navia-Urrutia	0007068286	NA24015394	19/02/2024	Nga Tran
TREC-ASHMex7	DNA			Elsinoe perseae			USA			Dr Romina Gaz	Lederson Gañán Betancur	0007068286	NA24015394	19/02/2024	Nga Tran
TREC-ASHDR2	DNA			Elsinoe perseae			USA			Dr Romina Gaz	Lederson Gañán Betancur	0007068286	NA24015394	19/02/2024	Nga Tran
TREC-MSVP1	DNA			Elsinoe mangiferea			USA			Dr Romina Gaz	Lederson Gañán Betancur	0007068286	NA24015394	19/02/2024	Nga Tran
TREC-YMS1	DNA			Elsinoe sp.			USA			Dr Romina Gaz	Lederson Gañán Betancur	0007068286	NA24015394	19/02/2024	Nga Tran
TREC-beetle1	95% ethanol			*Xyleborus glabratus*			USA			Dr Romina Gaz	Lederson Gañán Betancur	n/a	n/a	19/02/2024	Nga Tran
TREC-beetle2	95% ethanol			*Xyleborinus saxesenii*			USA			Dr Romina Gaz	Lederson Gañán Betancur	n/a	n/a	19/02/2024	Nga Tran
TREC-beetle3	95% ethanol			*Euplatypus parallelus*			USA			Dr Romina Gaz	Lederson Gañán Betancur	n/a	n/a	19/02/2024	Nga Tran
TREC-beetle4	95% ethanol			*Xyleborus affinis*			USA			Dr Romina Gaz	Lederson Gañán Betancur	n/a	n/a	19/02/2024	Nga Tran
TREC-beetle5	95% ethanol			*Xylosandius compactus*			USA			Dr Romina Gaz	Lederson Gañán Betancur	n/a	n/a	19/02/2024	Nga Tran
PL1004	DNA			*Harringtonia aguacate*			USA			Dr Romina Gaz	Lederson Gañán Betancur	0007068286	NA24015394	19/02/2024	Nga Tran
RL4	DNA			*Harringtonia lauricola*			USA			Dr Romina Gaz	Lederson Gañán Betancur	0007068286	NA24015394	19/02/2024	Nga Tran
LWP040	DNA			*Harringtonia lauricola*			Myan	mar		Dr Romina Gaz	Lederson Gañán Betancur	0007068286	NA24015394	19/02/2024	Nga Tran
L3WP04	DNA			*Harringtonia lauricola*			Myan	mar		Dr Romina Gaz	Lederson Gañán Betancur	0007068286	NA24015394	19/02/2024	Nga Tran
LWP665	DNA			*Raffaelea sulphurea*			USA			Dr Romina Gaz	Lederson Gañán Betancur	0007068286	NA24015394	19/02/2024	Nga Tran
Lula healthy A	DNA			*Persea americana*			USA			Dr Romina Gaz	Lederson Gañán Betancur	0007068286	NA24015394	19/02/2024	Nga Tran
BL1	DNA			*Persea americana*			USA			Dr Romina Gaz	Lederson Gañán Betancur	0007068286	NA24015394	19/02/2024	Nga Tran
BL5	DNA			*Persea americana*			USA			Dr Romina Gaz	Lederson Gañán Betancur	0007068286	NA24015394	19/02/2024	Nga Tran
PD1	DNA			*Persea americana*			USA			Dr Romina Gaz	Lederson Gañán Betancur	0007068286	NA24015394	19/02/2024	Nga Tran
PD5	DNA			*Persea americana*			USA			Dr Romina Gaz	Lederson Gañán Betancur	0007068286	NA24015394	19/02/2024	Nga Tran
ASLL3 TREC	DNA			*Elsinoe perseae*			USA			Dr Romina Gaz	Lederson Gañán Betancur	0007068286	NA24015394	19/02/2024	Nga Tran
LimateLate2	DNA			*Elsinoe* sp.			USA			Dr Romina Gaz	Lederson Gañán Betancur	0007068286	NA24015394	19/02/2024	Nga Tran
TREC-JPS7	DNA			*Elsinoe* sp.			USA			Dr Romina Gaz	Lederson Gañán Betancur	0007068286	NA24015394	19/02/2024	Nga Tran
TREC-JPSX	DNA			*Elsinoe* sp.			USA			Dr Romina Gaz	Lederson Gañán Betancur	0007068286	NA24015394	19/02/2024	Nga Tran
TREC-JMS3	DNA			*Elsinoe* sp.			USA			Dr Romina Gaz	Lederson Gañán Betancur	0007068286	NA24015394	19/02/2024	Nga Tran
TREC-SERT2	DNA			*Lasidioplodia* sp.			USA			Dr Romina Gaz	Lederson Gañán Betancur	0007068286	NA24015394	19/02/2024	Nga Tran
TREC-Ps-AvoEtt	DNA			*Pseudocercospora* sp.			USA			Dr Romina Gaz	Lederson Gañán Betancur	0007068286	NA24015394	19/02/2024	Nga Tran
TREC-Dent1	DNA			*Ceraceosorus* sp.			USA			Dr Romina Gaz	Lederson Gañán Betancur	0007068286	NA24015394	19/02/2024	Nga Tran

Q20309948

Raffaelea spp and _Xyleborus glabarotus_ DNA imported from USA October 2023

Provider University of Georgia, Drs Caterina Villari and Colton Meinecke

Biosecurity Entry Number: **Q20309948**
Released from Biosecurity: 06-Oct-23

DNA tube #	Isolate code	Species	Host/Source	Origin	Collector	Notes
1	CV-2017-004	*Harringtonia lauricola*	Xyleborus glabratus	Hunting Island, South Carolina, United States	Stephen Fraedrich, United States Forest Service	
2	CV-2017-004					
3	CV-2017-005	*Harringtonia lauricola*	Xyleborus glabratus	Hunting Island, South Carolina, United States	Stephen Fraedrich, United States Forest Service	
4	CV-2017-006	*Harringtonia lauricola*	Xyleborus glabratus	Hunting Island, South Carolina, United States	Stephen Fraedrich, United States Forest Service	
5	CV-2017-007	*Harringtonia lauricola*	Xyleborus glabratus	Hunting Island, South Carolina, United States	Stephen Fraedrich, United States Forest Service	
6	CV-2017-008	*Harringtonia lauricola*	Persea borbonia	Hilton Head, South Carolina, United States	Stephen Fraedrich, United States Forest Service	
7	CV-2017-009	*Harringtonia lauricola*	Persea borbonia	Hunting Island, South Carolina, United States	Stephen Fraedrich, United States Forest Service	
8	CV-2017-010	*Harringtonia lauricola*	Persea borbonia	Hunting Island, South Carolina, United States	Stephen Fraedrich, United States Forest Service	
9	CV-2017-010					
10	CV-2017-011	*Harringtonia lauricola*	Persea borbonia	Perry County, Mississippi, United States	Stephen Fraedrich, United States Forest Service	
11	CV-2017-011					
12	CV-2017-014	*Harringtonia lauricola*	Sassafras sp.	Early County, Georgia	Stephen Fraedrich, United States Forest Service	
13	CV-2017-014					
14	CV-2017-012	*Harringtonia lauricola*	Sassafras sp.	Miller County, Georgia, United States	Stephen Fraedrich, United States Forest Service	
15	CV-2017-013	*Harringtonia lauricola*	Sassafras sp.	Baker County, Georgia, United States	Stephen Fraedrich, United States Forest Service	
17	CV-2017-015	*Harringtonia lauricola*	Persea borbonia	Lumberton, Texas, United States	Stephen Fraedrich, United States Forest Service	
18	CV-2017-016	*Harringtonia lauricola*	Persea borbonia	Lumberton, Texas, United States	Stephen Fraedrich, United States Forest Service	
19	CV-2017-018	*Harringtonia lauricola*	Persea borbonia	Jesup, Georgia, United States	Stephen Fraedrich, United States Forest Service	
20	CV-2017-019	*Harringtonia lauricola*	Persea borbonia	Sapelo Island, Georgia, United States	Stephen Fraedrich, United States Forest Service	
21	CV-2017-020	*Harringtonia lauricola*	Persea borbonia	Pembroke, Georgia, United States	Stephen Fraedrich, United States Forest Service	
22	CV-2017-021	*Harringtonia lauricola*	Persea borbonia	Richmond Hills, Georgia, United States	Stephen Fraedrich, United States Forest Service	
23	CV-2017-023	*Raffaelea subfusca*	Xyleborus bispinatus	Florida, United States	Daniel Carillo and Luisa Cruz, University of Florida	
24	CV-2017-024	*Raffaelea subalba*	Xyleborus bispinatus	Florida, United States	Daniel Carillo and Luisa Cruz, University of Florida	
25	CV-2017-025	*Raffaelea arxii*	Xyleborus bispinatus	Florida, United States	Daniel Carillo and Luisa Cruz, University of Florida	
26	CV-2017-026	*Raffaelea arxii*	Xyleborus volvulus	Florida, United States	Daniel Carillo and Luisa Cruz, University of Florida	
27	CV-2017-027	*Raffaelea subfusca*	Xyleborus volvulus	Florida, United States	Daniel Carillo and Luisa Cruz, University of Florida	
28	CV-2017-028	*Raffaelea subfusca*	Xyleborus volvulus	Florida, United States	Daniel Carillo and Luisa Cruz, University of Florida	
29	CV-2017-029	*Raffaelea fusca*	Xyleborus volvulus	Florida, United States	Daniel Carillo and Luisa Cruz, University of Florida	
30	CV-2017-030	*Raffaelea fusca*	Xyleborus volvulus	Florida, United States	Daniel Carillo and Luisa Cruz, University of Florida	
31	CV-2017-031	*Raffaelea subfusca*	Xyleborus volvulus	Florida, United States	Daniel Carillo and Luisa Cruz, University of Florida	
32	CV-2017-032	*Raffaelea subalba*	Xyleborus volvulus	Florida, United States	Daniel Carillo and Luisa Cruz, University of Florida	
33	CV-2017-033	*Raffaelea sp. PL1001*	Xyleborus volvulus	Florida, United States	Daniel Carillo and Luisa Cruz, University of Florida	
34	CV-2017-035	*Raffaelea arxii*	Xyleborus volvulus	Florida, United States	Daniel Carillo and Luisa Cruz, University of Florida	
35	CV-2017-036	*Raffaelea arxii*	Xyleborus volvulus	Florida, United States	Daniel Carillo and Luisa Cruz, University of Florida	
36	CV-2017-037	*Raffaelea arxii*	Xyleborus volvulus	Florida, United States	Daniel Carillo and Luisa Cruz, University of Florida	
37	CV-2017-038	*Raffaelea arxii*	Xyleborus volvulus	Florida, United States	Daniel Carillo and Luisa Cruz, University of Florida	
38	CV-2017-039	*Raffaelea arxii*	Xyleborus volvulus	Florida, United States	Daniel Carillo and Luisa Cruz, University of Florida	
39	CV-2017-040	*Raffaelea arxii*	Xyleborus volvulus	Florida, United States	Daniel Carillo and Luisa Cruz, University of Florida	
40	CV-2017-041	*Raffaelea arxii*	Xyleborus volvulus	Florida, United States	Daniel Carillo and Luisa Cruz, University of Florida	
41	CV-2017-042	*Raffaelea PL6099 xylebor*	Xyleborus glabratus	Florida, United States	Daniel Carillo and Luisa Cruz, University of Florida	
42	CV-2017-064	*Raffaelea PL1004 aguacate*			Jason Smith, University of Florida	
43	CV-2017-065	*Harringtonia lauricola*	Persea borbonia	Pembroke, Georgia, United States	Thomas Harrington, Iowa State University	Unusual ifw allele
44	CV-2017-066	*Harringtonia lauricola*	Persea borbonia	Pembroke, Georgia, United States	Thomas Harrington, Iowa State University	Unusual ifw allele
45	CV-2017-067	*Harringtonia lauricola*	Sassafras sp.; Xyleborus g	Demepolis, Alabama, United States	Thomas Harrington, Iowa State University	Unusual ifw allele
46	CV-2017-068	*Harringtonia lauricola*	Persea borbonia	Halfmoon Landing, Georgia, United States	Thomas Harrington, Iowa State University	Unusual ifw allele
47	CV-2017-069	*Harringtonia lauricola*	Sassafras albidum	Halfmoon Landing, Georgia, United States	Thomas Harrington, Iowa State University	Unusual ifw allele
48	CV-2017-070	*Harringtonia lauricola*	Persea borbonia	Halfmoon Landing, Georgia, United States	Thomas Harrington, Iowa State University	Unusual ifw allele
49	CV-2017-096	*Raffaelea sulphurea*	Populus deltoides; Xyleb	Lawrence, Kansas, United States	Thomas Harrington, Iowa State University	
50	CV-2017-097	*Raffaelea gnathotrichi*	Picea engelmannii; Gnath	Fort Collins, Colorado, United States	Thomas Harrington, Iowa State University	
51	CV-2017-098	*Raffaelea montetyi*	Quercus suber; Platypus	Masif des Maures, France	Thomas Harrington, Iowa State University	
52	CV-2017-099	*Raffaelea tritirachium*	Quercus sp.	Livonia, Pennsylvania, United States	Thomas Harrington, Iowa State University	
53	CV-2017-100	*Raffaelea albimanens*	Ficus sycomo; Platypus e	Dukuduku, Kwa-Zulu Natal, South Africa	Thomas Harrington, Iowa State University	
54	CV-2017-101	*Raffaelea ambrosiae*	Quercus sp.; Platypus cyl	Southern England	Thomas Harrington, Iowa State University	
55	CV-2017-102	*Raffaelea canadensis*	Pseudotsuga menziesii; P	Canada	Thomas Harrington, Iowa State University	
56	CV-2017-103	*Raffaelea sulcati*	Pseudotsuga menziesii; G	Vancouver Island, British Columbia, Canada	Thomas Harrington, Iowa State University	
57	CV-2017-104	*Raffaelea ellipticospora*	Xyleborus glabratus	Hunting Island, South Carolina, United States	Thomas Harrington, Iowa State University	
58	CV-2017-105	*Raffaelea quercivora x2*	Platypus quercivorus	Japan	Thomas Harrington, Iowa State University	
59	CV-2017-106	*Raffaelea santori*	Platypus mutatus	Argentina	Thomas Harrington, Iowa State University	
60	CV-2017-107	*Raffaelea amasae x2*	Amasa concitatus	Taiwan	Thomas Harrington, Iowa State University	
61	CV-2018-003	*Harringtonia lauricola*	Sassafras sp.	Duplin County, North Carolina, United States	Stephen Fraedrich, United States Forest Service	*Freshly extracted
62	CV-2018-004	*Harringtonia lauricola*	Sassafras sp.	Duplin County, North Carolina, United States	Stephen Fraedrich, United States Forest Service	
63	CV-2018-005	*Raffaelea sulphurea*	Sassafras sp.	Duplin County, North Carolina, United States	Stephen Fraedrich, United States Forest Service	
64	CV-2018-026	*Raffaelea brunnea*		Florida, United States	Jiri Hulcr, University of Florida	
65	Beetle-01	*Xyleborus glabratus*				*tested positive for H. lauricola by LAMP
66	Beetle-02	*Xyleborus glabratus*				*tested positive for H. lauricola by LAMP
67	Beetle-03	*Xyleborus glabratus*				*tested positive for H. lauricola by LAMP
68	Beetle-04	*Xyleborus glabratus*				*tested positive for H. lauricola by LAMP
69	Beetle-05	*Xyleborus glabratus*				*tested positive for H. lauricola by LAMP
70	Beetle-06	*Xyleborus glabratus*				*tested positive for H. lauricola by LAMP
71	Beetle-07	*Xyleborus glabratus*				*tested positive for H. lauricola by LAMP
72	Beetle-08	*Xyleborus glabratus*				*tested positive for H. lauricola by LAMP
73	Beetle-09	*Xyleborus glabratus*				*tested positive for H. lauricola by LAMP
74	Beetle-10	*Xyleborus glabratus*				*tested positive for H. lauricola by LAMP
75	Beetle-11	*Xyleborus glabratus*				*tested positive for H. lauricola by LAMP
76	Beetle-12	*Xyleborus glabratus*				
77	Beetle-13	*Xyleborus glabratus*				

Mexico and Florida biosecurity study tour

By Andrew Geering and Nga Tran – University of Queensland

Avocados are native to Mexico. It therefore comes as no surprise that about half of the high priority exotic pests and pathogens (EPPs) for the Australian avocado industry also originate from Mexico. The tables would be turned if we were talking about eucalyptus pests and diseases.

We lead a project called 'Biosecurity Capacity Building for the Australian Avocado Industry', which is funded by grower levies and Australian Government contributions, all managed by Hort Innovation Australia. Two of the major priorities in this current project are the Persea mite (*Oligonychus perseae*) and the avocado seed moth (*Stenoma catenifer*). If one or the other was introduced into Australia, then there would be a tremendous economic impact through damage to the fruit or tree, and because of trade restrictions that would be immediately imposed on the trade of fruit.

The Persea mite is indigenous to Mexico but has now spread and established in Costa Rica, the USA (California, Florida, and Hawaii), Morocco, southern Europe (Portugal, Italy, France, mainland Spain and Canary Islands), and Israel. This mite can cause severe defoliation of the tree, which in turn leads to yield reductions (smaller and fewer fruit) and sunburn-like symptoms. Like most spider mites, the Persea mite is favoured by hot, dry conditions and would cause most damage in our southern production regions (south-west Western Australia and the Tristates region) compared to the more humid production regions of Queensland and northern NSW. While avocado is considered the primary host of the Persea mite, it has been found feeding and breeding on a diverse range of plant species from 17 botanical families.

The avocado seed moth has also invaded new territories, but mostly along the north-south axis of the Americas. From Central America, this pest has spread to Venezuela, Guyana, Brazil, Colombia, Peru and Argentina but not yet to Chile. However, it has jumped across the ocean to the Galapagos Islands, and there was a quarantine intercept of adults and pupae of this moth in Peruvian fruit that was imported into China in 2018. The main economic impact of the avocado seed moth is caused by the larvae (grubs), which burrow into the fruit and cause premature fruit drop. Yield losses of 60–80% have been recorded in Brazil and Venezuela.

Mites are a challenge even for the experts to identify, and the grubs of the avocado seed moth have few distinguishing features. We therefore are taking molecular approaches to identify these pest species, as DNA barcodes provide unambiguous identifications. We have developed a new type of diagnostic assay, called a LAMP assay, for the Persea mite, and using this assay an identification can be made within 15 minutes. This LAMP assay is field deployable without needing specialised laboratory equipment.

To validate our LAMP assay for the Persea mite, it was necessary to travel to Mexico to sample and then test fresh specimens. We visited the Colegio de Postgraduados at Texcoco de Moro, a city 25 km northeast of Mexico City, for a week-long visit to do some experimental work. The LAMP assay proved its worth, successfully detecting the Persea mite but not non-target species. Informal collaborations were established with the Mexican researchers, and ethanol-preserved and pinned specimens of a range of pest insects including the avocado seed moth were imported back to the laboratory in Australia for future research.

Nga Tran continued to Florida after Mexico, while I (Andrew Geering) returned to Brisbane.

The Floridian avocado industry is being devastated by laurel wilt disease, which is caused by a beetle-transmitted fungus. The beetle is *Xyleborus glabratus*, and it transmits the fungus *Harringtonia lauricola* in its mouthparts. The beetle is native to Asia, including India, Japan, Myanmar, and Taiwan, and was introduced into south-eastern USA in 2002 after hitchhiking in wooden shipping crates. The avocado tree is hypersensitive to infection by *Harringtonia lauricola*, and this fungus causes the water conducting elements (the xylem) of the tree to become clogged though overgrowth of the lining cells – a phenomenon called tyloses. The symptoms of laurel wilt are those of water stress. The tree rapidly dies after being attacked by the beetle, and the fungus travels between trees in the row along natural graft unions of the roots of neighbouring trees.

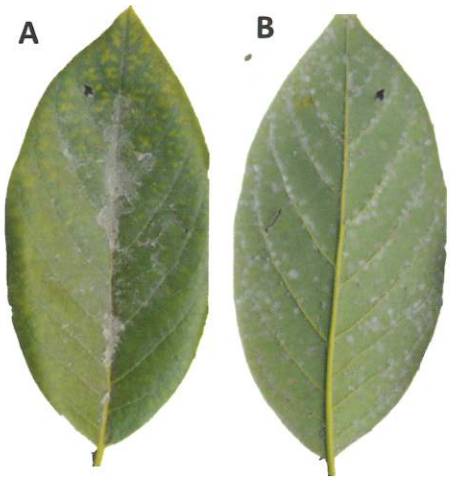


Figure 1. Avocado leaf infested by mites (A) *Oligonychus punicae* on the upper side, and (B) Persea mite (*O. perseae*) on the underside in Mexico.

In a second whirlwind week, Nga was trained in the various diagnostic techniques for laurel wilt and saw firsthand the damage being done by the disease in the orchards. A LAMP assay for *Harringtonia lauricola* was validated using fresh samples. A second benefit of the Florida trip was a chance to road test a new diagnostic test developed by us for another high priority exotic pathogen, *Elsinoë perseae*, which causes avocado scab. Specimens of *H. lauricola* vector beetles preserved in ethanol and DNA of laurel wilt and scab pathogens were also imported to Australia for future research and to use as positive controls for the diagnostic tests. One requirement of a newly developed diagnostic assay is that it needs to be validated by different operators at another laboratory, and one immediate outcome of this visit was that the University of Florida team agreed to collaborate on validating our diagnostic assays and be international contacts for the National Diagnostic Protocols.

This study tour will contribute to improving the biosecurity diagnostic capacity and capability of the Australian avocado industry through enhancing hands-on experience of the diagnosticians, the connections and collaborations with overseas experts who have expertise on the EPPs, and the materials imported that cannot be obtained domestically but are vital to biosecurity research.

Figure 2. Andrew Geering (second from right) with the Mexican team on a field trip to avocado orchard to collect mite and avocado seed moth specimens.

Figure 3. Symptoms of laurel wilt including (A) dead avocado trees and (B) cross section of avocado trunk showing damage (browning) to the xylem (top) compared to healthy trunk (bottom).

Innovations in plant biosecurity research for the Australian avocado industry

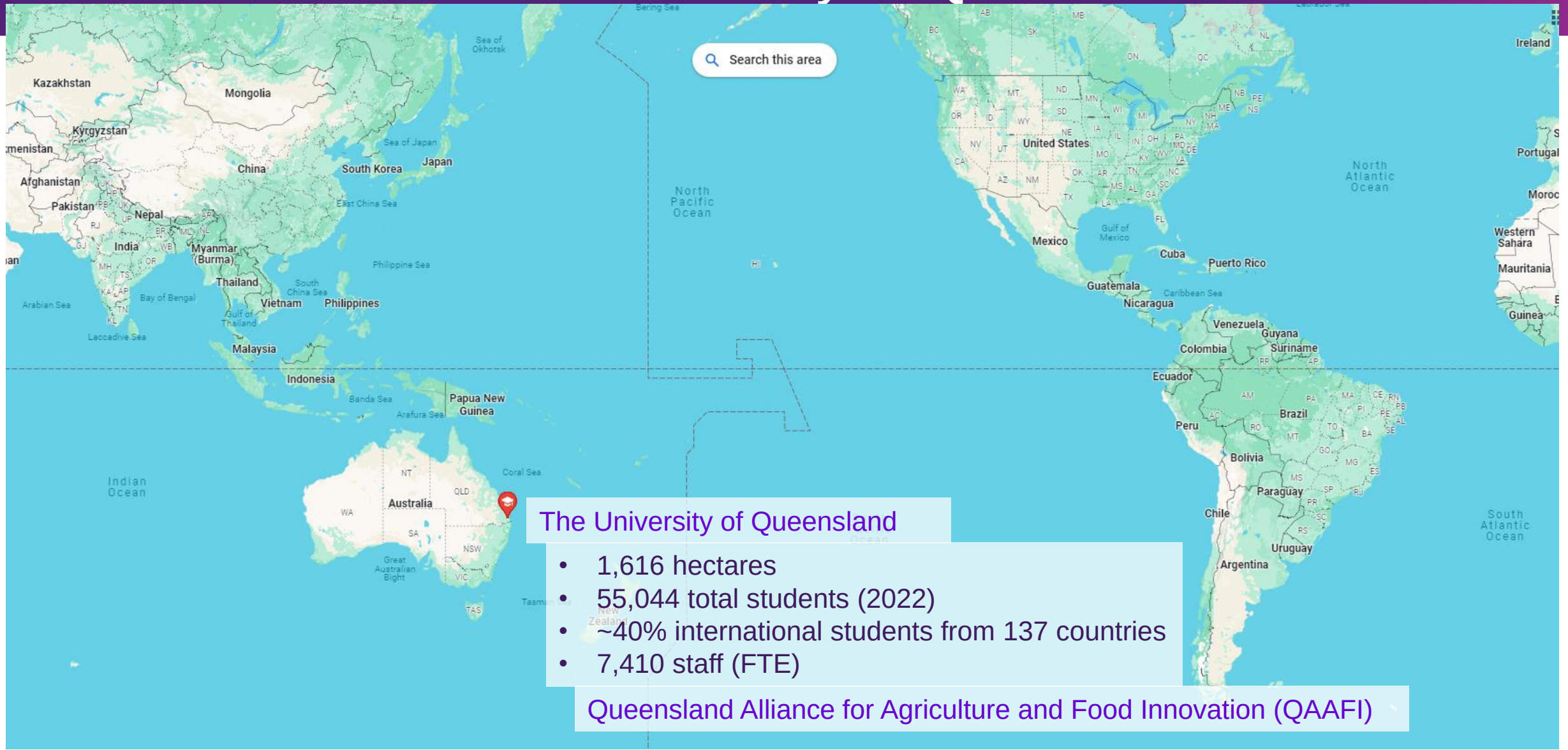
Nga Tran, Lara Pretorius, Louisa Parkinson, and Andrew Geering

Queensland Alliance for Agriculture and Food Innovation (QAAFI)

The University of Queensland, Australia

And collaborators

About the University of Queensland



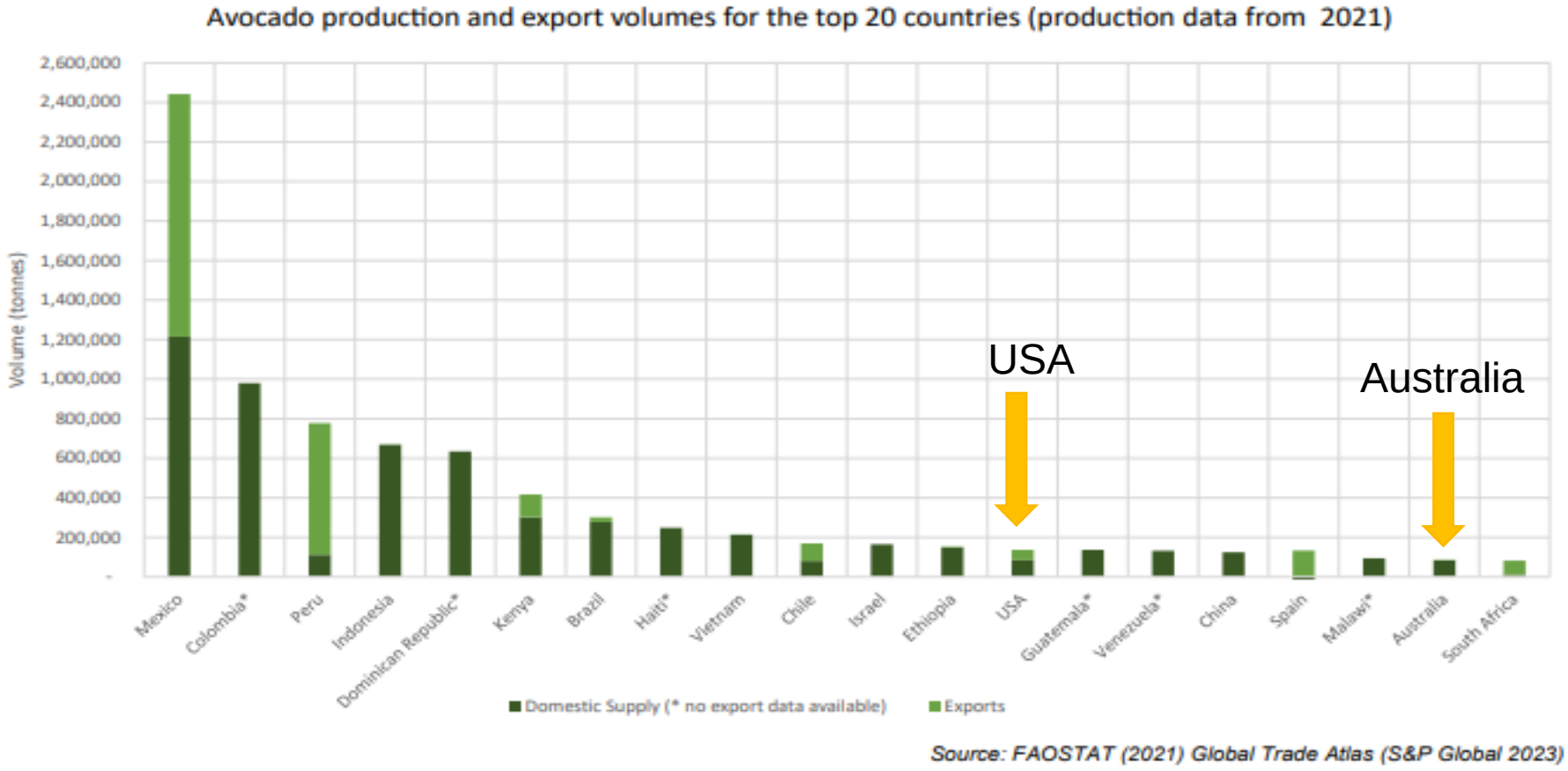
The University of Queensland

- 1,616 hectares
- 55,044 total students (2022)
- ~40% international students from 137 countries
- 7,410 staff (FTE)

Queensland Alliance for Agriculture and Food Innovation (QAAFI)

The Australian avocado industry at a glance

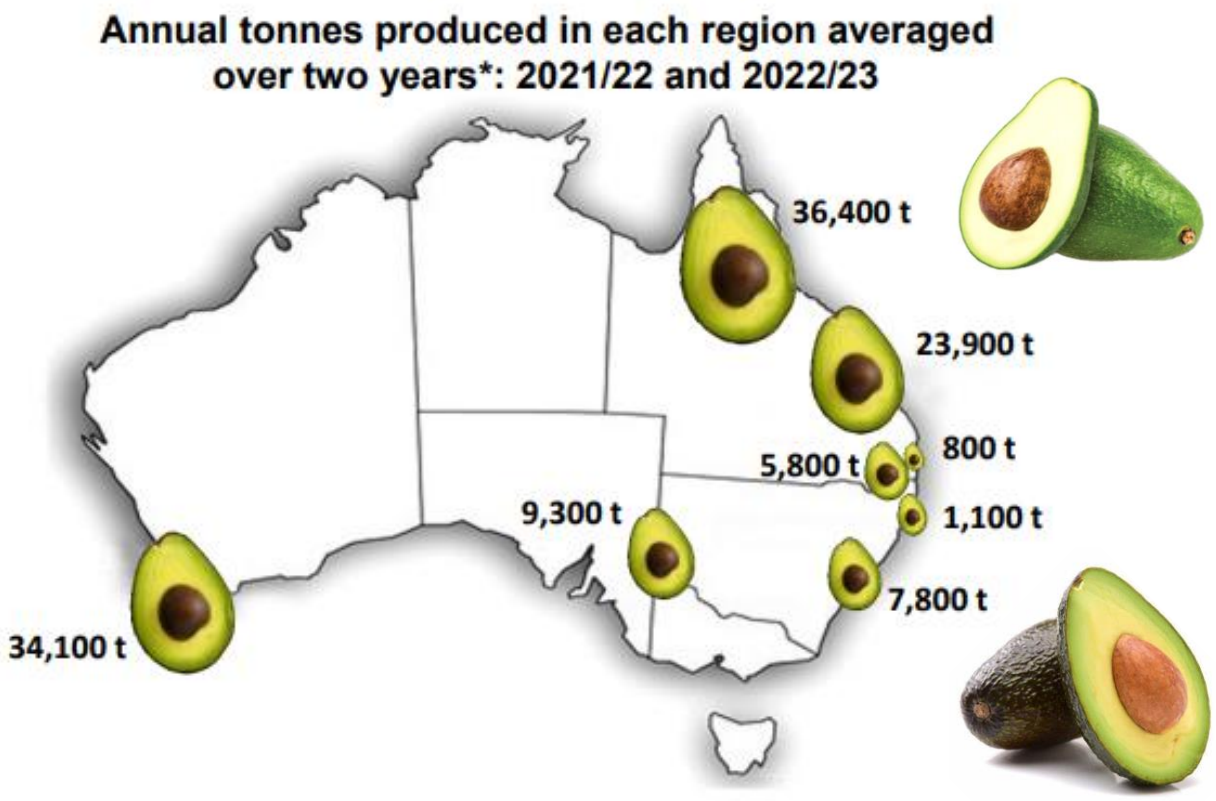
Top 20 largest global producers



Source: FAOSTAT (2021) Global Trade Atlas (S&P Global 2023)

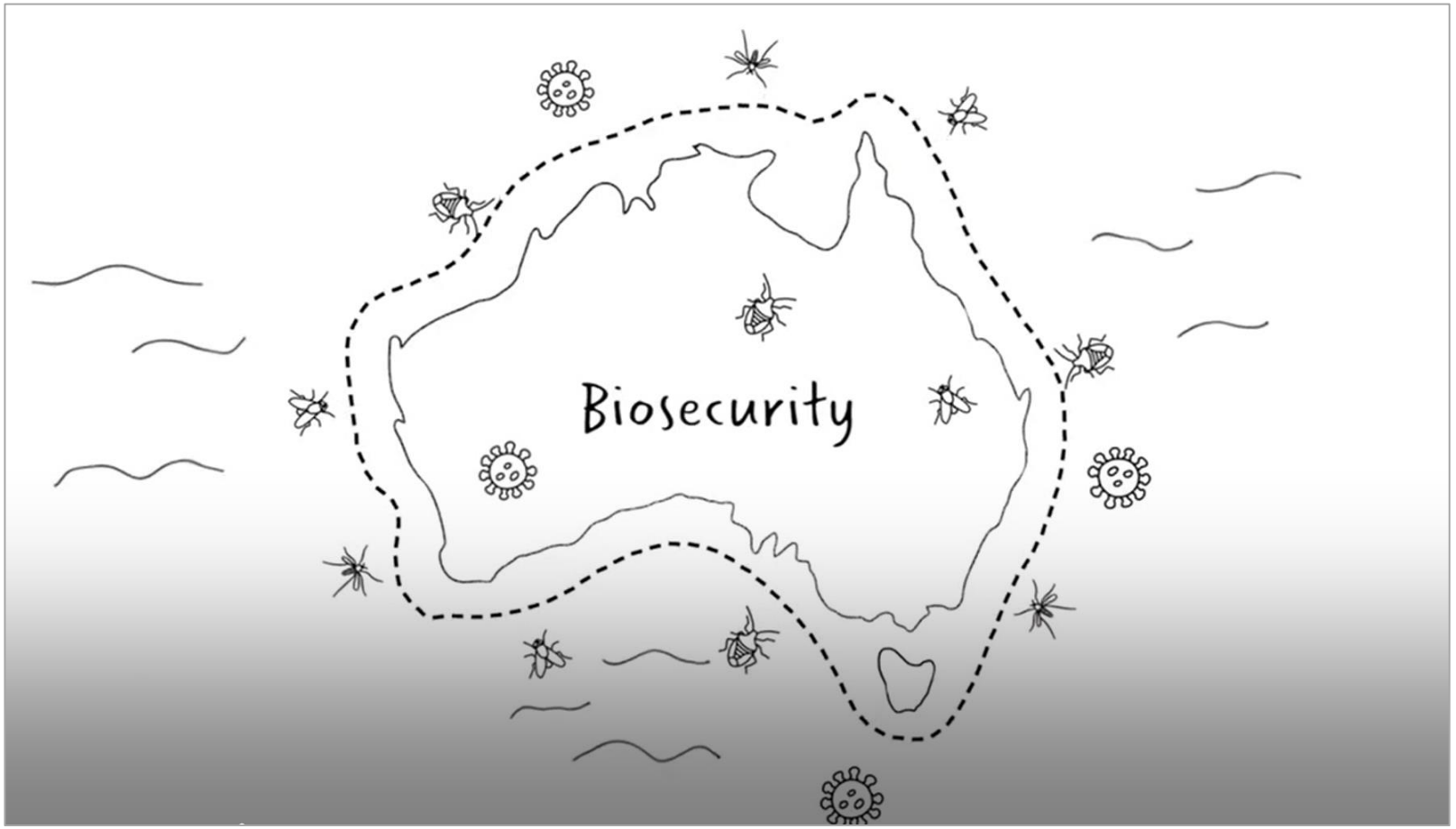
The Australian avocado industry at a glance

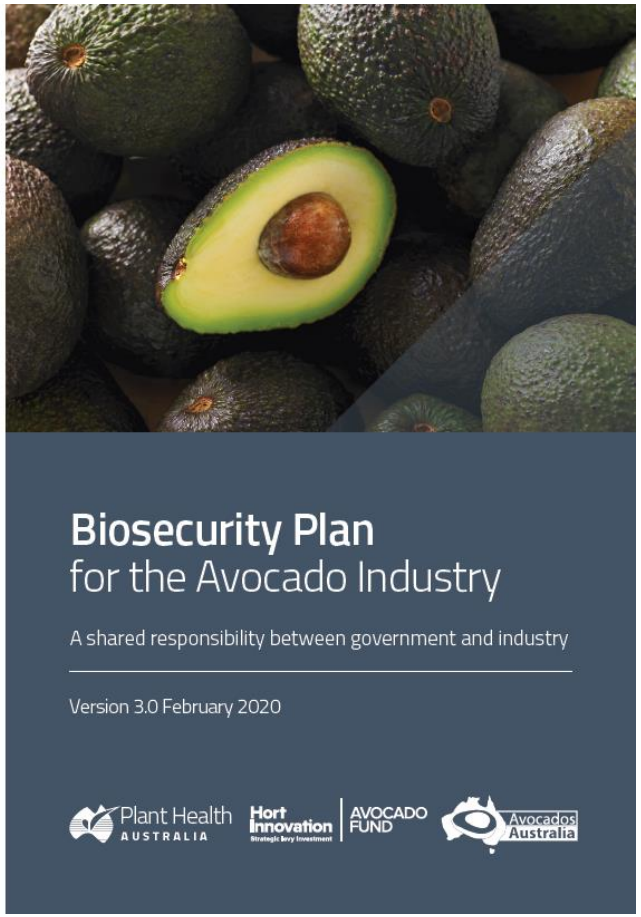
- Farmgate value (2022/23) = $522 million
- Production mainly for domestic market, 9.5% of total crop exported
- Two main varieties:
  - Hass (purple skin), grown everywhere, 70% of production
  - Shepard (green skin), grown on Atherton Tableland, 25% of production.
- Nearly all-year-round production – shortage of Hass from Feb–April. Imports from New Zealand and Chile during this time
- Saturation of market is a problem – industry strategy to increase exports



✓ Total: 18,000 ha (2022)
✓ Average yield: 8.2 t/ha

A rigorous biosecurity system of Australia, keeping many important pests and pathogen out





High priority EPPs for avocado industry

Common name	Scientific name	Common name	Scientific name
Pests		**Pathogens**	
Fruit flies (10 species)	*Bactrocera* and others	Avocado scab	*Elsinoe perseae*
Brown-headed leafroller	*Ctenopseutis* spp.	Bark canker	*Phytophthora mengei*
Avocado seed moth	*Stenoma catenifer*	Sudden oak death	*Phytophthora ramorum*
Avocado thrips	*Scirtothrips perseae*	Laurel wilt	*Harringtonia lauricola*
Small seed weevils	*Conotrachelus* spp.	Bacterial canker complex	*Pseudomonas syringae* *Xanthomonas campestris*
Large seed weevil	*Heilipus lauri*		
Papaya mealybug	*Paracoccus marginatus*		
Persea mite	*Oligonychus perseae*		

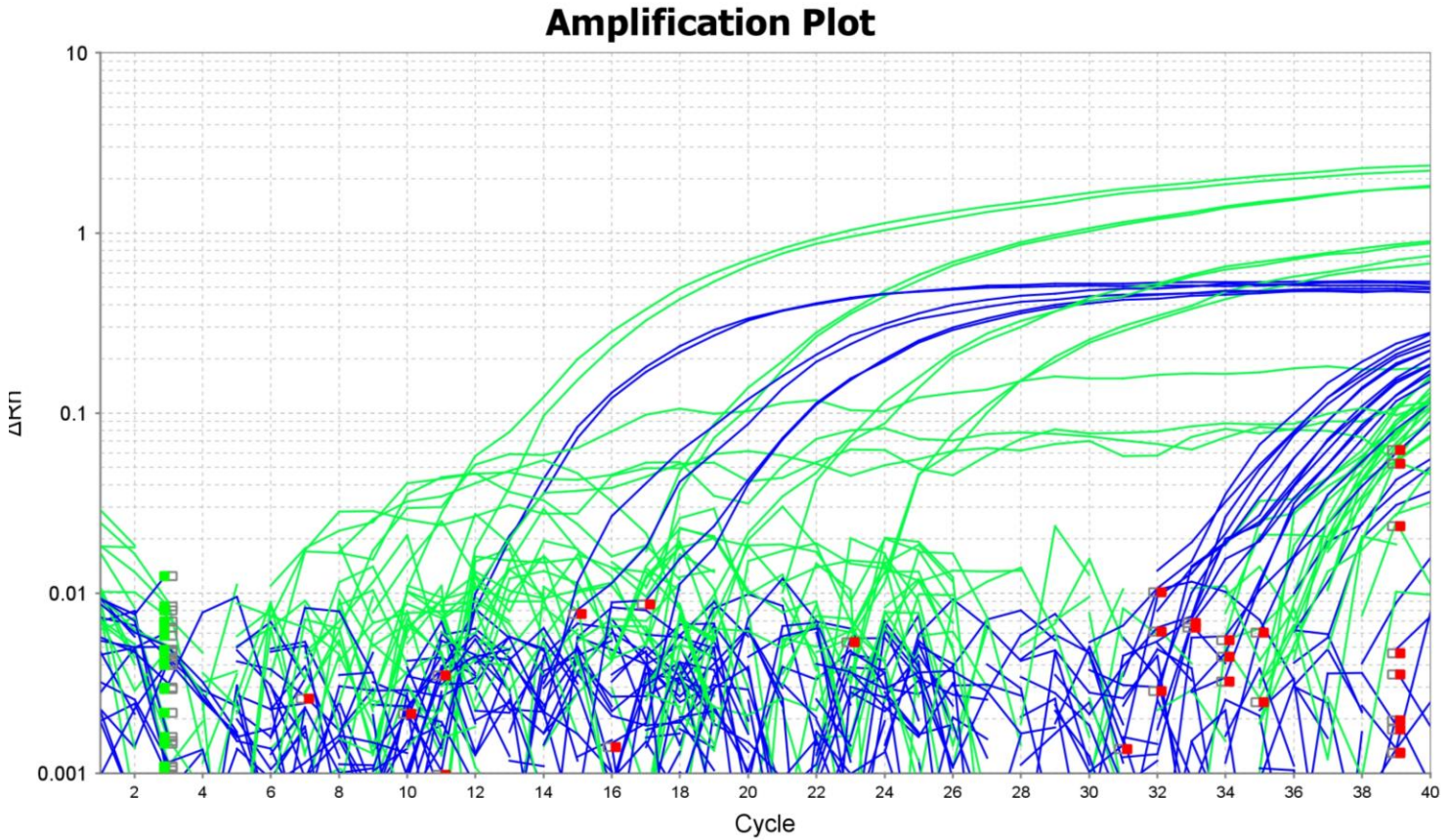
- **Established pests of biosecurity significance:** Avocado sunblotch viroid

Biosecurity pathways

- Fresh fruit imports into Australia from New Zealand and Chile.
- Indonesia is fourth largest producer of avocados in the world, very close to Australia.
  - Most production is in Java but minor production throughout archipelago.
- EPPs can come in through other agricultural commodities,
  - e.g. scolytid beetles in timber.

Our research in avocado biosecurity

Surveillance for early detection and establishment of pest freedom status	Diagnostic support for clean planting material scheme	Diagnostic support for quarantine services



The Queensland Alliance for Agriculture and Food Innovation (QAAFI) is

Phytopathology® • 2023 • 113:559-566 • https://doi.org/10.1094/PHYTO-08-22-0295-R

Virology

Surveillance for Avocado Sunblotch Viroid Utilizing the European Honey Bee (*Apis mellifera*)

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[3] Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, St. Lucia, Queensland 4072, Australia
Accepted for publication 3 November 2022.

Avocado sunblotch viroid (ASBVd)

- Circular, single-stranded RNA molecule, 247 nucleotides.
- Natural host range is limited to avocado.
- Seed-transmitted, root to root & mechanically transmissible, no vectors.
- Reduces quantity and size of fruit and affects marketability through unsightly scarring on skin.
- Major trade impediment because of quarantine restrictions on fresh fruit.
- Very rare in Australia due to clean planting material scheme (ANVAS).

Costa Rica bans avocado imports from 9 countries

By *The Tico Times* May 12, 2015

Share

Surveillance for ASBVd

Evidence of pest-freedom required to facilitate trade.

Question: How can an orchard containing 30,000 trees, each 10 x 5 m in size, be rapidly and inexpensively surveyed for ASBVd?

Potential solution: Bee-assisted surveillance!

- ASBVd is pollen-transmitted.
- Bee hives brought in to boost pollination.
- ASBVd is a very resilient molecule in the environment.
- Worker bees forage on thousands of flowers and store pollen in hive.

Leaf stored at room temp for 4 weeks and still strongly positive (Ct = 17.6)

Methods

- Study done in Australia and South Africa, but only Australian results presented here.
- Block of avocados containing 343 trees, including a tight clump of four infected trees.
- Bee hives at *c*. 100, 200, 300 and 400 m distance from infected trees.
- Sampling done at end of avocado flowering period – 28 Sep 2020.
- Worker bees collected from flowers on infected tree.
- Worker bees and pollen samples collected from hives.
- Diagnosis done using three methods:
  - RT-qPCR using Taqman™ probe.
  - Illumina sequencing of ribo-depleted total RNA and small RNA (21-22 nt) – *de novo* assemblies and mapping of reads to reference genomes (CLC Genomics Workbench v20).

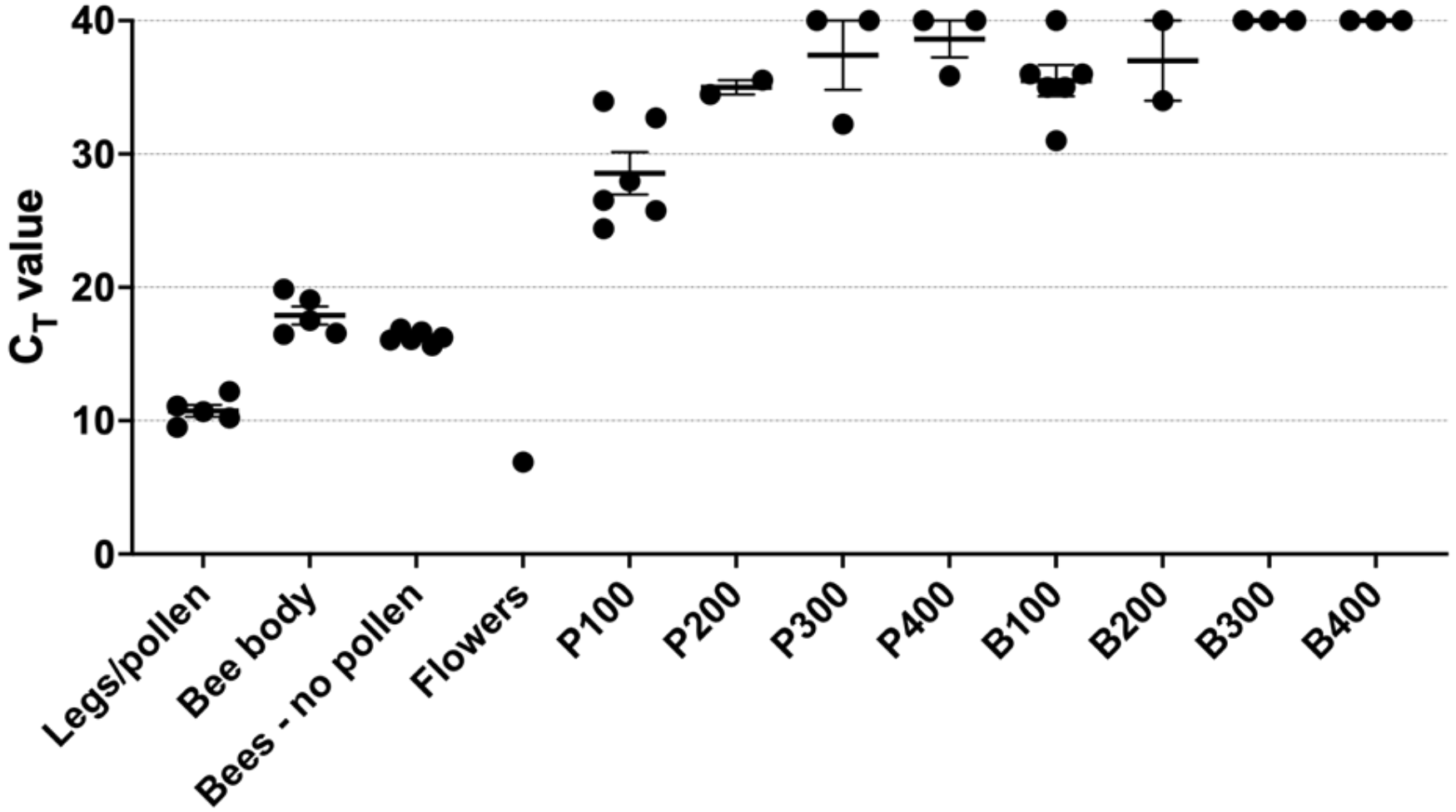
Arrangement of hives (QLD1-4) relative to clump of four infected trees (red dot)

Methods

Identification of most common plant species represented in hive pollen (# sequence contigs)

Plant species (closest match)	QLD1 hive	QLD2 hive	QLD3 hive	QLD4 hive
Eucalyptus grandis	4,143	3,638	3,672	2,927
Syzigium oleosum	701	220	557	562
Citrus spp.	440	227	638	8
Raphanus sativus	738	138	127	99
Rhodamnea argentea	396	82	276	284
Brassica spp.	31	136	13	48
Medicago truncatula	64	2	56	6
Camellia sinensis	47	0	6	0
Gossypium spp.	0	32	13	2
Cicer arietinum	19	2	21	4
Pyrus x *bretschneideri*	11	0	8	24
Persea americana	**2**	**0**	**0**	**20**

RT-qPCR detection of ASBVd



High throughput sequencing (HTS) detection of ASBVd in hive samples

Sample name	Sample type	HTS type	Normalized sequence copy no.
QLDforagers	Forager bees on infected tree	RNA-seq	0.6
QLDforagers	Forager bees on infected tree	Small RNA-seq	289
QLD1	Hive bees	Small RNA-seq	0.3
QLD1	Hive pollen	Small RNA-seq	8.7
QLD2	Hive pollen	Small RNA-seq	0
QLD3	Hive pollen	Small RNA-seq	0
QLD4	Hive pollen	Small RNA-seq	0

Other viruses and viroids that were detected in hives

Virus/viroid	Family	% max nt identity	% genome coverage	RNAseq		sRNA	
				Bees	Pollen	Bees	Pollen
Persea americana alphaendornavirus 1**	*Endornaviridae*	99	99	✓	✓	✓	✓
Persea americana chrysovirus**	*Chrysoviridae*	99	98	✓	✓	✓	✓
Pelargonium zonate spot virus	*Bromoviridae*	99	98	✓	✓	✓	✓
Tomato ringspot virus**	*Secoviridae*	97	63	✓	✓		
Solanum nigrum ilarvirus**	*Bromoviridae*	99	16	✓	✓		
Peanut stunt virus**	*Bromoviridae*	99	78	✓			
Turnip mosaic virus	*Potyviridae*	91	37	✓			
Pear blister canker viroid*	*Pospiviroidae*	99	100	✓			

Status
**Exotic
*New for QLD

Conclusions

- Bee-assisted surveillance is a valuable tool for determining ASBVd pest status of an avocado orchard.
- Stored pollen is the best sample type.
- Optimal when trees are no further than 100 m from any tree (normal stocking rate is 2-3 hives/ha).
- Multi-purpose surveillance method – plant and bee viruses, viroids, maybe fungi, and varroa mites.
- Biased surveillance method.
  - Plants must be flowering at the time of sample collection.
  - Bees prefer some flowers over others and you can't dictate where they go!
  - Favours pollen-transmitted viruses/viroids and high titre mechanically transmissible viruses (e.g. ilarviruses, sobemoviruses and tobamoviruses).
- Best done by specialists!

Rapid Sample Preparation for High Throughput Diagnosis of Avocado Sunblotch Viroid

The University Of Queensland
AUSTRALIA

QAAFI
Queensland Alliance for Agriculture and Food Innovation

Queensland Government

Lara Pretorius

ASBVd Management - The road to pest freedom

- Eradication effort in 1970s-Prevented spread
- Eradication phase
  - Avocado Nursery Voluntary Accreditation Scheme (ANVAS)
  - Surveys in older blocks
  - Implementing rigorous testing regimes
    - In-line testing
    - Multiplication blocks
    - Production blocks- this is the tricky one
- Filter disc extraction method developed due to large number of avocado trees tested
- Diagnostic assay: reverse transcription quantitative PCR (RT-qPCR) (Geering et al, 2006)

Filter disc extraction method

- An easy, rapid and inexpensive method of preparing template for a reverse transcription qPCR
- Reversible binding of viroid RNA to filter paper

Journal of Virological Methods 301 (2022) 114455

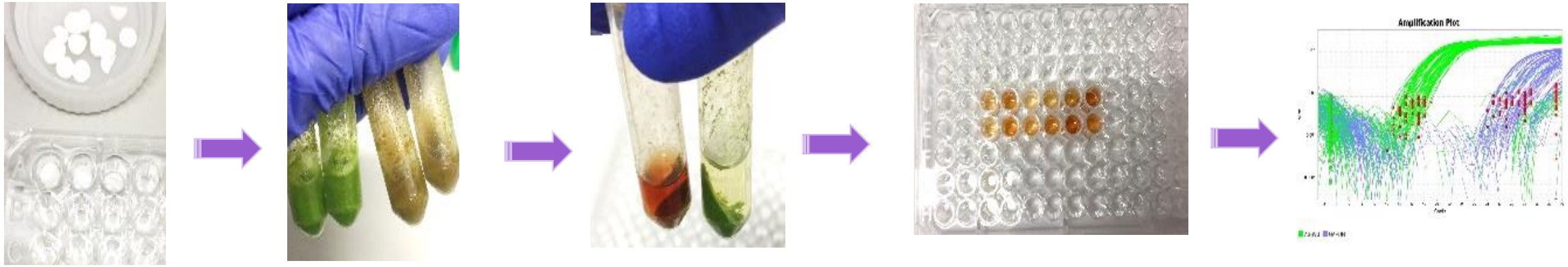
Contents lists available at ScienceDirect

Journal of Virological Methods

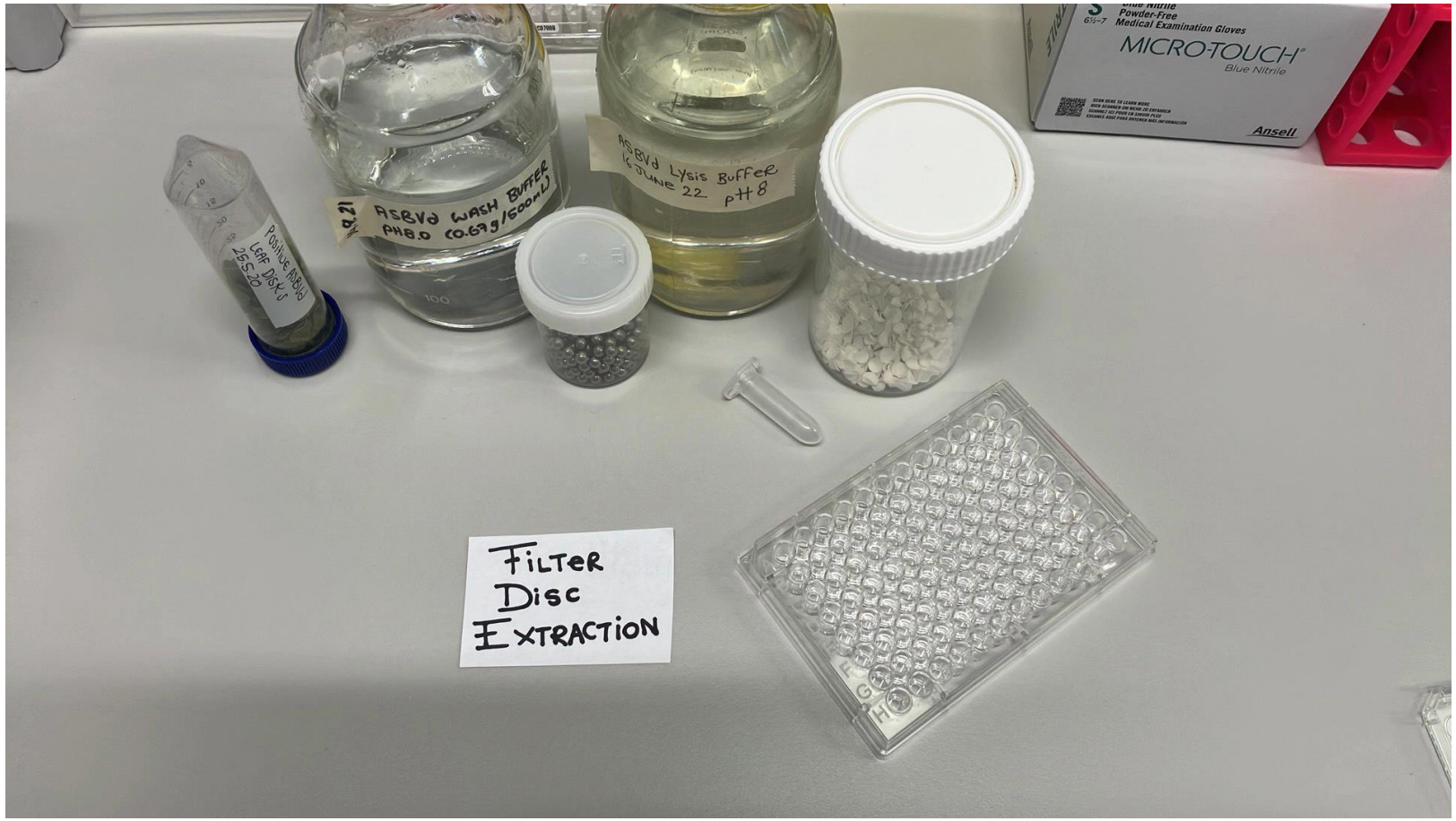
journal homepage: www.elsevier.com/locate/jviromet

Adaptation of a filter paper method for RNA template preparation for the detection of avocado sunblotch viroid by reverse transcription qPCR

Lara-Simone Pretorius [a], Kerri A. Chandra [b], Anna E.C. Jooste [c], Lebogang C. Motaung [c], Louisamarie E. Parkinson [a], Andrew D.W. Geering [a,*]

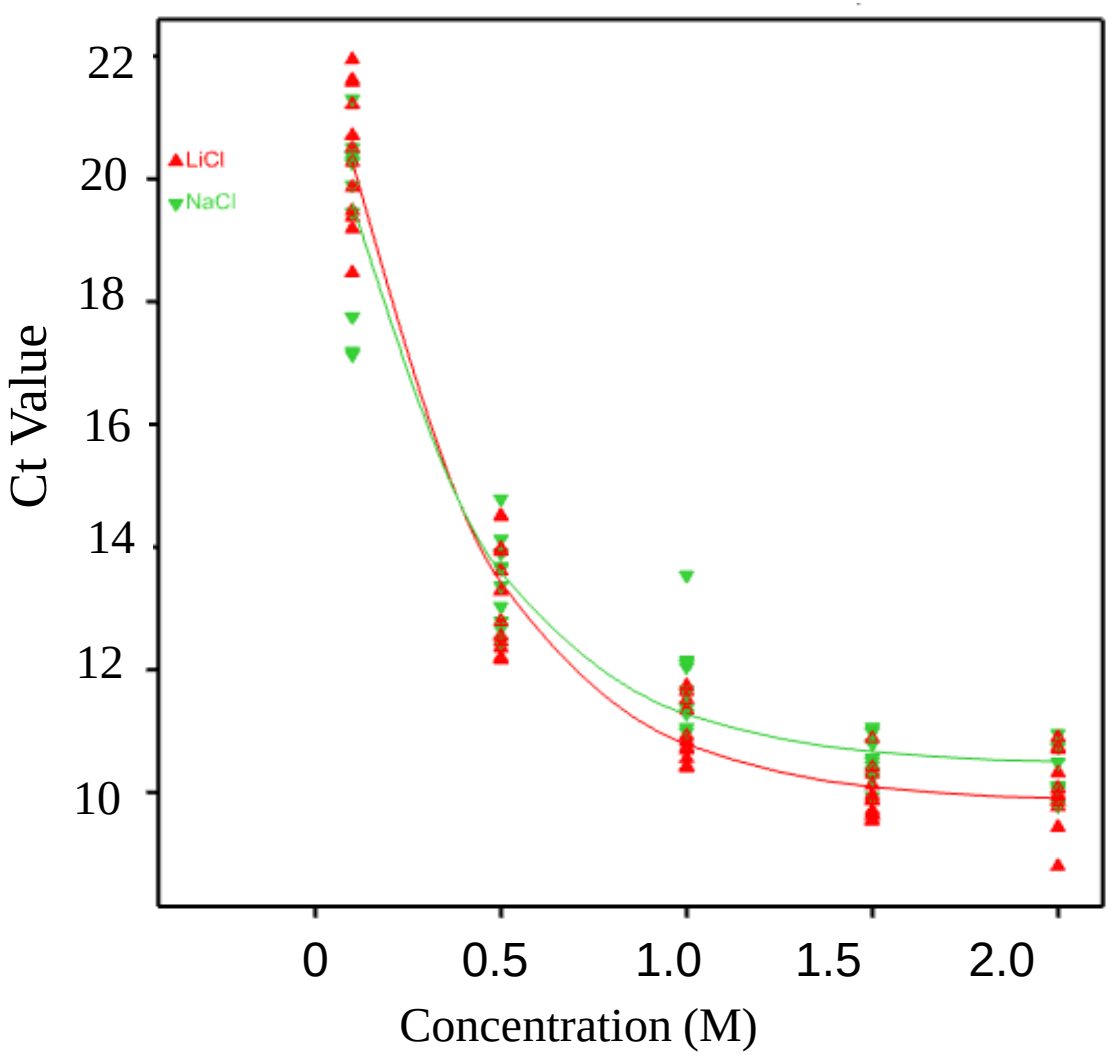


Filter disc extraction method

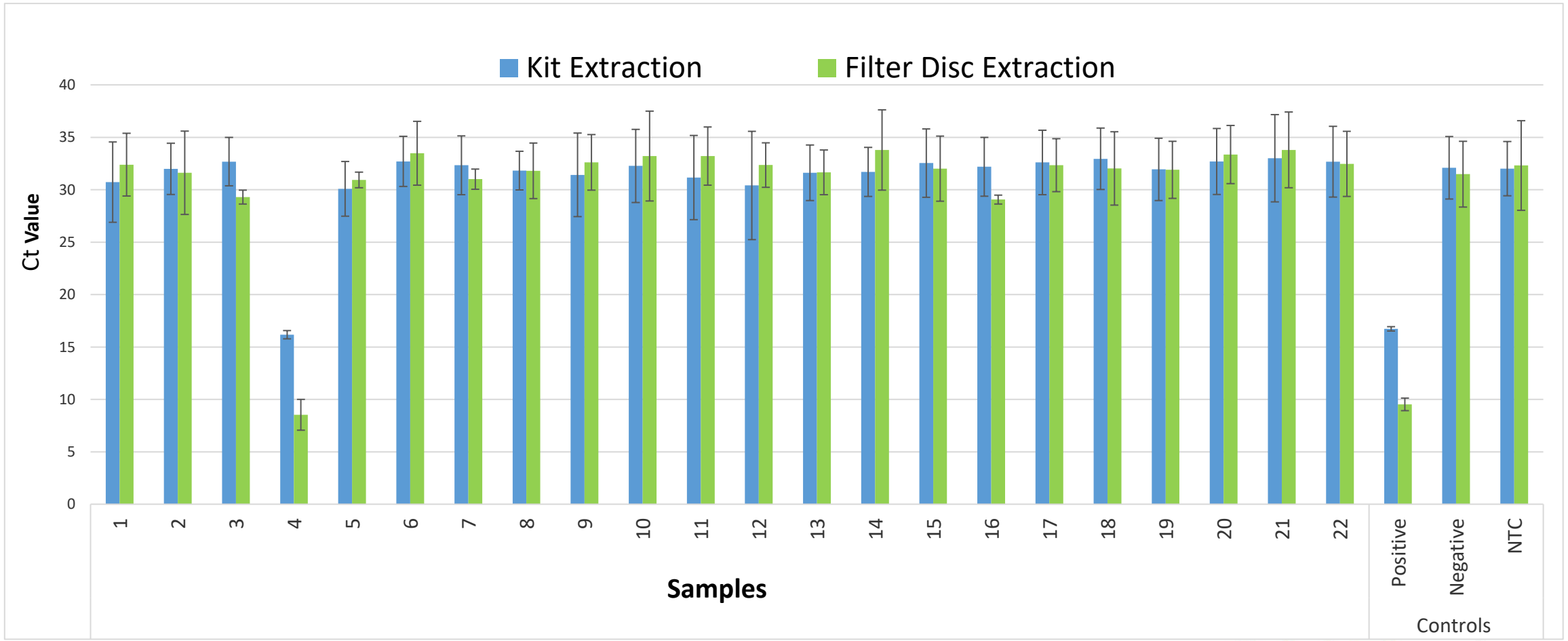


Optimising the filter disc extraction method

- **Testing lysis buffers: NaCl and LiCl, varying concentrations**
  - No statistically significant differences between salt types – Only concentration
  - Increasing salt = decreasing Ct value = Increase yield of nucleic acids
  - **LiCl 1.5 M was optimal for ASBVd RNA extraction**



Filter disc extraction vs commercial RNA extraction kit



Resilience of avocado sunblotch viroid RNA

Leaves and fruits

Stored at 4°C

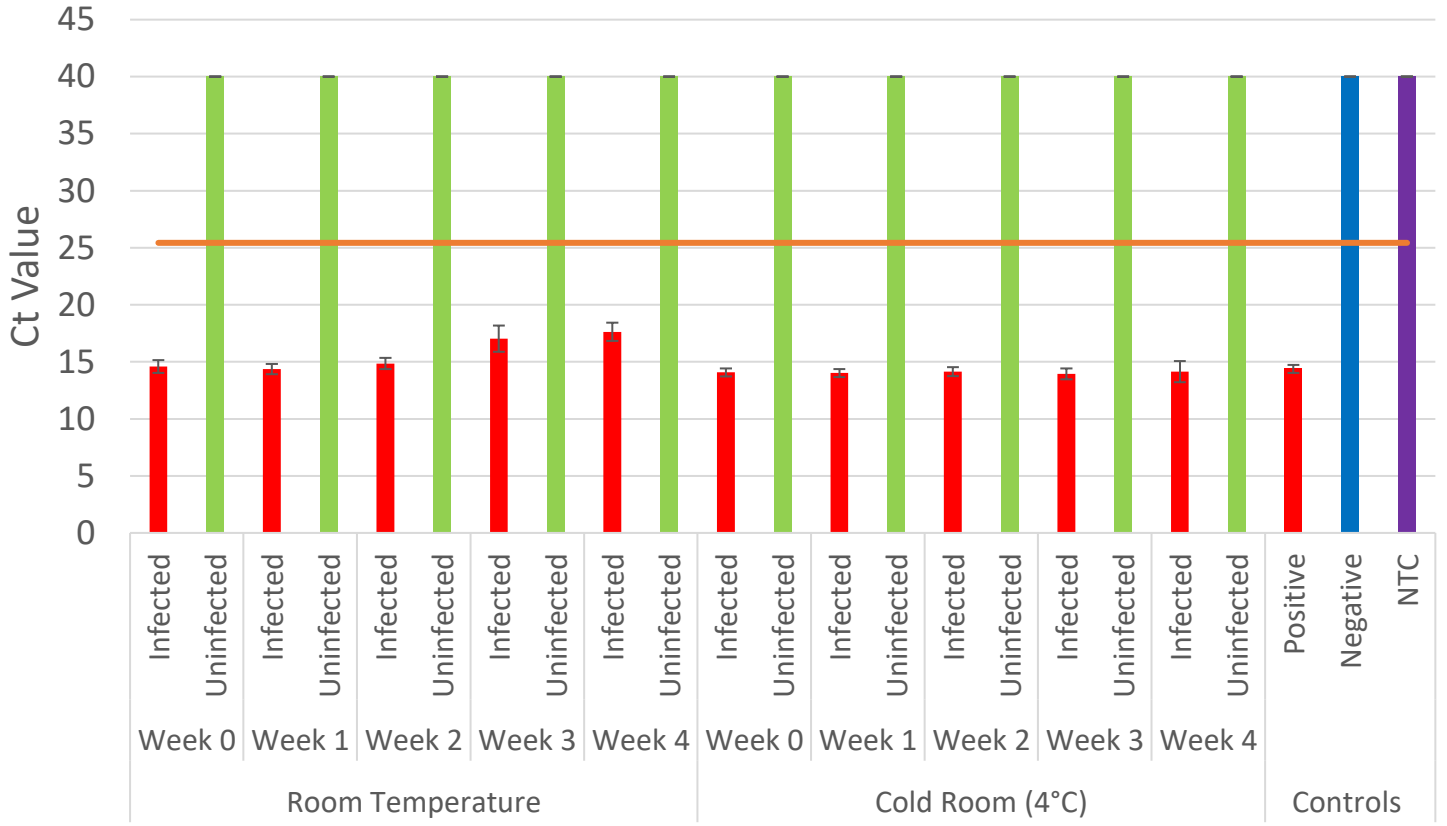
Stored at RT

- Biopsied weekly in triplicate for RT-qPCR
- For 4 Weeks

Resilience of avocado sunblotch viroid RNA

Day of Collection

4 Weeks at RT



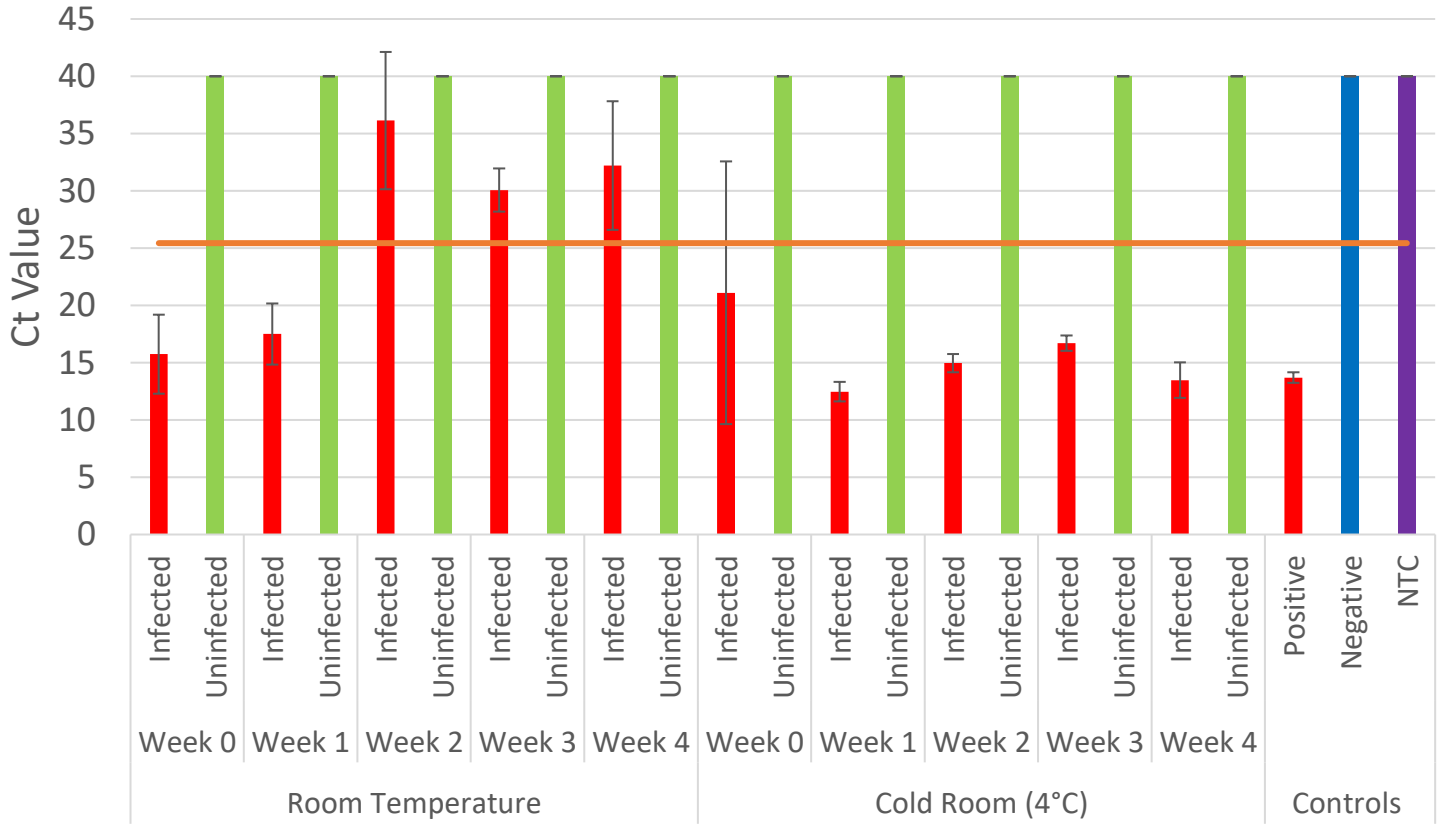
van Brunschot, Bergervoet et al. 2014

The Queensland Alliance for Agriculture and Food Innovation (QAAFI) is a research institute of The University of Queensland (UQ), supported by the Queensland Government.

Resilience of avocado sunblotch viroid RNA

Day of Collection

4 Weeks at RT



Conclusions

- Filter disc extraction method is cost effective, ½ cost of conventional RNA extraction kit
- Time saving - decreasing time actively performing extraction ultimately decreasing the labour cost and possibility of human error
- Increased yields of viroid RNA using the filter paper method than that obtained using a commercial kit
- Ideally suited for large-scale surveillance for ASBVd
- Applicable in other pathogen diagnostics, e.g. fungi and arrange of RNA viruses

Diagnostics for avocado biosecurity

Nga Tran, Lara Pretorius, Louisa Parkinson, and Andrew Geering

Queensland Alliance for Agriculture and Food Innovation (QAAFI)

The University of Queensland, Australia

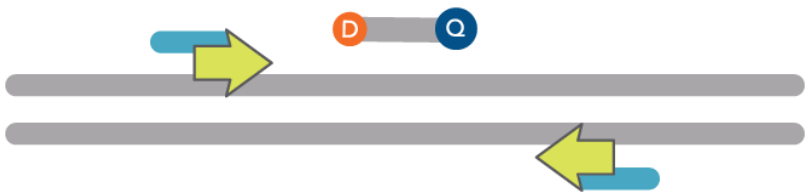
Diagnostic for exotic pests and pathogens

- Initial identification of an EPP is based on:

1. International Plant Protection Convention (IPPC) protocols
2. National Diagnostic Protocols (NDPs)
3. Peer reviewed published procedures
4. Best practice diagnostic techniques

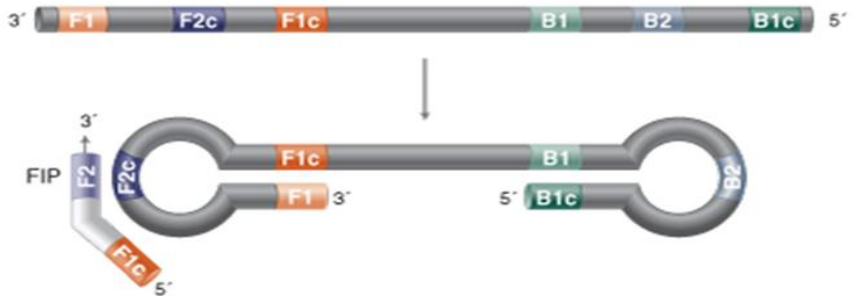
Our preferred assays

Quantitative real time PCR (qPCR)



- ✓ 2 primers and a fluorescently labelled probe
- ✓ Real time detection & sensitive
- ✓ Quantitative
- ✓ Detection methods: probe (TaqMan), florescent dye (SYBR Green)
- ✓ Multiplexing options
- ✓ Allow downstream application of products (sequencing etc.)
- × Lab based & requires experience

Loop-mediated Isothermal Amplification (LAMP)



- ✓ 4-6 primers, thus some gene loci not suitable
- ✓ Real time detection & highly sensitive
- ✓ Quantitative
- ✓ Multiple detection methods: probe, florescent dye, lateral flow, colorimetric
- ✓ Multiplexing options
- × Product not ideal for downstream application
- ✓ Portable & doesn't require expertise

Developing/adopting diagnostic tests for EPPs

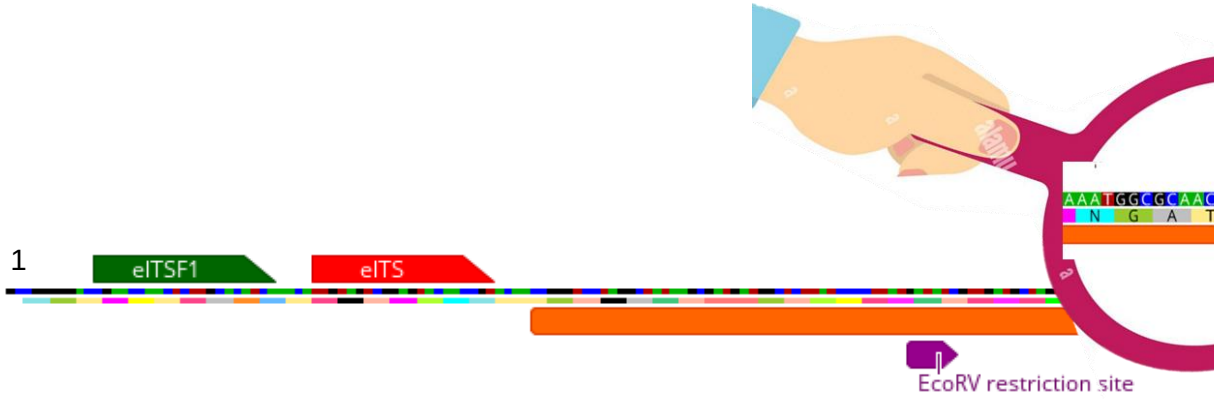
- **Validation parameters following EPPO and NATA standards:**

- **Positive controls (actual and synthetic)**

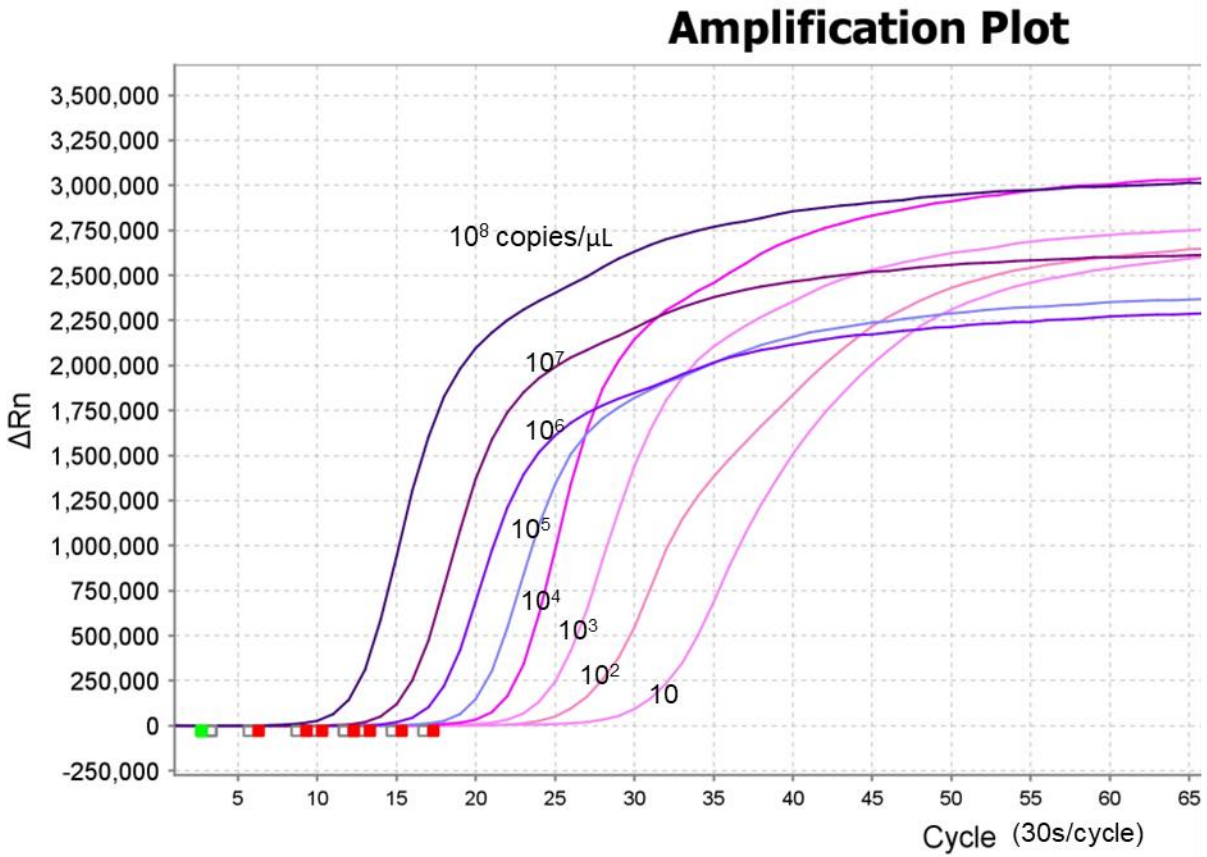
Developing/adopting diagnostic tests for EPPs

Using synthetic positive controls

- dsDNA fragment artificially synthesized, including primer binding sites
- block of random sequence added to:
  - ✓ distinguish native from synthetic DNA in ca
  - ✓ include a restriction enzyme substrate site
  - ✓ adjust GC content to increase Tm of synth
  - ✓ standardization of assay (sensitivity etc.)
  - ✓ synthetic controls not subject to quarantin



gBlock of 274 bp as positive control for the ITS qPCR of *E. perseae*



Diagnosis of avocado scab (*Elsinoë perseae*)

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Avocado scab caused by *Elsinoe perseae*: A diagnostic guide

Christ Mane Belizaire, Lederson Gañán-Betancur, and Romina Gazis

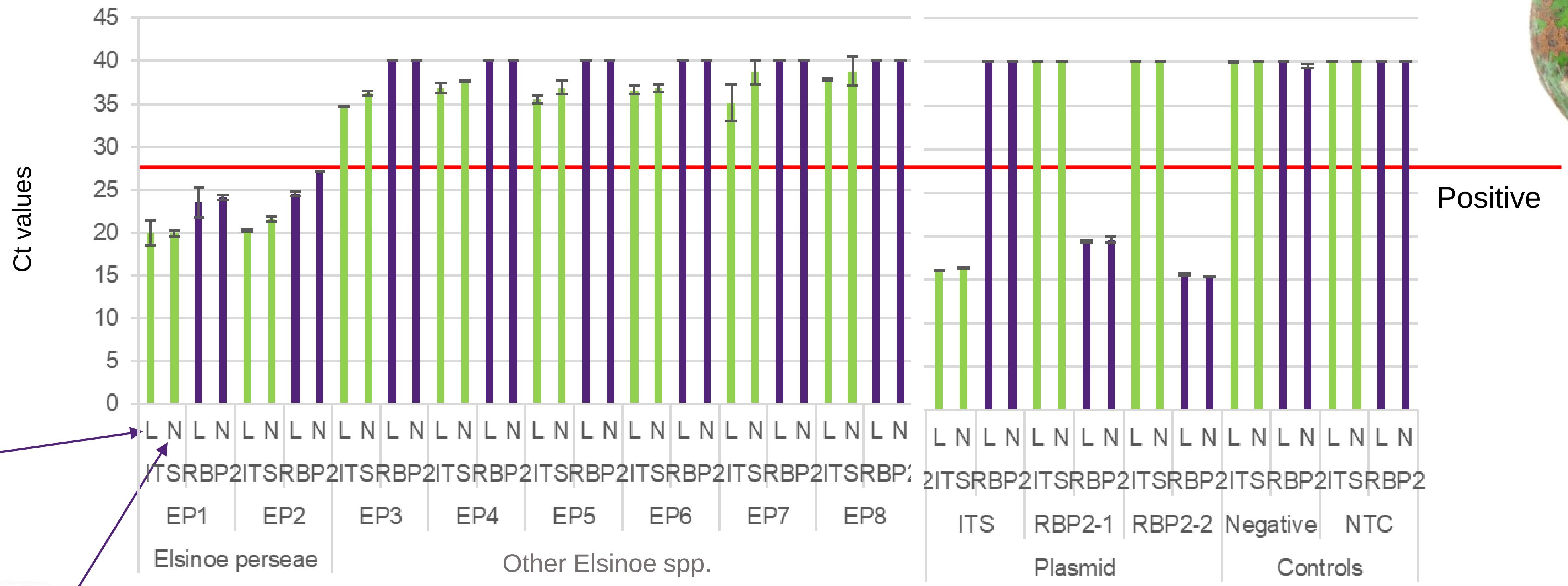
Published Online: 19 Dec 2023 | https://doi.org/10.1094/PHP-10-23-0084-DG

TOOLS SHARE

Abstract

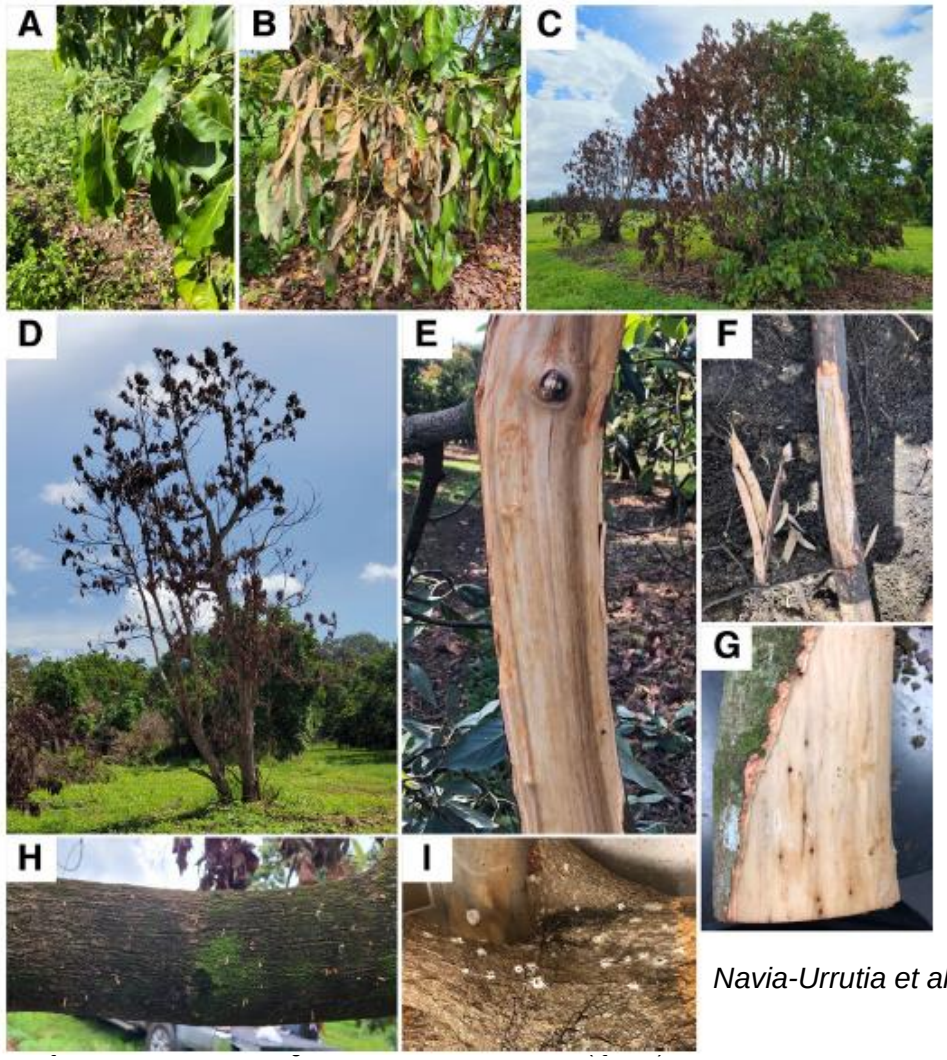
Avocado scab, caused by the fungus *Elsinoe perseae*, is a major disease of avocado (*Persea americana*). Diagnosis of avocado scab is hampered by the similarity that advanced symptoms have with fruit defects caused by other etiologies (e.g., mechanical injuries and insect feeding damage). This diagnostic guide aims to establish key aspects of the identification of symptoms and signs of avocado scab in the field and laboratory, and to provide guidance when conducting isolation, identification (i.e., based on morphological characters, molecular-based assays), maintenance, storage, and pathogenicity tests of the fungus *E. perseae*.

Novel duplex qPCR assay for avocado scab



qPCR results for *Elsinoe perseae* – **Reproducibility** & **repeatability** validation

Diagnosis of laurel wilt (*Harringtonia lauricola*)



Navia-Urrutia et al. 2022

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Diagnostic Guide

A Diagnostic Guide for Laurel Wilt Disease in Avocado

Monica Navia-Urrutia, Laura Sánchez-Pinzón, Pedro Pablo Parra, and Romina Gazis

Affiliations

Published Online: 15 Sep 2022 | https://doi.org/10.1094/PHP-12-21-0149-DG

SECTIONS ABSTRACT PDF e-Xtra TOOLS

Plant Disease • 2020 • 104:3151-3158 • https://doi.org/10.1094/PDIS-02-20-0422-RE

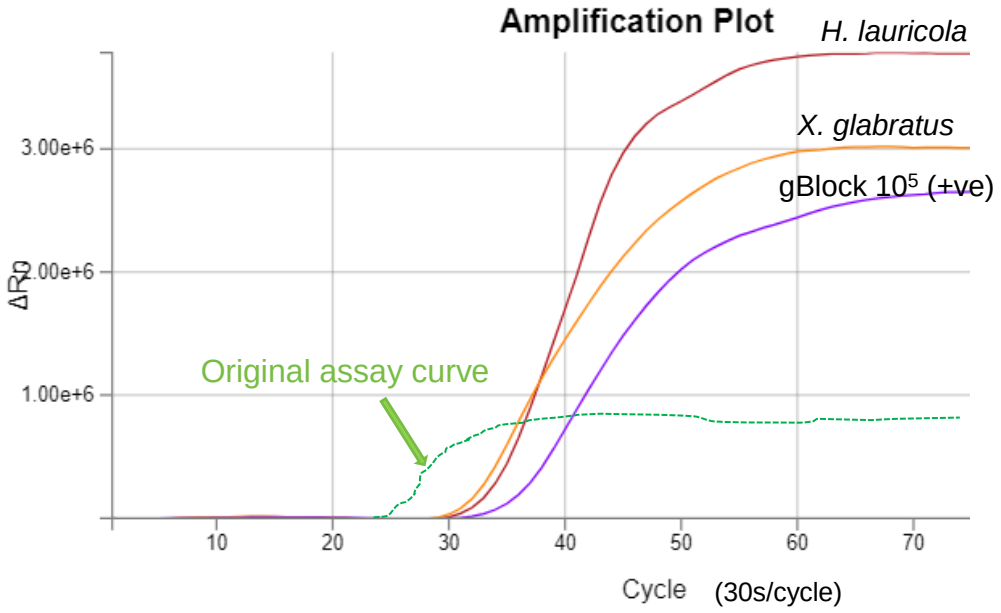
Rapid Detection of *Raffaelea lauricola* Directly from Host Plant and Beetle Vector Tissues Using Loop-Mediated Isothermal Amplification

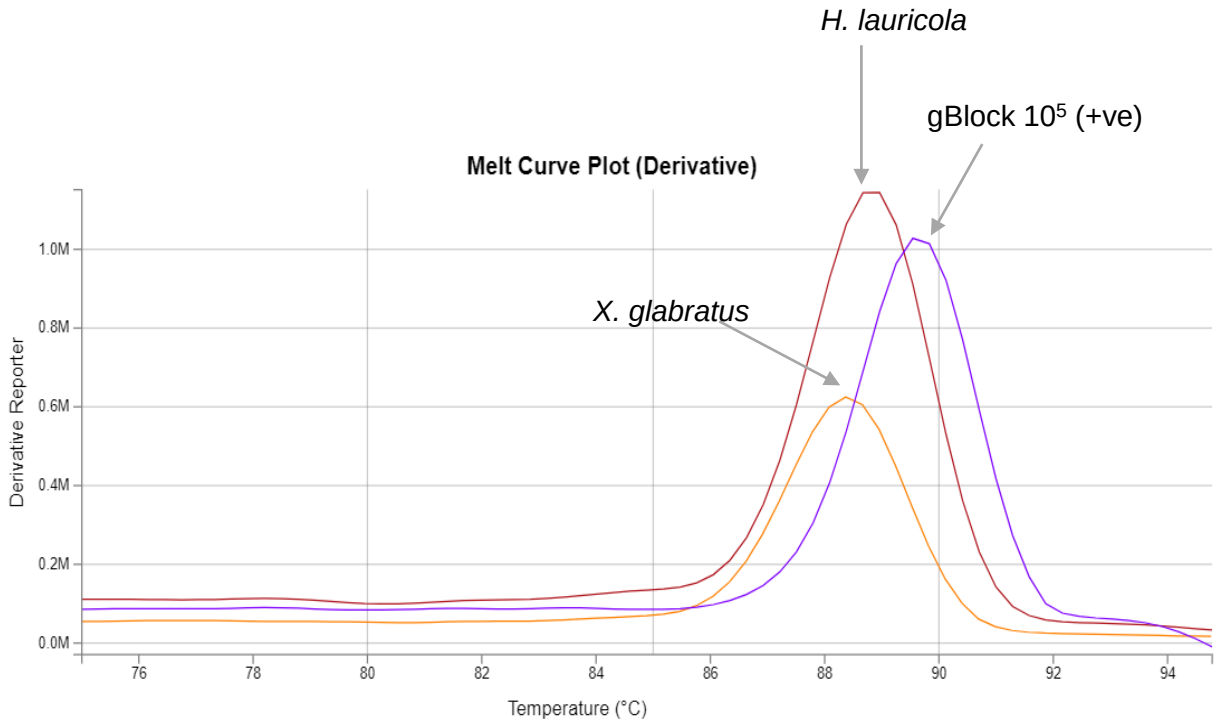
Jeffrey L. Hamilton,[1,†] J. Noah Workman,[1,2] Campbell J. Nairn,[1] Stephen W. Fraedrich[3] and Caterina Villari[1,†]

[1] Warnell School of Forestry & Natural Resources, University of Georgia, Athens, GA 30602, U.S.A.
[2] Department of Genetics, Harvard Medical School, Boston, MA 02115, U.S.A.
[3] United States Forest Service, Southern Research Station, Athens, GA 30602, U.S.A.

Laurel wilt LAMP – our modifications

- Florescent dye instead of probe based detection:
  - Stronger detection (but slightly late)
  - Melt curve analysis option
- Newly designed gBlock used as positive control
- Colorimetric





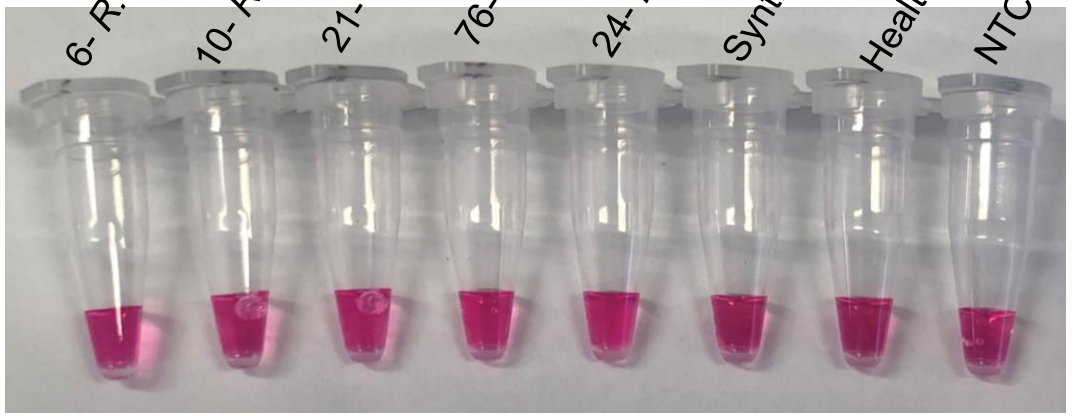
Laurel wilt LAMP – Colorimetric

Colorimetric master mix

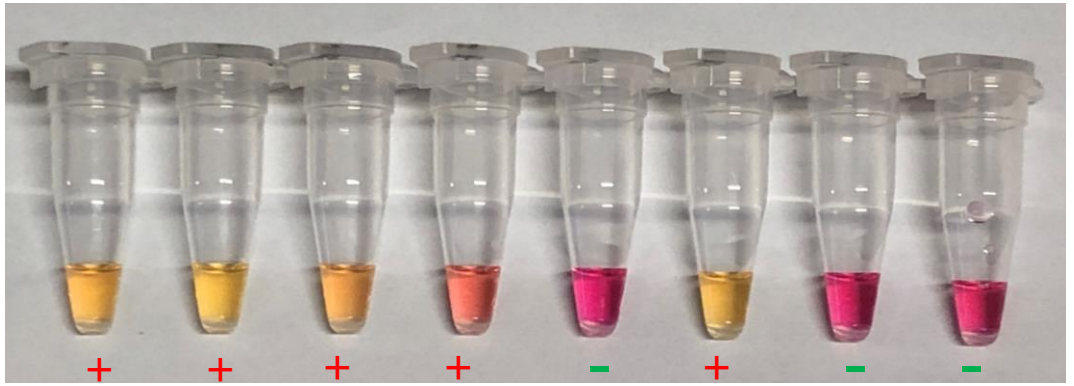
Primer mix

DNA/RNA

Start



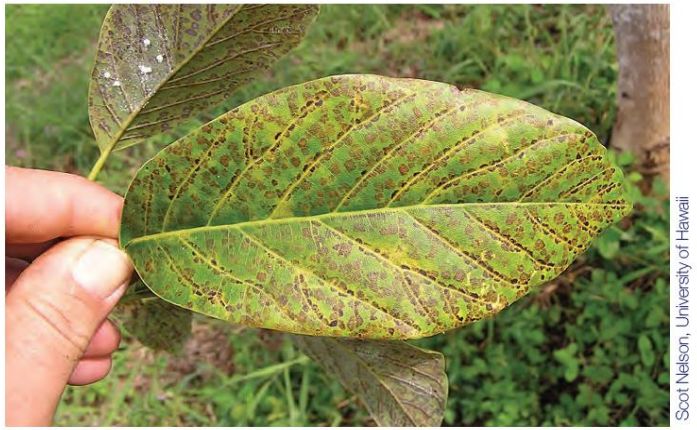
30 mins at 65°C



(weak)

Diagnosis of Persea mite

- Symptoms are distinctive.
- Morphological identification difficult for novices, like us!
- DNA barcoding completed using ITS2, 28S and COI gene loci.
- Three genetic lineages in Mexico, but only one is invasive.
- No information available as to whether genetic lineages differ in biology.



Scot Nelson, University of Hawaii

Feeding on upper leaf surfaces causes a yellow spotting pattern.

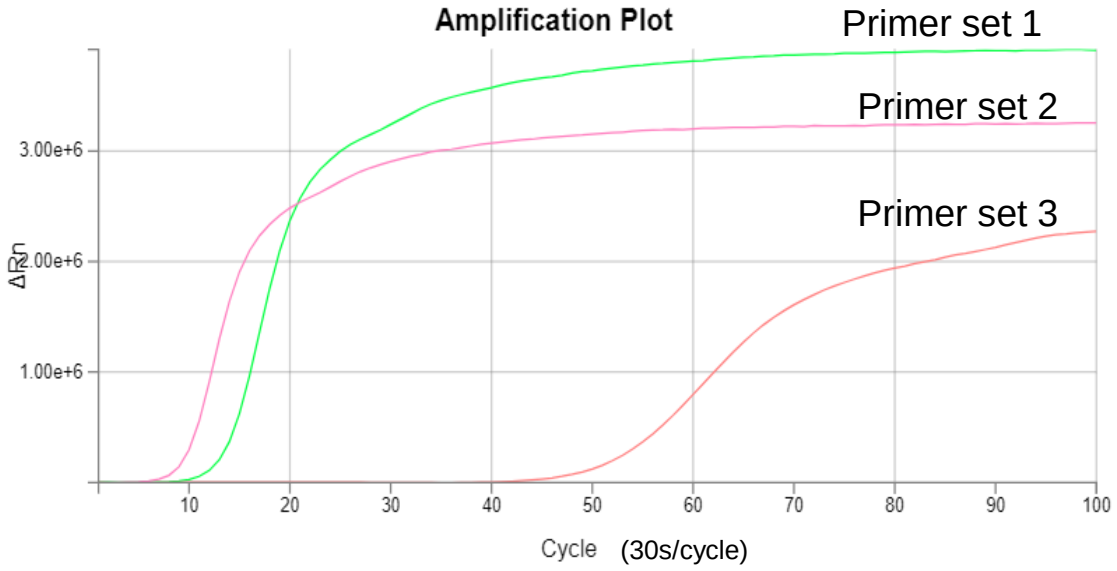
Jack Kelly Clark, University of California Statewide IPM Project

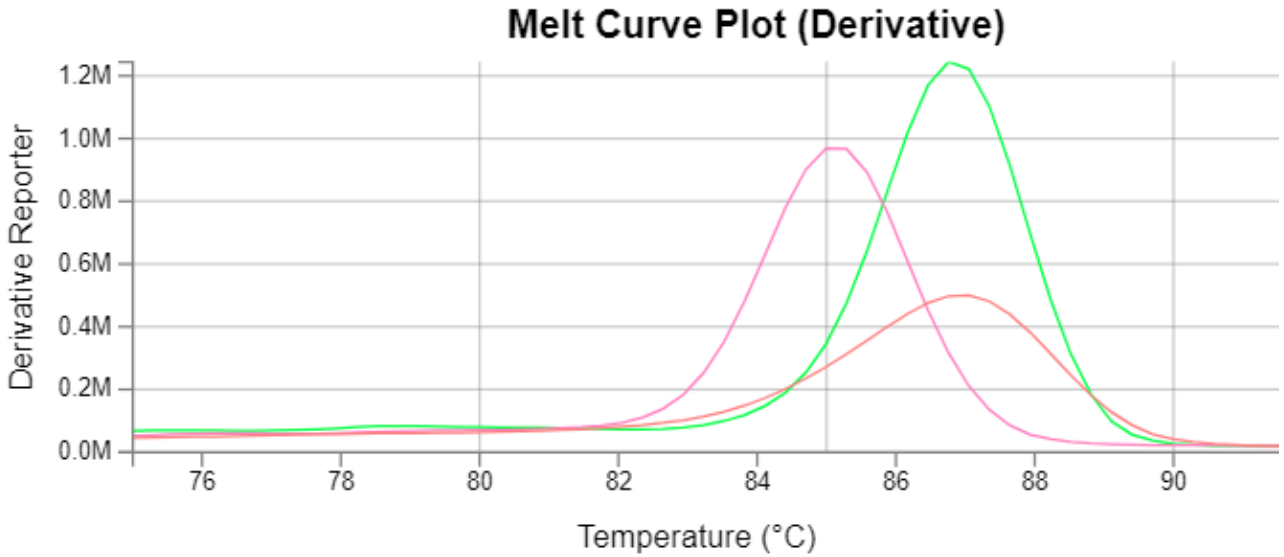
Immature stages are yellowish or greenish in colour

Source: Perseae mite factsheet, Plant Health Australia

Novel LAMP assay for Persea mite

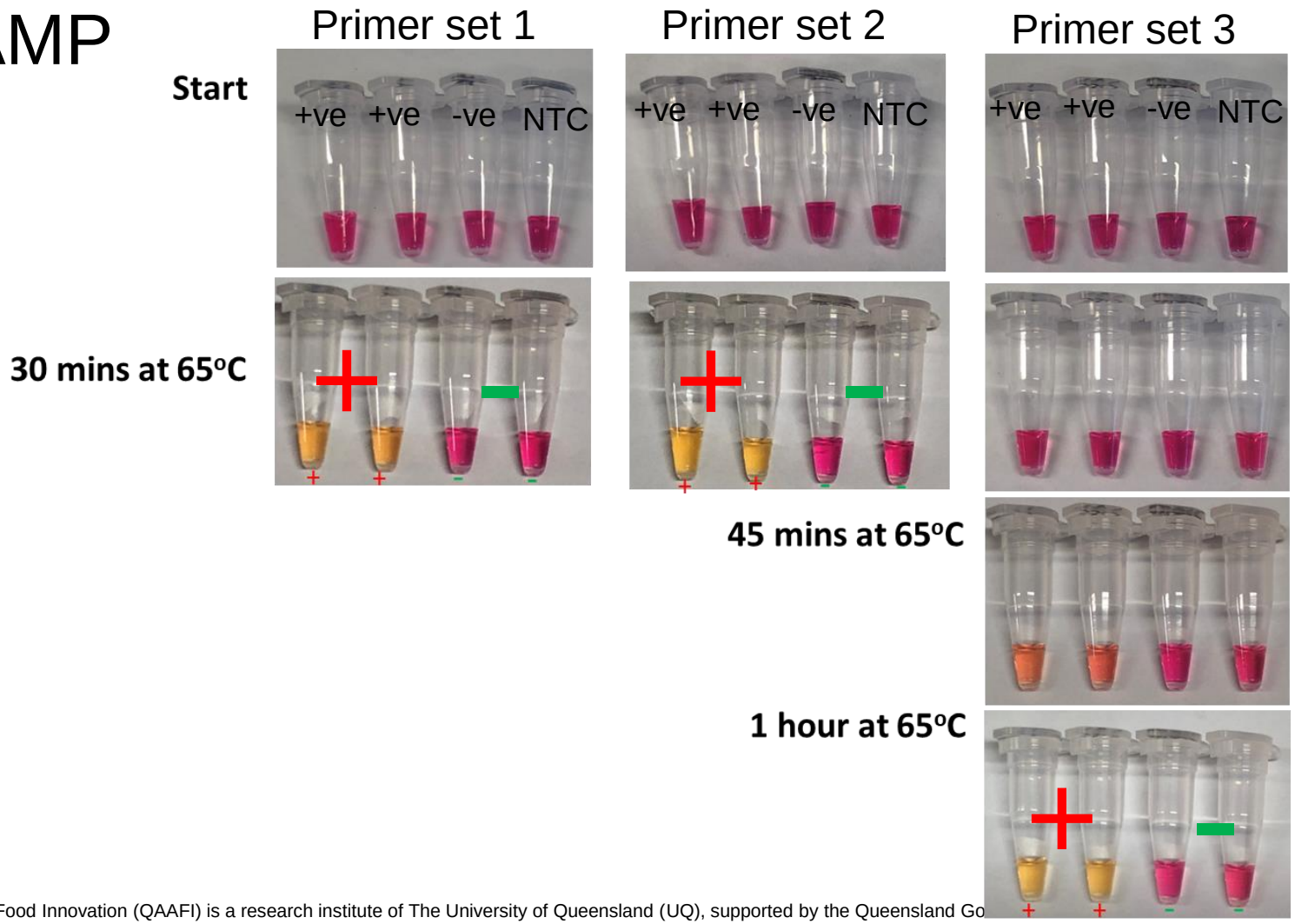
- Three sets of primers have been designed and tested:





Novel LAMP assay for Persea mite

Colorimetric LAMP



Further thoughts…

- Need thorough understanding of population genetics of a pest organism before diagnostic assay is developed.
- Important to test specificity of assay using related organisms found in each individual environment.
- Diagnostic assays need to be validated in different laboratories.

Acknowledgements

- University of Georgia (A/Prof. Caterina Villari's team) for providing us *Raffaelea* spp. DNA
- University of Florida (A/Prof. Romina Gazis's team) for validating the *Elsinoë* qPCR assay and for generously hosting us.
- Dr Elize Jooste (Agricultural Research Council, South Africa) and Dr John Roberts (CSIRO Australia) for collaboration in developing bee-assisted surveillance method.
- Australian avocado growers and Hort Innovation for funding this research.
- Plant Health Australian for the Diagnostic Residential Grant.

Thank you ☺

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The Queensland Alliance for Agriculture and Food Innovation (QAAFI) is a research institute of The University of Queensland (UQ), supported by the Queensland Gover

Diagnostic Residential 2023

Towards finalising NDPs for avocado scab and laurel wilt

Nga Tran
The University of Queensland

THE UNIVERSITY OF QUEENSLAND
AUSTRALIA

QAAFI
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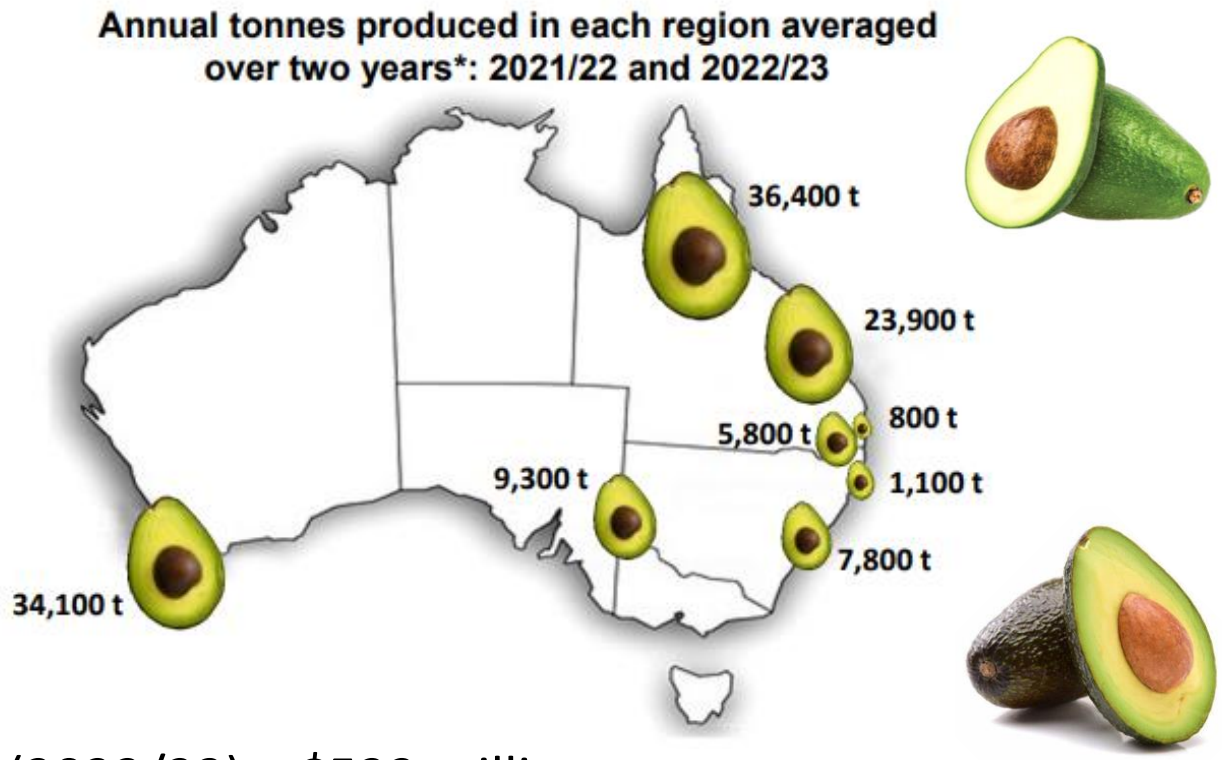
Queensland Government

The Australian avocado industry at a glance



- ✓ Farmgate value (2022/23) = $522 million
- ✓ Total: 18,000 ha (2022)
- ✓ Average yield: 8.2 t/ha

High priority pathogens (HPPs) – Diagnostic gaps

Avocado scab (*Elsinoë perseae)*

Laurel wilt (*Harringtonia lauricola, Xyleborus glabratus)*

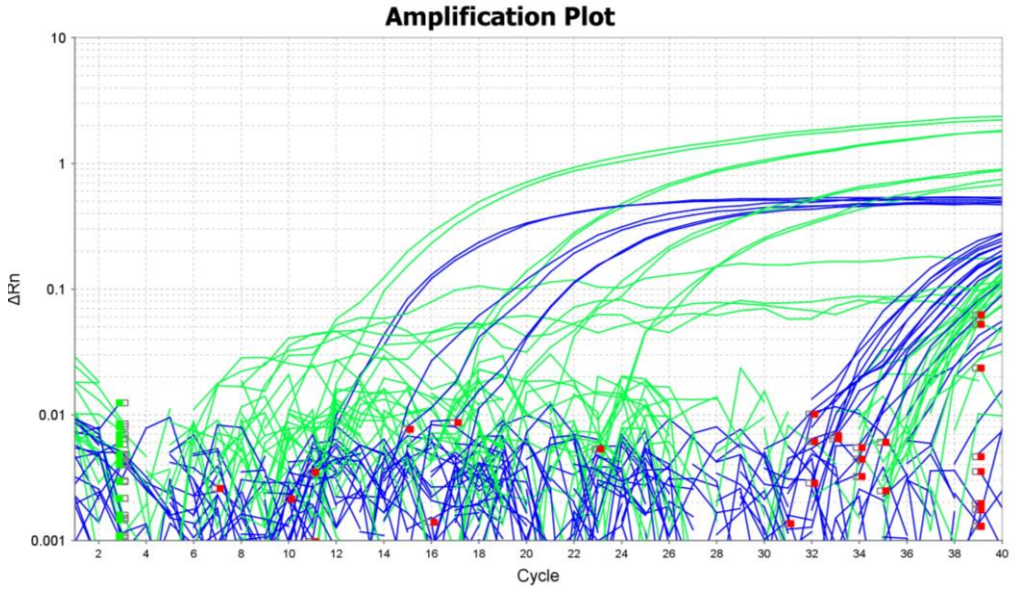
- No International Plant Protection Convention (IPPC) diagnostic protocols
- No National Diagnostic Protocols

COMMON NAME (SCIENTIFIC NAME)	NATIONAL DIAGNOSTIC PROTOCOL[24]	SURVEILLANCE PROGRAMS	FACT SHEETS[25]	CONTINGENCY PLAN	EPPRD CATEGORY	NATIONAL PRIORITY PEST	COLLABORATORS[26]
Avocado scab (*Elsinoe perseae (syn. Sphaceloma perseae)*)	In development	Not covered by a pest specific surveillance program	Yes- Avocado industry	Not developed	Not categorised	Not listed	-
Laurel wilt (*Raffaelea lauricola*)	Draft	Not covered by a pest specific surveillance program	Yes- Avocado industry	Not developed	Not categorised	Not listed	-

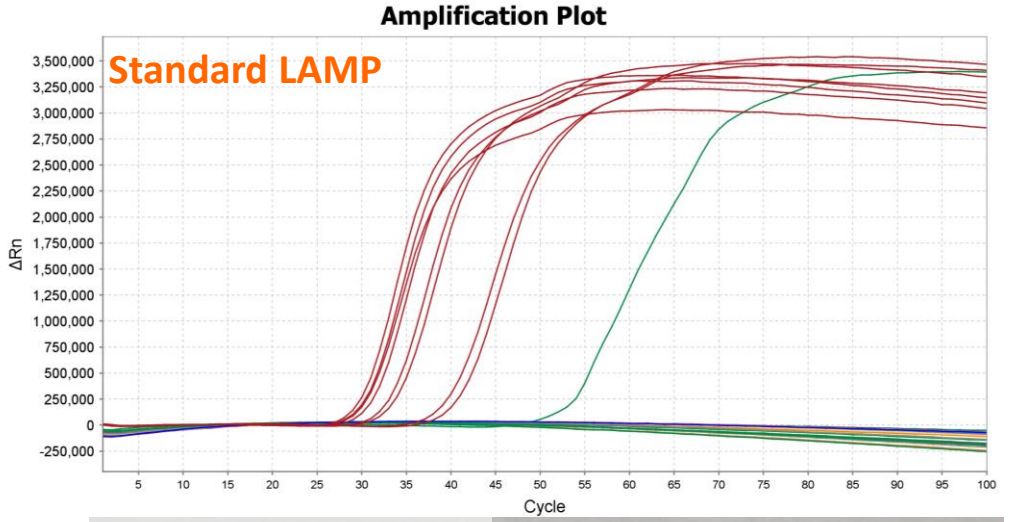
(Source: Avocado Biosecurity Plan v3.0)

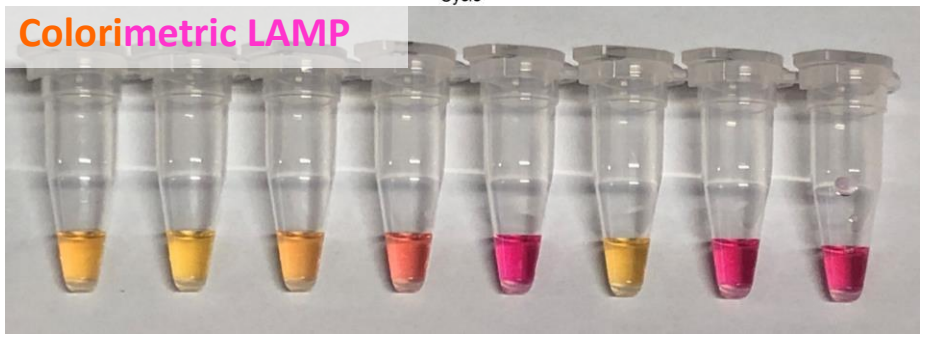
Diagnostic assays

E. perseae: Novel duplex TaqMan qPCR



H. lauricola: Adopted LAMP assay (Hamilton et al, 2020)

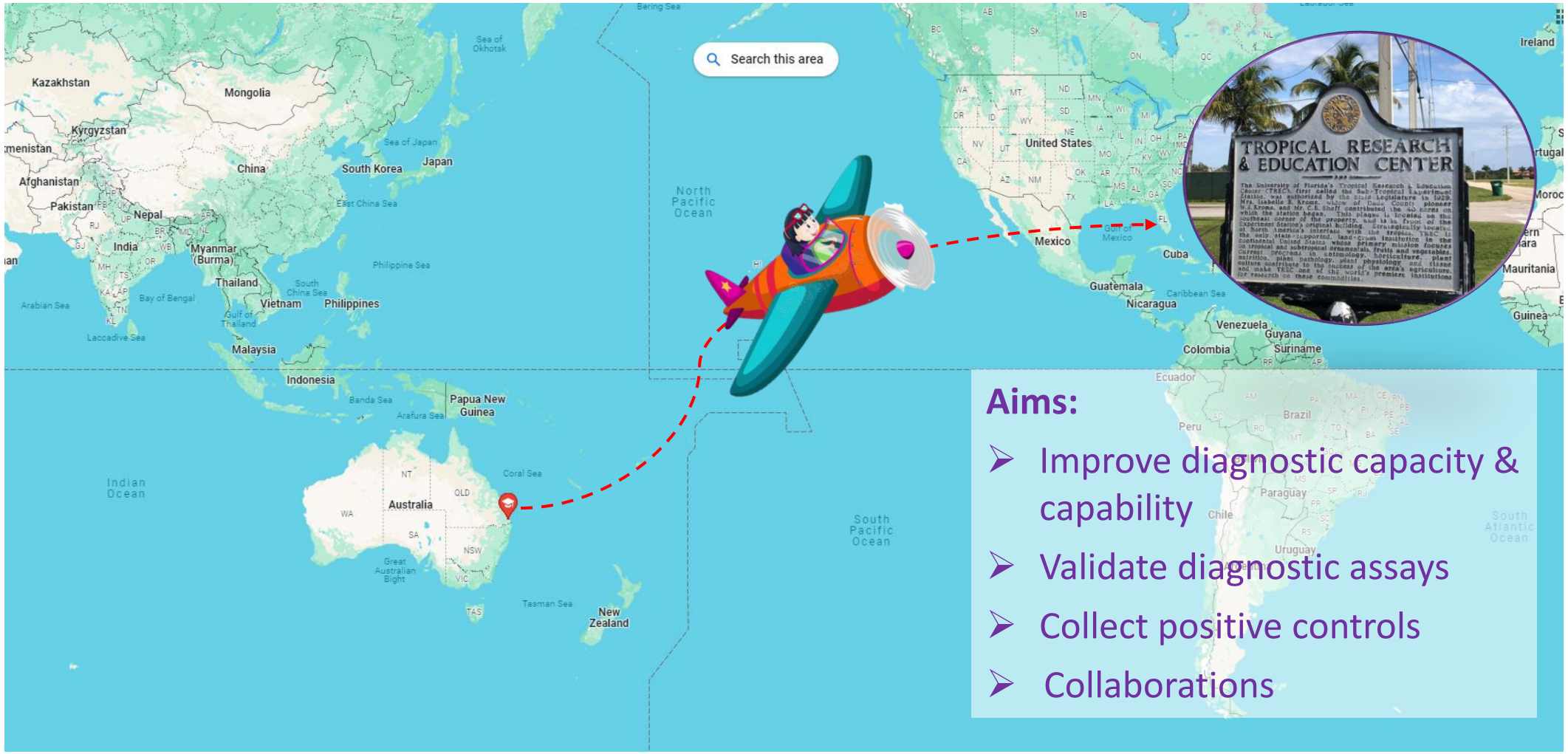




- Validation following EPPO and NATA standards…
- …using imported DNA and synthetic positive controls
- Diagnostic gap: Validate on infected tissues???

Residential project: Finalising NDPs for avocado scab and laurel wilt

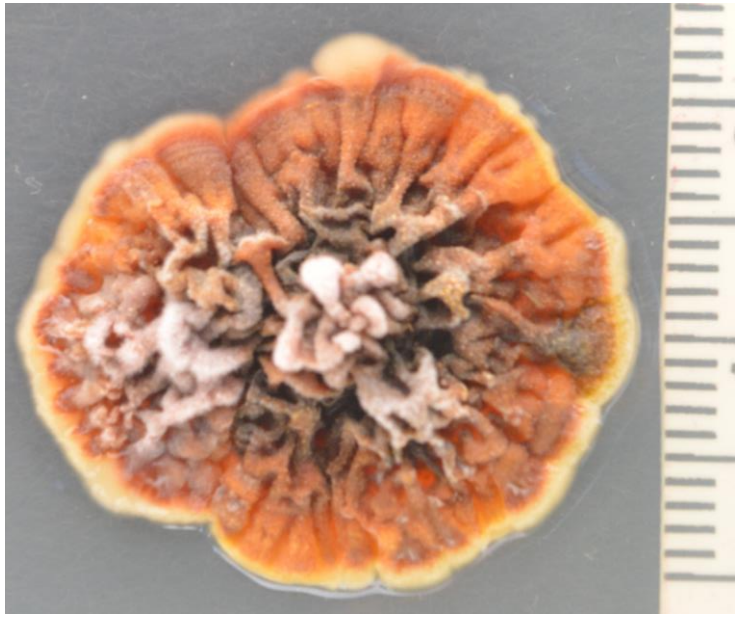
TREC, University of Florida, Homestead

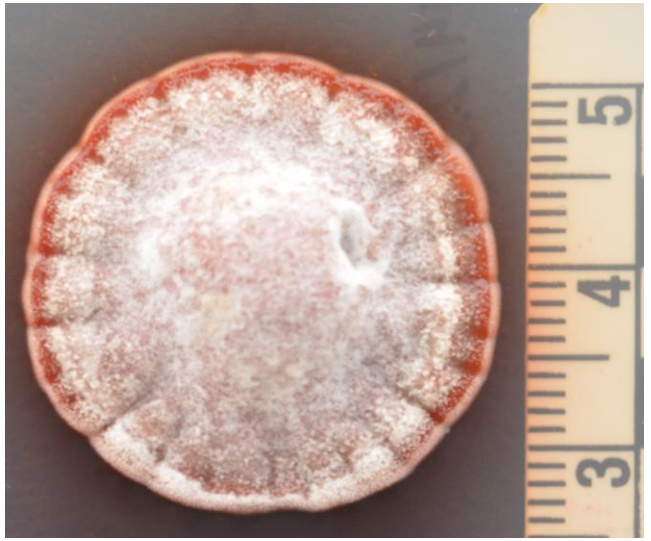


Aims:

- Improve diagnostic capacity & capability
- Validate diagnostic assays
- Collect positive controls
- Collaborations

Avocado scab (*Elsinoë perseae*)

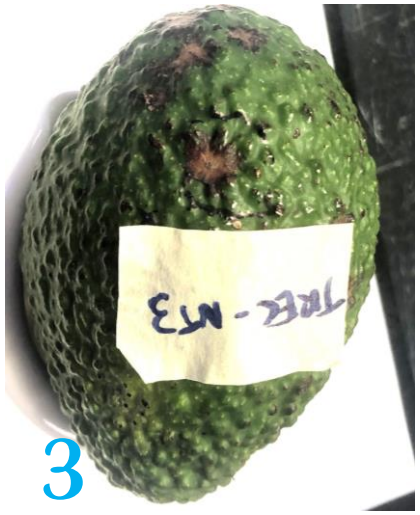




Scab, or not scab, that is the question!


1


2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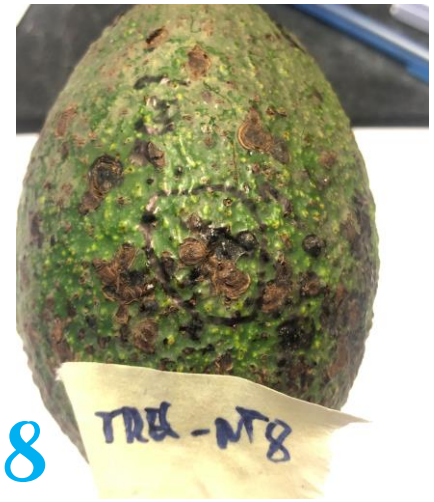
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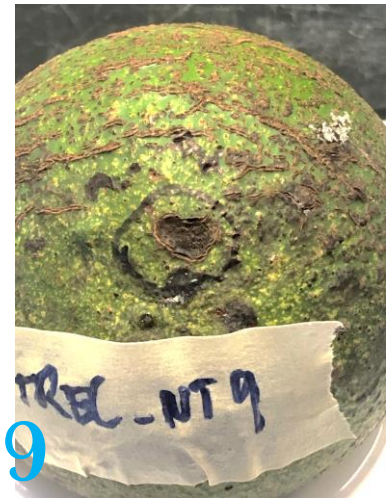
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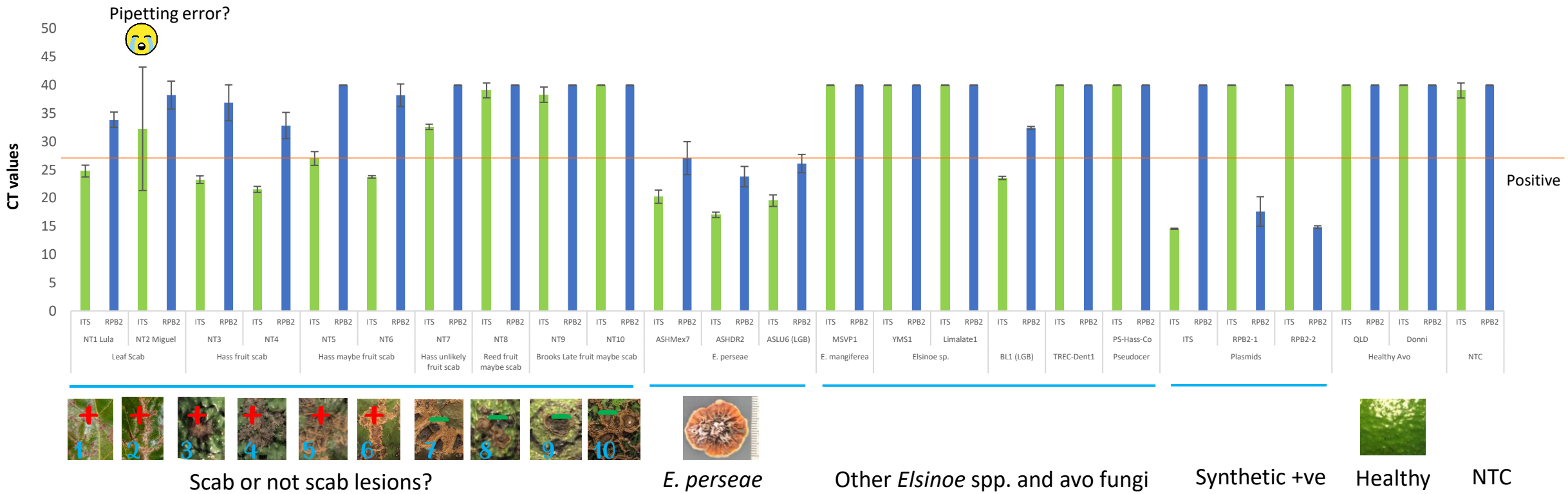
10


Healthy

Validating *Elsinoe* qPCR – Scab or not scab??



Laurel wilt: Symptoms








Laurel wilt = huge damage

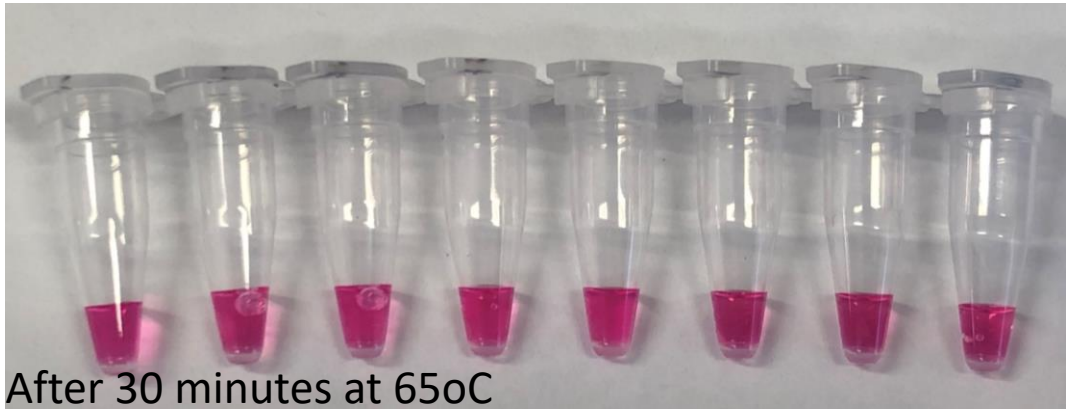
Laurel wilt: Samplings

Validating LAMP assay for *H. lauricola*

Standard LAMP

No.	Sample Name	CT
1	Avo laurel wilt symptom 1	95.526
2	Avo laurel wilt symptom 2	68.943
3	Raffaelea sp. PL1004	Undetermined
4	Raffaelea sp. LWP665	15.347
5	*H. lauricola*	41.646
6	gBlock (+VE)	56.657
		51.461
		Undetermined

Colorimetric LAMP



After 30 minutes at 65oC

Probably reagents brought from Brisbane had gone off? ☹

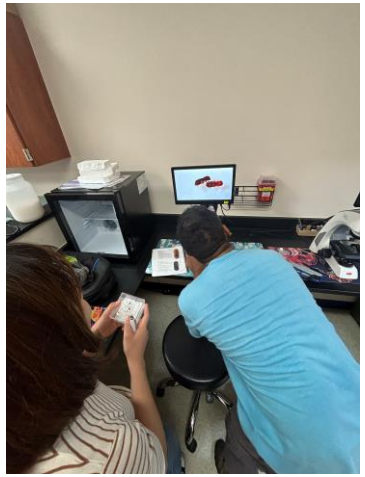
Samples brought back from TREC

Name	# specimens	Sample type	Location
Ambrosia beetles	5	Dead specimens	USA
Elsinoe perseae	8	DNA from pure culture	USA
E. perseae	17	DNA from avocado scab lesions	USA
Elsinoe spp.	6	DNA from pure culture	USA
Avo fungi	5		USA
Harringtonia lauricola	5		USA/Myanmar
Raffaelea sulphurea	1		USA

Summary

- Avocado scab and laurel wilt cause huge losses in Florida
- *E. perseae* qPCR assay validated at a different lab, on avocado infected tissues
  - ITS primers/probes worked well ☺
- *H. lauricola* LAMP didn't work, likely due to reagents have gone off ☹
  - More than 1 week would've allowed troubleshooting
- Positive controls brought back
- Improved networks
- NDPs to be finalised

Professional interactions ☺ ☺



Acknowledgements

- A/Prof. Andrew Geering, Dr Lara Pretorius, Dr Louisa Parkinson, Dr Paul Campbell
- A/Prof. Romina Gazis and team (TREC/UF): Maria, Sara, Carlos, Monica, Shelby for generously hosting me and for validating Elsinoe qPCR assay
- University of Georgia (A/Prof. Caterina Villari's team) for providing us *Raffaelea* spp. DNA
- Australian avocado growers and Hort Innovation for funding this research.
- Plant Health Australian for the Diagnostic Residential Grant.

Hort Innovation
Strategic levy investment

AVOCADO FUND

THE UNIVERSITY OF QUEENSLAND
AUSTRALIA

QAAFI
Queensland Alliance for Agriculture and Food Innovation

Queensland Government

Plant Health AUSTRALIA

Rapid detection of avocado biosecurity threats

Dr Nga Tran, Dr Lara Pretorius, Dr Louisa Parkinson, and Prof Andrew Geering

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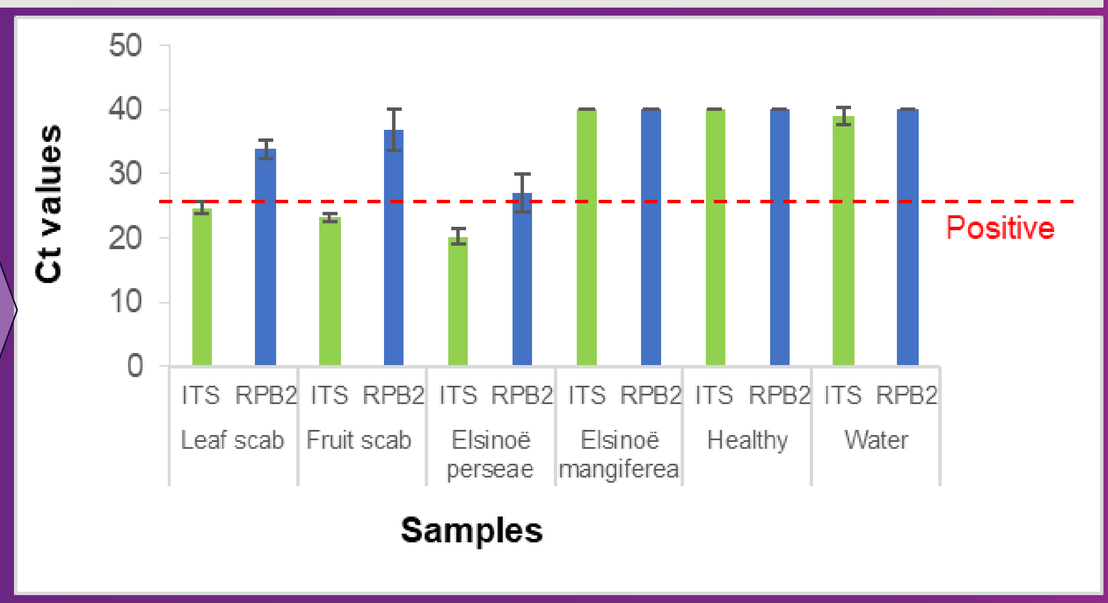
Queensland Alliance for Agriculture and Food Innovation, The University of Queensland

The economic impacts following an incursion of a new pest or pathogen are often dramatic, through yield reductions, additional expenses associated with plant protection, and potentially the loss of domestic or export markets. The University of Queensland avocado pathology team has developed new diagnostic tools for several high priority exotic pests and pathogens to allow rapid responses in the event of incursions, and to facilitate the export of Australian fruit by demonstrating pest and pathogen freedom.

Duplex quantitative PCR assay to diagnose avocado scab

Avocado scab symptoms

qPCR machine



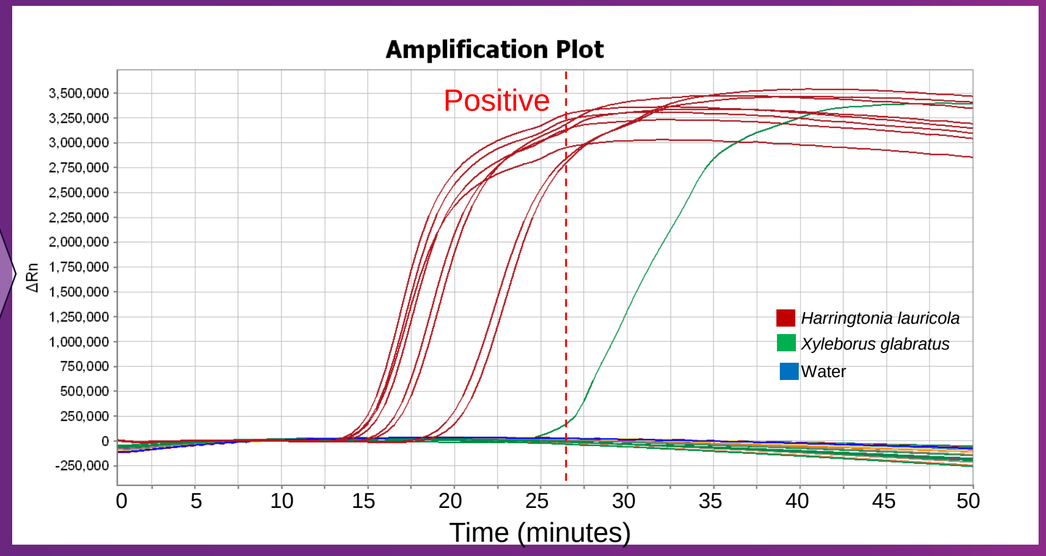
Test results obtained within 20 minutes with cycle threshold (Ct) values ≤ 25 are positives. The duplex assay detects ITS and RPB2 genes simultaneously for increased confidence of the results

LAMP and colorimetric LAMP assays for laurel wilt and Persea mite

Laurel wilt (*Harringtonia lauricola*) in Florida. The vector is the redbay ambrosia beetle *Xyleborus glabratus* (inset)

LAMP assays

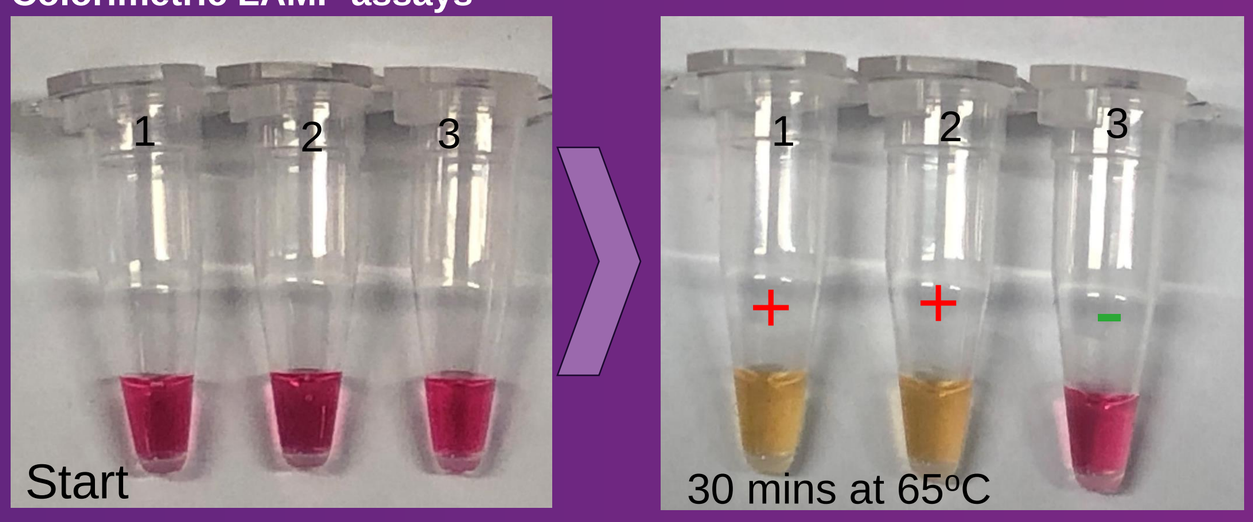
Genie® III, portable LAMP device



The laurel wilt fungus, *H. lauricola* was reliably detected from fungal cultures, infected plant tissues and the vector beetles within 26 minutes

Persea mite (*Oligonychus perseae*) symptoms

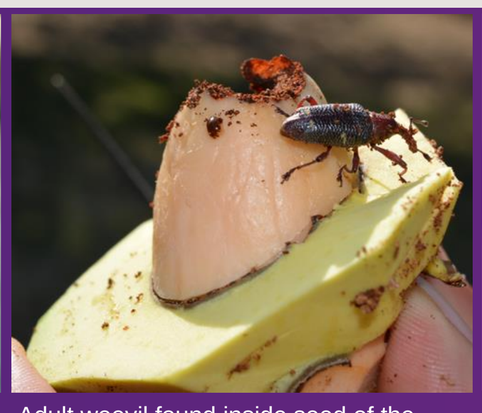
Colorimetric LAMP assays



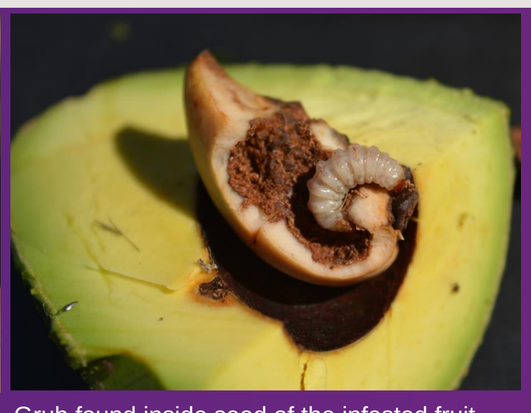
Colour changes in the reaction tubes from pink to yellow are positives while negatives remain pink

PCR assays for large seed weevils

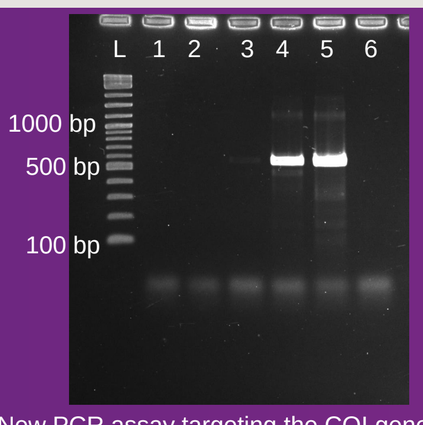
Typical symptoms (entry holes) observed on fruit infested by large seed weevils (*Heilipus lauri*)



Adult weevil found inside seed of the infested fruit



Grub found inside seed of the infested fruit



New PCR assay targeting the COI gene for identification of *H. lauri*. Lane L = ladder, lanes 1-3 = non-target species, lanes 4-5 *H. lauri*, and lane 6 = water (non-template control)

Project AV21003 entitled 'Avocado Industry Biosecurity Capacity Building: Phase II' has been funded by Hort Innovation, using the avocado research and development levy and contributions from the Australian Government. Hort Innovation is the grower-owned, not-for-profit research and development corporation for Australian horticulture.

Queensland Alliance for Agriculture and Food Innovation

Shining a Light on the Avocado Mite

Novel LAMP assay for rapid detection of biosecurity pest *Oligonychus punicae*

Samuel Garty[1], Nga Tran[2], Lara Pretorious[2], and Andrew Geering[2]

1. School of Chemistry and Molecular Biosciences, University of Queensland, St Lucia

2. Queensland Alliance for Agriculture and Food Innovation, Ecosciences Precinct, Dutton Park

Introduction:

- The avocado brown mite, *Oligonychus punicae ,* family Tetranychidae feeds upon the upper surface of avocado leaves, causing bronzing, reduction in photosynthetic activity, defoliation and yield reduction.
- *O. punicae* is exotic to Australia and is rated as a high priority pest (HPP) which poses a high risk of causing significant harm to the industry if an incursion was to occur[1].
- Rapid and accurate detection of a HPP is paramount to successful eradiation and early incursion responses, however, morphological identification of *O. punicae* is challenging due to their minute size, limited number of taxonomically informative features- requiring technical expertise and experience.
- Loop-mediated isothermal amplification (LAMP) is a molecular technique that is rapid and highly specific- achieved by using three primer pairs binding to different regions of the target DNA fragment. LAMP is suitable for laboratory and in-field diagnostics, for high quality and crude DNA extracts.

Aims:

The aims of the research were to: (i) develop a LAMP assay and vigorously validate it following international standards[2,3] to allow rapid and accurate detection of *O. punicae,* and (ii) design and evaluate a gBlock dsDNA fragment for use as a reliable synthetic positive control in the new LAMP assay.

(L) Browning spots seen on avocado leaf from Opun feeding. (R) Mite is microscopic, credit: Jack Kelly Clark, University of California

Materials and Methods:

Mite specimens and LAMP setup

- WarmStart LAMP (NEB# E1700) and WarmStart Colorimetric LAMP (NEB #M1800) kits were used with reaction setups as per manufacturer's instructions except volumes reduced to 10 µL.
- Twenty specimens, including 8 of *O. punicae* imported from Mexico, and other non-targets of species in the family Tetranychidae were used.

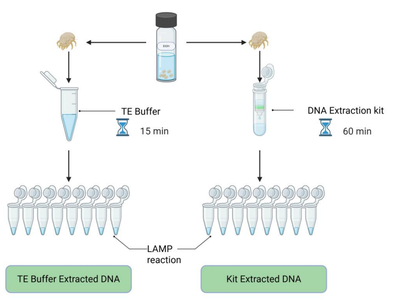
LAMP Primers and gBlock Design

- Primers were designed targeting the ITS1 using the NEB LAMP primer design tool, validated *in silico*.
- A gBlock gene fragment was designed to use as a synthetic positive control, to include all primer binding sites and randomly generated sequences with similar length but higher GC content than the target species sequence fragment.

A subset of the sequence alignment showing the LAMP target region on the ITS1 of *O. punicae* and closely related species and the gBlock, with the primer binding sites.

DNA Extraction

- DNA was extraction from a single mite by crude TE buffer extraction method (Pretorius, unpublished) or following kit methodology (Zymo Quick-DNA Insect Miniprep)



Validation Parameters

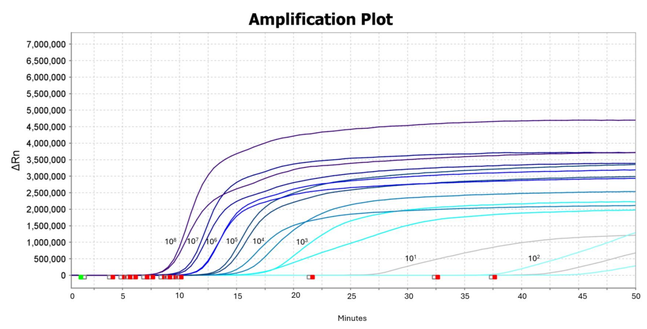
- Assay was validated against **specificity** (all target and non-targets), **sensitivity** (serial dilutions of gBlock and DNA), **accuracy** (reproducible and repeatable between occasions and users), and **robustness** (including different LAMP kits, DNA extraction methods, LAMP machines) [2,3]

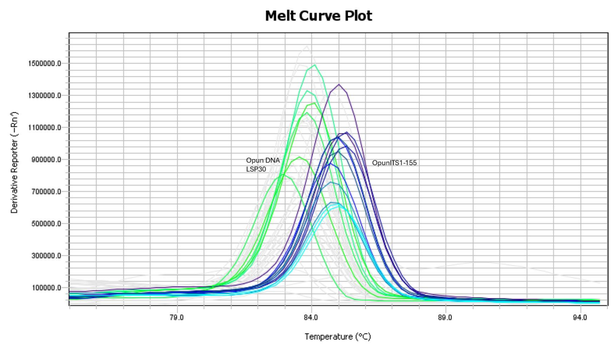
Conclusions and next steps:

- The newly developed LAMP assay allowed rapid detection of the Australian avocado biosecurity threat, *O. punicae,* within 25 minutes and is applicable in-field using a portable LAMP device, colorimetric detection methods and crude extracted DNA.
- The gBlock was found to be a reliable positive control, producing a different melting temperature to allow differentiation from amplification of the target mite DNA.
- Further validation is required to investigate the false positive for one non-target, and to test probe-based detection method for multiplexing with the simultaneously developed LAMP assay for Persea mite.

Results:

gBlock Evaluation and Sensitivity

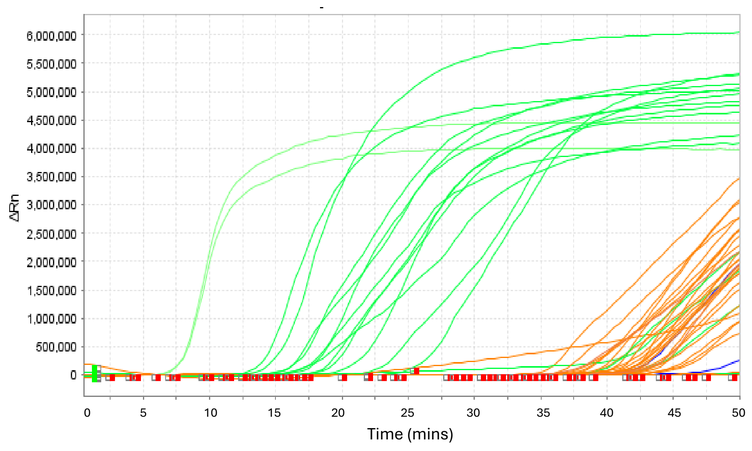




- The gBlock was reliably detected at as low as 10^3 copies/µL for both florescent dye and colorimetric detection methods. DNA extracted from a single mite was detectable at 1:100 dilutions (concentration to be quantified) (left).
- Anneal derivatives (right)- mite DNA (green) was 84 °C and gBlock (blue) was 85.5 °C.

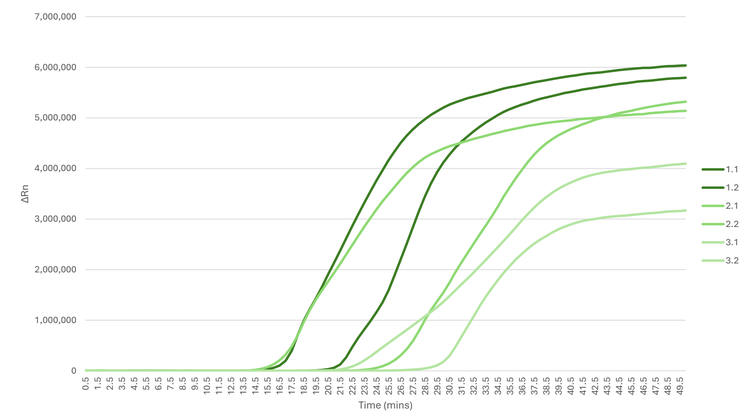
Specificity

- Successful amplification of 6/8 *O. punicae* specimens (green curves) across two trials.
- False positive amplification for one non-target specimen (orange curves). Non-specific amplification occurs >35 mins.
- All detection of target species *O. punicae* seen within 25mins.

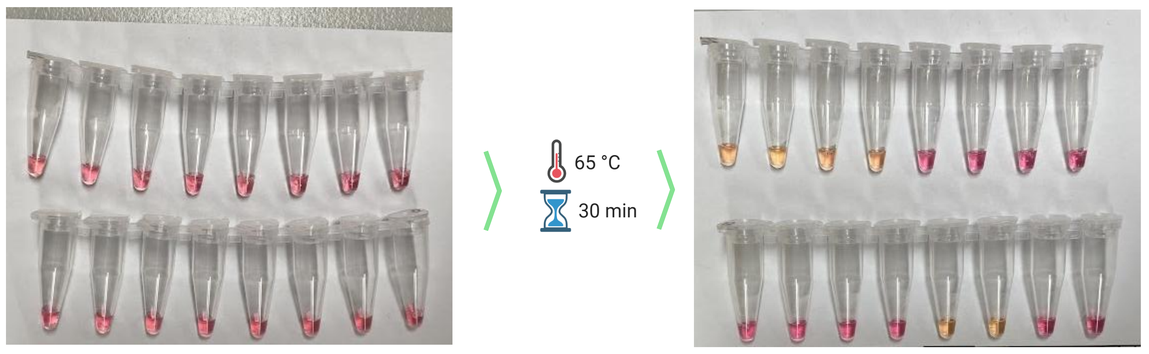


Accuracy

- Repeated assays of the same users but on different occasions (right), and between two users elucidated similar amplification results
- Variation within samples and duplicates warrant further trials
- DNA quantification of dilutions would confirm source of variation



Robustness



Colorimetric detection of target DNA-positive samples are yellow following incubation

- Assays trialled with modifications to specific variables all produced similar results, demonstrating usability in varying conditions:
  - Isothermal equipment (PCR thermocycler, Genie II, QuantStudio qPCR)
  - Detection methods (fluorescent and colorimetric)
  - DNA extraction methods (TE buffer or commercial kit)

[1]Plant Health Australia, 2020. Biosecurity Plant for the Avocado Industry v3.0. pp. 138.
[2]EPPO 2009. Standards: Diagnostic protocols for regulated pests: PM 7/17(2): Guignardia citricarpa. EPPO Bulletin 39:318-327.
[3]Growth-Helms, D., Rivera, Y., Martin, F. N., Arif, M., Sharma, P., Castlebury, L. 2023. Terminology and Guidelines for Diagnostic Assay Development and Validation: Best Practices for Molecular Tests. *PhytoFrontiers. 3*(1)
Schematics produced in BioRender