Hort Innovation

Final Report

Avocado sunblotch viroid survey

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Delivery partner:

The University of Queensland

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AV18007

Project:

Avocado sunblotch viroid survey (AV18007)

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Public summary

Avocado sunblotch viroid (ASBVd) is one of the smallest disease-causing agents in the world but carries a large punch, as it can reduce the yield of an avocado tree by as much as 80%. Fortunately, ASBVd is relatively easily controlled by ensuring only cleaning planting material is delivered to growers. The avocado nursery industry in Australia is self-regulated through operation of the Avocado Nursery Voluntary Accreditation Scheme (ANVAS), and part of the obligations of belonging to this scheme is ensuring that all propagation material is tested and shown to be free of the pathogen. ANVAS has operated for 40 years and ASBVd has progressively been eradicated from Australia. Although ASBVd has become exceedingly rare in Australia, it still creates a problem for avocado growers through impeding exports of fresh fruit. Importing countries require quarantine declarations that the fruit originates from orchards that are free of ASBVd, and these declarations need to be evidence-based.

This project has addressed the problem of how to facilitate the trade of fresh fruit by developing rigorous methods to demonstrate pest freedom in an orchard. A component of the project was to develop a theoretical framework for surveys that met the regulatory requirements of our trading partners. To translate theory into practice, a software app was developed, which allows selection of the optimal number of trees for testing. Once the trees have been sampled, the leaves need to be processed in the laboratory and this step can cause delays in the diagnostic process. To speed up processing of the leaves, a novel 'filter paper' sample extraction method was developed, which reduces the processing time for each sample from 2 hours to 15 minutes and dramatically decreases costs. Avocado sunblotch viroid is pollen-transmitted, which led to the novel idea that pollen stores in beehives could be tested for the presence of the viroid, and in so doing, allow the bees to do the hard work of sampling the trees instead of humans. This novel surveillance method was successfully demonstrated in Australia and South Africa and shows much promise for future disease surveys. Finally, disease surveys were done on the Atherton Tableland, South-east Queensland and in the Riverina region and ASBVd not found at any site, except one, supporting the notion that the avocado industry is largely free of this pathogen. The orchard where ASBVd was found was comprehensively surveyed and four infected trees in a tight cluster found, but with no evidence of further spread. Furthermore, over 8,000 trees and nursery plants were tested for ASBVd as part of ANVAS requirements, and the pathogen never detected, suggesting that this important disease management program is working very effectively.

Keywords

Avocado, *Persea americana*, sunblotch disease, ANVAS, nursery accreditation scheme, surveys, diagnostics, detection assay, pest free place of production, exports, international phytosanitary measure, ISPM 10

Introduction

Avocado sunblotch viroid (ASBVd) is a sub-cellular pathogen of avocado (*Persea americana*), which likely originated in Central America but has been transported to many avocado-producing countries around the world (Geering 2018; Kuhn et al. 2017). Avocado was first developed as a commercial crop in California in the early twentieth century using germplasm that was sourced from Mexico, where the plant is indigenous and had been cultivated by aboriginal Americans for thousands of years (Geering 2018). It is thought that the viroid was inadvertently introduced into California via seed and budwood that was brought back by the early botanical explorers. Without proper knowledge of control methods, the pathogen soon became common within nurseries and then orchards in southern California (Whitsell 1952). Farmers in other countries looked to California to provide improved varieties and it was via latently infected propagules that ASBVd was introduced into South Africa, Israel and Australia (Geering 2018; Whitsell 1952). ASBVd was first noted in Australia in 1967 (Trochoulias and Allen 1970) but was likely present well before this date.

Australia has a proud tradition of research on ASBVd. Sunblotch disease was first described in California in 1928 (Coit 1928) but it was not until scientists in Brisbane and Adelaide began researching the problem about 50 years later that the cause of the disease, ASBVd, was discovered. Pioneering discoveries made in Australia included sequencing of the viroid genome, development of the first molecular detection assays and elucidation of the viroid's replication cycle. An outcome of this pioneering research was that Australia was one of the first countries to implement an industry-wide control program, principally the establishment of a clean planting material scheme (Geering 2018). As early as the mid-1970s, an avocado foundation tree block was established at Somersby Horticultural Research Station, just north of Sydney, containing trees that had tested negative for ASBVd. The 'Virus-Tested Tree Registration Programme' began in December 1980, which amalgamated with the Avocado Nursery Voluntary Accreditation Scheme (ANVAS) during the 1990s. Efforts were also made to index the avocado germplasm collection held at Merbein, Victoria. Given that less than 1% of the Australian avocado tree population predates 1980, it could be expected that the vast majority of trees in orchards have been propagated using viroid-tested propagules.

To gauge the success of the ASBVd control program and to provide guidance for future surveys, Geering (2018) reviewed all academic and grey literature containing information on the distribution of ASBVd in Australia. It was concluded that ASBVd had never been found in the states of Western Australia or South Australia, and the pathogen was very rare elsewhere in the country. All records came from three regions, (i) the Tristates irrigation area near Mildura, (ii) northern NSW and south-east Queensland, and (iii) the Atherton Tableland. The greatest number of records in commercial orchards were from the Atherton Tableland, and although these records were not published, farm addresses where the infections had been found were available through records in a disease accession book held at Mareeba Department of Agriculture and Fisheries.

The involvement of the Australian avocado industry in the international market is growing, through efforts to expand exports of Australian fruit into Asia and the Middle East, as well as reciprocal interest by Central and South American countries to import into Australia. ASBVd is seed-transmitted at rates of 86-100%, hence fresh fruit represents a movement pathway for the pathogen. Exports from Australia may be impeded by quarantine conditions pertaining to ASBVd, and there is a risk of ASBVd being brought into pest-free regions within Australia through imports. Consequently, great benefit to the Australian avocado industry could be obtained through better knowledge of the distribution of ASBVd in Australia, and the collection of data to support pest-freedom status.

Methodology, results, and discussion

All methods are fully described in Appendices 1 – 7 with brief summaries provided below.

Improvements to the diagnostic protocol to allow high throughput processing of samples from surveys

At the beginning of this project, it was realized that a major rate-limiting step in the diagnostic flow that prevented large numbers of survey samples being processed was the RNA extraction step from leaves. To remove this bottleneck, a filter paper method of RNA extraction was developed and validated in two independent laboratories, those of the Queensland Alliance for Agriculture and Food Innovation and the Agricultural Research Council-Tropical and Subtropical Crops in South Africa (see **Appendix 1**). This method is based on reversible binding of RNA to the cellulose fibers in Millipore filter paper in the presence of a monovalent cation such as lithium or sodium. Very large improvements in RNA yield were obtained by increasing the salt concentration (either lithium chloride or sodium chloride) in the tissue lysis buffer up to a maximum of 1.5 M. The filter paper method of RNA extraction provided equivalent yields of viroid RNA to commercially available RNA extraction kits or more elaborate and time-consuming dsRNA enrichment methods used in South Africa. There were several benefits to using the filter extraction method other than the speed with which samples could be processed. By minimizing the number of steps in the RNA extraction protocol, the risk of operator error and nucleic acid contamination was reduced. The reagents needed for the filter paper method are also commonly available in any laboratory and are much cheaper than a commercial RNA extraction kit.

The filter paper method of RNA extraction was adopted for all ASBVd testing done is this project. The method was provided to Prof Adéle McLeod, who is now using it for the ANVAS-equivalent scheme in South Africa, the Avocado Plant Improvement Scheme.

During the COVID lockdowns of 2020, delays were experienced in the delivery of avocado leaf samples by courier and post. Fears were held that these delays would compromise the samples and result in false negative diagnostic results. Experiments were therefore done to compare the effect of storage of fruit and leaf samples at room temperature and 4°C on ability to detect ASBVd by RT-qPCR (see **Appendix 2**). Remarkably, detection sensitivity only declined slightly over a 4-week period when the leaves were stored at room temperature, despite the leaves becoming necrotic, desiccated and brittle. ASBVd could also easily be detected in the fruit while the skin remained green but detectability declined as the fruit ripened, coinciding with the skin turning a brownish-purple colour. Importantly, it should be possible to test fruit that is imported into Australia that has been subject to cold chain storage.

Evaluation of bee-assisted surveillance for ASBVd

Mature avocado trees pose many technical challenges for pathogen surveillance because of the large size of the trees. Bee-assisted surveillance was evaluated as a means of reducing the physical challenges, labour costs and safety issues related to inspecting and sampling avocado trees to determine their infection status. Avocado sunblotch viroid is transmitted in pollen, and it was hypothesized that the viroid would be detectable in pollen samples collected by bees and stored in the beehives.

A series of experiments were done to evaluate bee-assisted surveillance at sites in south-east Queensland and South Africa, representing low and high incidence sites for ASBVd, respectively (see **Appendix 3** for a detailed description of the research). To test the basic premise that pollen carried by bees contained detectable ASBVd, foraging bees were collected from the flowers of infected trees at the Queensland site and various parts of the bee were tested for the presence of the viroid by RT-qPCR. ASBVd-levels in the pollen loads of foraging bees were significantly higher than bee bodies ($p = 0.003$) and were similarly high to levels in the flowers ($p=0.952$). Worker bees and pollen samples were then collected from hives at varying distances from the infected trees. Only pollen from hives (QLD1) within 100 m of infected trees gave a positive detection of ASBVd based on a conservative threshold of $CT < 30$ and was significant compared to hives at further distances ($p < 0.05$).

ASBVd was consistently detected in pollen and bees from pollination hives at four South African orchard sites in 2020. Detection was similar in pollen and bees at each site, except for the SA4 hives where there were no ASBVd detections in any bee sample. The prevalence of ASBVd-infected trees at the orchard where the SA4 hives were positioned was unknown but is presumed to be low because the site belongs to a certified nursery that undergoes regular indexing of orchards. ASBVd levels in hive pollen at the SA1 hives were significantly higher than other sites ($p=0.0112 – 0.0001$), reflecting the higher prevalence of ASBVd-infected trees within 100-200 m from these hives.

Pollen and bee samples were also analyzed by high throughput sequencing (HTS) for detection of ASBVd and other viruses. Using both total RNA sequencing and small RNA sequencing approaches, ASBVd was detectable in hive pollen but was not reliably found in bee samples. Results from the HTS analyses correlated with those obtained by RT-qPCR, with ASBVd confidently detected in foraging bees collected from infected plants, in pollen from the closest pollination hives

(QLD1), and in all South African pollen samples. Small RNA sequencing recovered a higher number of reads for ASBVd than total RNA sequencing, including a single read in the QLD1 bee sample that was negative in real-time PCR testing. Other than ASBVd, a range of other plant viruses was detected including two cryptic viruses associated with avocado, namely Persea americana alphaendornavirus 1 and Persea americana chrysovirus. These cryptic viruses are non-pathogenic and therefore do not pose a biosecurity concern, nor would they affect productivity of the trees. However, detection of these viruses does serve a useful purpose by confirming that the bees have been foraging on avocado trees, irrespective of whether ASBVd is detected.

This study demonstrates that bee-assisted surveillance is a useful tool to test for the presence or absence of ASBVd within an orchard. At the low prevalence orchard in Queensland, ASBVd was detectable in a pollen sample but not from bees from the pollination hives, and the failure to detect the viroid in the bees probably reflects the limited retention time of pollinia on the mouthparts and feet of the bee and the low probability that a bee had recently visited an infected tree within this maximum 25 retention time. By contrast, in the higher prevalence orchards of South Africa, ASBVd was detectable in both the pollen and bee samples from the pollination hives. ASBVd was also detectable in pollen samples in South Africa about three months after the end of flowering, attesting to the extreme resilience of the viroid's genomic RNA.

Using the ASBVd study system we gained important insights for how best to implement honeybee surveillance at the orchard level. Our data from Australia and South Africa showed that hives positions within 100 m of infected trees contained detectable levels of ASBVd. This is consistent with field observations reporting that honeybees are more abundant within 100 m of hives during avocado pollination. Hive stocking rates of 2-3 hives/ha are recommended for effective pollination, which would theoretically put all trees within 100 m of a hive and deliver orchard-wide surveillance.

Development of a survey protocol to demonstrate pest freedom in an orchard

Nationwide pest freedom from ASBVd is a difficult status to achieve and the immediate priority should be to establish pest free places of production, which may be whole orchards or parts thereof. According to international conventions, declarations of pest freedom should be evidence-based but no guidance is given as to how the surveys should be conducted or the level of confidence needed to support a claim of pest freedom. As a starting point, we consulted the survey protocol developed by the New Zealand Ministry of Agriculture and Forestry (NZMAF), which is the only biosecurity authority in the world to have undertaken surveys for ASBVd with the aim of declaring pest freedom. For example, an assumption of the NZMAF protocol is that if a tree is infected, then two-thirds of the leaves on that tree will contain detectable levels of viroid. We then undertook a series of experiments to estimate other parameters needed to develop a survey protocol. Leaves from different quadrants of an infected tree were tested by RT-qPCR and no preferential areas of viroid accumulation were identified. The distribution of infection in a leaf was also examined and again no consistent patterns of infection were identified. Hence, a leaf could be collected from any position on the tree with an equal chance of detecting the viroid; therefore, there is no justification to sample from the top of the tree. The dilution effect of batch testing was modelled in order that an optimal batch testing number could be selected given a target RT-qPCR Ct value. Finally, parameters relating to the cost of collecting the leaves and running the RT-qPCR assays were included in a final equation to calculate the optimal allocation of samples to demonstrate pest freedom (see **Appendix 4** for a full description of the study). To make it easier for statistically non-inclined people to design a survey protocol, a software app has been developed and can be accessed by clicking on the following hyperlink https://danielbonnery.shinyapps.io/ASBVd_Sampling/

Surveys for ASBVd

Disease surveys during this project were curtailed by the COVID pandemic. During this time there were bans on interstate travel and even when travelling within Queensland, surveys were not possible due to a desire of the orchard owners to restrict visitation to minimize the risk of introducing COVID19 into the worker population. Nevertheless, time was found to do surveys in South-east Queensland, on the Atherton Tableland and in the Tristates Production region, which were the highest priority places to visit.

At the beginning of the project, symptomatic fruit were submitted to our laboratory from a farm in South-east Queensland and the presence of ASBVd confirmed by RT-PCR. In April 2020, all trees in the orchard block from which the fruit originated were tested for ASBVd by RT-qPCR. Four of the trees tested positive for ASBVd and all were located in a tight cluster (Fig. 1). This orchard block contained old trees (>30 years old), which were mostly cv. Hass. With permission from the farmer and Biosecurity Queensland, these infected trees were retained for the duration of the project and were used as experimental subjects for the bee-surveillance and modelling studies described above.

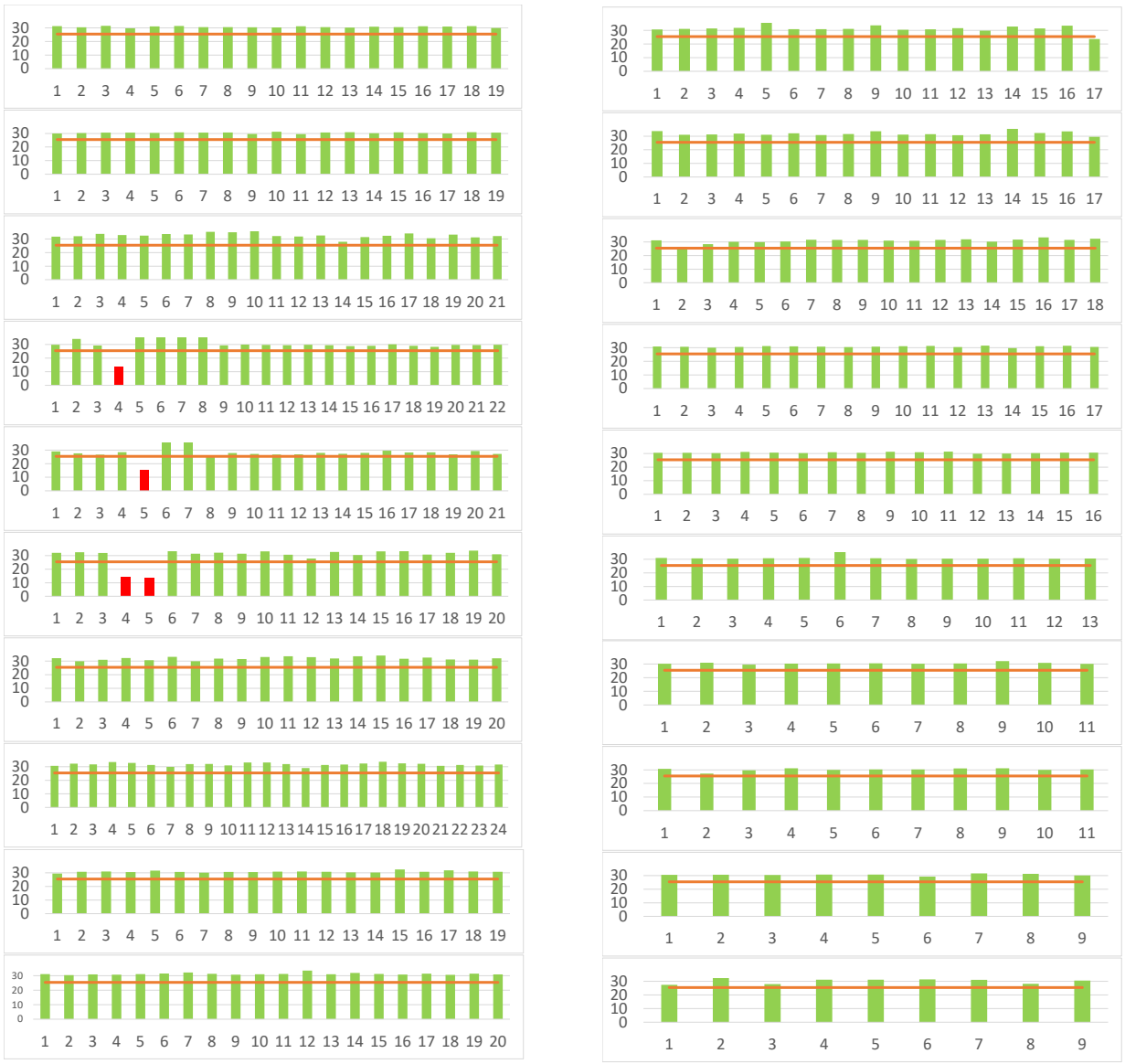

Fig. 1. Distribution of avocado sunblotch viroid in the orchard block in South-east Queensland. Green and red columns represent healthy an infected trees, respectively, and the orange line is the threshold of detection, below which the diagnostic results are considered positive.

Sunblotch disease surveys were also done on the Atherton Tableland and in the Tristates production region (see **Appendices 5 and 6** for detailed descriptions of these surveys. The surveys on the Atherton Tableland were guided by historical records of ASBVd from the 1980s that are catalogued in the Queensland Department of Agriculture and Fisheries disease accession book that is maintained at the Mareeba Research station. Unfortunately, precise locations of the historical records of ASBVd from the Tristates production region are not available, and therefore the focus of the surveys was the very oldest orchards, as advised by Mr Kym Thiel, Tristate Director for Avocados Australia. At each orchard, the growers were interviewed as to whether they had ever seen sunblotch symptoms either on the tree or in the packing shed, and the orchards were also inspected by foot or in a slowly moving vehicle. No symptoms of sunblotch disease were noted in any of the orchards that were visited in these two production regions.

Diagnostic support for the avocado industry

The research team associated with project AV18007 has acted as the national reference laboratory for ASBVd and as such

has provided diagnostic support for the Avocado Nursery Accreditation Scheme (ANVAS) and avocado industry in general.

Samples were regularly submitted by avocado nurseries that are registered with ANVAS for testing for ASBVd, and in total 8,320 field trees or nursery plants were processed and demonstrated to be free of this pathogen (see **Appendix 7**). The nurseries from which the samples were submitted are located in the Riverina, northern NSW, south-eastern Queensland, and on the Atherton Tableland. These results provide confidence that ASBVd is being effectively controlled in Australia though use of high health status planting material and add to the pool of evidence supporting claims of pest freedom in Australia. An example of a diagnostic report, reproduced with the permission of the nursery, is given in **Appendix 8**). High praise was received from the ANVAS nurseries for the diagnostic service that was provided (see Appendix 7).

Given the glut of avocado fruit on the domestic market, new export markets for fresh fruit are being explored, for example, New Caledonia. To satisfy quarantine conditions imposed by the importing country, declarations on the status of ASBVd in Queensland were provided as requested by the company seeking to export (see **Appendices 9 and 10**). These trade requests were successful, in part due to the work done in this and previous projects.

Photos/images/other audio-visual material

Photographs are embedded within the publications that are provided as Appendices.

Outputs

Table 1. Output summary

Output	Description	Detail
Cheaper, more rapid diagnostic protocols for avocado sunblotch viroid.	New diagnostic protocol developed, which high throughput processing of survey samples.	A new method of viroid RNA extraction was developed, which greatly increases sample throughput and decreases testing costs. This method has been published in the Journal of Virological Methods.
Cheaper, more rapid diagnostic protocols for avocado sunblotch viroid.	Experimental data provided to test the hypothesis that the presence of ASBVd in an orchard could be evaluated by testing pollen samples from hives.	Data was obtained to support the hypothesis that bee-assisted surveillance could be used to survey for the presence of ASBVd in an orchard. The study has been written up and submitted for publication in the international scientific journal *Phytopathology*.
Surveys of avocado orchards and nursery multiplication blocks for avocado sunblotch viroid	Data on the ASBVd infection status of nursery propagules to satisfy ANVAS Best Management Guidelines. Data on the presence of ASBVd in farming regions to support claims of pest freedom.	Survey results are presented in the main body of this report. Diagnostic reports are sent to the nurseries and must be archived by these nurseries for auditing purposes. Copies of the diagnostic reports are held by UQ.
Statistically robust survey strategies for avocado sunblotch viroid	New survey protocol that is both pragmatic and meets internationally accepted standards for demonstrating pest freedom. Protocol intended to be used by biosecurity agencies.	A new survey protocol has been developed and implemented in a software app. The theoretical framework for the survey protocol and empirical estimates of each survey parameter have been provided and the work written up and will be submitted for publication in the international scientific journal *PLoS One*.

Outcomes

Table 2. Outcome summary

Outcome	Alignment to fund outcome, strategy and KPI	Description	Evidence
New knowledge, practice change, commercialisation	Industry supply, productivity and sustainability. Building capability and innovative culture.	Effective crop protection solutions for avocado sunblotch viroid. Adoption of best practices through use of superior planting material.	Number of nurseries participating in ANVAS has doubled; over 8,000 samples tested for ASBVd; high level of stakeholder satisfaction with viroid indexing service provided (see Appendix 7).
New knowledge	Industry supply, productivity and sustainability.	Growth in avocado exports	Provision of expert knowledge on distribution of ASBVd to support granting of plant health certificates. Export permit to New Caledonia granted for Queensland avocado growers. Development of new survey protocol for ASBVd to be used to obtain access to New Zealand market.
New knowledge	Industry supply, productivity and sustainability.	Access to a broader suite of crop protection solutions through successful demonstration of bee-assisted surveillance.	Manuscript describing research submitted to high impact scientific journal.

Monitoring and evaluation

Table 3. Key Evaluation Questions

Key Evaluation Question	Project performance	Continuous improvement opportunities
To what extent has the project achieved its expected outcomes?	The project has met many of its expected outcomes. The initial objective of collecting enough survey data to support claims of pest freedom at a national scale was always overly ambitious for such a small research team.	It is imperative that a registry of orchards established with ANVAS planting material is created. It will always be easier to certify an orchard as a pest free place of production at the beginning, rather than doing surveys once an orchard is mature.
How relevant was the project to the needs of intended beneficiaries?	Very relevant. The domestic avocado market has become saturated with fruit and it is essential that new export markets are opened.	The development of export protocols with regards to ASBVd has been slow and this process must be continued.
How well have intended beneficiaries been engaged in the project?	There has been very good collaboration with the ANVAS nurseries and Avocados Australia Limited (AAL). A three-way discussion between the project team, AAL and the Department of Agriculture, Fisheries and Forestry (DAFF) has begun.	Now that this project has finished and a survey protocol has been developed, discussions with DAFF need to ramp up.
To what extent were engagement processes appropriate to the target audience/s of the project?	Email and telephone were mostly used to communicate with the target audience. During surveys, there were opportunities for face-to-face communications.	The engagement processes were satisfactory.
What efforts did the project make to improve efficiency?	Viroid RNA extraction was identified as a major bottleneck for high throughput diagnostics and a major source of error due to risks of mixing samples and introducing other operator errors. To address this limitation, the 'filter paper method' of viroid RNA extraction was developed, which significantly reduced the number of steps in the RNA extraction protocol and dramatically cut the costs of reagents. Without this efficiency improvement, it would have been exceedingly difficult to process the number of avocado samples that were done.	The RT-qPCR assay has been optimized about as much as possible with current technologies. Further improvements in the diagnostic process will require adoption of completely new technologies such as digital PCR. Incremental improvements, particularly reducing the cost of the diagnostic assay, may be possible as cheaper RT-qPCR kits are released by companies.

Recommendations

The recommendations from this project are:

1) A registry of pest free places of production needs to be created for avocados and Avocados Australia Limited is best placed to build and administer this database. This registry should hold aerial maps of the orchards, including GPS coordinates to mark the corners of the orchards, as well as layers of metadata to capture details of the planting material that was used and any surveys that have subsequently been done during the life of the orchard. New Zealand has an export registration scheme https://industry.nzavocado.co.nz/exporter-registration/ and this would be a good starting point for building a similar registry in Australia.

2) ANVAS is operating as effectively as it has done for the last 20 years, and the protocols for testing for ASBVd are well bedded in. The viroid indexing work for ANVAS consumes about 0.5 FTE and it is important that funding for this position continues, and expertise is not lost through gaps in funding.

3) Export protocols for fresh fruit to New Zealand need to be renegotiated with MAF New Zealand. Currently, the export protocol states that exports are permitted as long as the fruit has been "sourced from an approved orchard which has been inspected and found free from symptoms of Avocado sunblotch viroid" https://www.avocado.org.au/wp-content/uploads/2017/02/Avocado-Export-Development-Plan-2014-2019-Appendix-B-1.pdf. An "approved orchard" is one that has been established with plants from an ANVAS nursery. Unfortunately, many avocado growers have lost records of what planting material was used, and this may preclude approval of the orchard for exports. Even now there are concerns that some farmers are not keeping records of the identification numbers for the planting material, which link back to nursery production records and viroid indexing results. This information should be retained in the registry of pest free places of production. There also needs to be a provision to retrospectively certify orchards as pest free places of production, and the survey protocol presented in this final report could be used for this purpose. Surveying for symptoms on trees in an orchard has very limited value as infections are normally asymptomatic; it would be desirable if this condition in the export permit was removed or replaced with a condition that the fruit is inspected for symptoms.

4) Bee-assisted surveillance for ASBVd is showing a lot of promise and there would be a lot of value in expanding this program across the industry, starting with a discrete production region such as the Atherton Tableland. Bees also collected fungal spores, so the technique might have value for surveying for flower and fruit-borne fungi such as *Elsinöe persicae* and *Pseudocercospora purpurea*. This project would also contribute to national surveillance efforts for bee pathogens and the varroa mite.

5) Accumulation of the body of evidence needed to support a declaration of pest freedom from ASBVd at the national scale will be a long and expensive process. The immediate priority should be to certify pest free places of production and as the dots on a map join together, then regional and nationwide pest freedom will gradually be achieved.

Refereed scientific publications

Journal article

Pretorius, L.-S., Chandra, KA, Jooste, A.E.C., Motaung, L.C., Parkinson, L.E., Geering, A.D.W., 2022. Adaptation of a filter paper method for RNA template preparation for the detection of avocado sunblotch viroid by reverse transcription qPCR. Journal of Virological Methods 301, 114455. https://doi.org/10.1016/j.jviromet.2022.114455

Pretorius, L.-S., Geering, A.D.W., 2022. Extreme resilience of avocado sunblotch viroid RNA in sampled avocado leaves and fruit. Australasian Plant Pathology (in press).

Roberts, J.M.K., Jooste, A.E.C., Pretorius, L.-S., Geering, A.D.W., 2022. Surveillance for Avocado Sunblotch Viroid Utilizing the European Honeybee (*Apis mellifera* L.). Phytopathology (approved subject to revisions).

Bonnéry, D.B., Pretorius, L.-S., Jooste, A.E.C., Geering, A.D.W., Gilligan, C.A., 2022. Rational design of a sampling protocol for detection of a subcellular plant pathogen to demonstrate area freedom in commercial orchards. PLoS One (submitted to journal on 21 Sep 2022).

Intellectual property

No project IP or commercialisation to report.

Acknowledgements

We thank the anonymous avocado growers who have provided access to their properties for the experiments and surveys described in this report. We also acknowledge the cooperation of the beekeepers, who assisted with the bee-assisted surveillance component of the project.

Appendices

Appendix 1. Reprint of Pretorius et al. (2022). Adaptation of a filter paper method for RNA template preparation for the detection of avocado sunblotch viroid by reverse transcription qPCR. J. Virol. Methods 301, 114455.

Appendix 2. Australasian Plant Pathology Manuscript (Brief note): Extreme resilience of avocado sunblotch viroid RNA in sampled avocado leaves and fruit

Appendix 3. Phytopathology manuscript: Surveillance for avocado sunblotch viroid utilizing the European Honeybee (*Apis mellifera* L.)

Appendix 4. PLoS One manuscript: Rational design of a sampling protocol for detection of a subcellular plant pathogen to demonstrate area freedom in commercial orchards

Appendix 5. Survey Report for Atherton Tableland production area

Appendix 6. Survey Report for Tristates production area

Appendix 7. Avocado sunblotch viroid surveys as part of the Avocado Nursery Accreditation Scheme

Appendix 8. Avocado sunblotch testing report for Turkinjie Nursery

Appendix 9. Plant health status declaration, Atherton Tableland

Appendix 10. Plant health status declaration, Childers production region

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

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ABSTRACT

An easy, rapid and inexpensive method of preparing RNA template for a reverse transcription qPCR assay for avocado sunblotch viroid (ASBVd) is described. This method depends on the principle of reversible binding of viroid RNA to filter paper under different concentrations of monovalent cation. Lysis buffers containing either sodium chloride or lithium chloride were compared, and 1.5 M lithium chloride was shown to be optimal for the adsorption of the viroid RNA to the filter paper. The extraction method was validated using field samples and equivalent yields of viroid RNA were obtained using this method and either a commercial RNA extraction kit or a dsRNA chromatography method. The filter paper method of RNA extraction is ideally suited for the large-scale surveillance for ASBVd.

Avocado sunblotch viroid (ASBVd; genus *Avsunviroid*) has a circular, single-stranded RNA genome of 247–251 nucleotides (nt), which has extensive internal base-pairing, causing the molecule to assume a double-stranded, rod-shaped conformation (Di Serio et al., 2018; Lopez-Carrasco and Flores, 2017). ASBVd is conventionally divided into three strains based on foliar symptoms, namely, bleached, variegated or symptomless carrier (Semancik and Szychowski, 1994). The natural host range of ASBVd is restricted to avocado (*Persea americana*), although the pathogen has been experimentally transmitted to other plant species in the family Lauraceae (Kuhn et al., 2017).

The economic impacts of ASBVd are several-fold. There are direct yield impacts of infection: canopy thinning is observed, the tree produces fewer fruit and the average size of the fruit is smaller (Saucedo-Carabez et al., 2014). Even when the tree is infected with a symptomless carrier strain, yield can be reduced by as much as 30–58 % depending on the plant variety (Saucedo-Carabez et al., 2014). There are also qualitative effects of infection, as more than half of the fruit can be made unmarketable through unsightly scarring (Saucedo-Carabez et al., 2014). An indirect impact of ASBVd is the quarantine conditions that are imposed on trade, which can range from a complete ban on imports through to the imposition of stringent testing regimes to ensure that there is an acceptable risk from importing the fruit (Kuhn et al., 2017). The symptomless carrier strain of ASBVd is seed-transmitted at rates of 86–100 %, and thus movement of fresh fruit represents a pathway for the pathogen to spread (Wallace and Drake, 1962).

ASBVd was never very common in Australia and has now become exceedingly rare because of the plant health scheme that was first adopted by the avocado nursery industry in the mid-1970s (Geering, 2018). It is anticipated that ASBVd will soon be eradicated from the country through natural attrition of the older avocado trees that predate the plant health scheme and replacement of these trees with clean planting material. The rate of field spread of ASBVd to new trees is less than 1% per annum and most spread occurs over very short distances, possibly a consequence of the natural union of roots from neighbouring trees (Schnell et al., 2011). ASBVd has no known arthropod vectors (Kuhn et al., 2017). Hence, if a farm block can be established using clean planting material and appropriate phytosanitation measures are practiced, for example by the use of dedicated pruning equipment for the block, then it is likely that block will remain viroid-free for the remainder of its commercial life.

The Australian avocado industry has adopted an eradication strategy for ASBVd, beginning with the establishment of pest-free places of production and scaling up to regional or national pest-freedom (IPPC, 2016). To provide evidence of pest-freedom, thousands of trees will need

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to tested and it is common practice to sample leaves or flowers from several positions around the tree to account for a potentially uneven distribution of the viroid (Allen and Dale, 1981). Reverse transcription (RT-) PCR in either a conventional or quantitative (q) format is now the preferred method for testing for ASBVd because of the sensitivity, specificity and speed of the assay compared to older technologies such as dot-blot hybridisation or electrophoresis (Geering et al., 2006; Kuhn et al., 2019; Schnell et al., 1997). While doing an RT-PCR assay is a relatively straightforward matter and can even be automated using a liquid handling robot, the two steps that remain time-limiting are collecting the leaf samples and then extracting the viroid RNA from these samples. For example, the RNA extraction method adopted by Kuhn et al. (2019) involves two overnight incubations and multiple pipetting and micro-centrifugation steps. Every manual-handling step increases the risk of an operator error or nucleic acid contamination and reduces the number of samples that can be processed at any one time by a small team.

To streamline a testing procedure for ASBVd, a fast and cheap method of RNA extraction is needed. The filter paper method of nucleic acid extraction developed by Zou et al. (2017) has the potential to reduce sample processing times down to less than 30 s, which would be a significant improvement upon existing methods such as that of Kuhn et al. (2019). The method of Zou et al. (2017) is based on the principle that the cellulose fibers present in a range of types of paper are able to bind, or at least entrap, RNA and DNA efficiently, but do not bind compounds that inhibit PCR. The inclusion of sodium chloride in the tissue lysis buffer also increases the yield of nucleic acids, supposedly as a result of the sodium cations neutralizing the negatively charged groups on the surface of the cellulose fibres that could repel nucleic acids, which also have a net negative charge.

The overarching objective of the experiments described in this paper was to validate the filter paper method of nucleic extraction for use to prepare template for RT-qPCR detection of ASBVd in avocado leaves. Experiments were also done to optimise the leaf tissue lysis buffer and significant improvements in viroid RNA yields obtained by changing from sodium chloride to lithium chloride and using a higher salt concentration.

Unless otherwise indicated, all experiments were done using lyophilized leaves of ASBVd isolate SB-1 (ASBVd-SB1; Symons, 1981) as the positive control and fresh leaves of a healthy 'Hass' avocado plant grown in the glasshouse as the negative control. To detect ASBVd, a one-step RT-qPCR assay was done using the primers and probe shown in Table 1 (Geering et al., 2006). Each 10 µL reaction contained 2.5 µL of TaqMan Fast Virus 1-Step Master Mix (ThermoFisher Scientific), 0.15 µl of a mixture of each primer at 10 µM concentration, 0.2 µL of the probe at 10 µM, 1 µL of viroid RNA template and water to the final reaction volume. To guard against false negative results, each reaction was also spiked with an internal control RNA and complementary primers and probe (RT-qPCR Extraction Control Orange, Meridian Bioscience), as per the manufacturer's instructions. The assays were carried out using the QuantStudio™ 5 Real-Time PCR System (Applied Biosystems) using the fast thermocycling setting with two stages, the first for reverse transcription and the second for PCR cycling. The reverse transcription stage was one cycle at 50 °C for 5 min followed by 95 °C for 20 s. The PCR stage comprised 40 cycles, each with two steps, a denaturation step at 95 °C for 3 s and a combined annealing and polymerisation step at 60 °C for 30 s. The results were analysed using the QuantStudio™ Design and Analysis Software v1.5.1 and subsequently exported to Microsoft Excel for further analyses.

Table 1
Primers and probe sequences for the avocado sunblotch viroid reverse transcription qPCR assay.

Name	Sequence[1]	Position[2]
ASBTM-F1	TTCCGACTCTGAGTTTCGACTT	66–87
ASBTM-R1	GTTCTTCCCATCTTTCCCTGA	168–189
ASBTM-probe	6FAM-TGAGAGAAGGAGGAGTCGT-MGBNFQ	89–107

[1] MGBNFQ represents a 3′ modification of the probe with a minor groove binding protein and non-fluorescent quencher (Thermo Fisher Scientific).
[2] Position in ASBVd genome (GenBank accession J02020).

In the first experiment, the pipette-free method of Zou et al. (2017) was tried, utilizing dipsticks made from Whatman No.1 filter paper that had a small 8 mm^2 nucleic acid binding surface and a long water repellent handle made by coating the paper with paraplast wax. ASBVd-infected leaf was macerated in 20 mM Tris−HCl, pH 8.0, 50 mM NaCl, 2 % (w/v) polyvinylpyrrolidone average mol. wt 40,000, 5 mM EDTA, 0.05 % SDS and 1 mM dithiothreitol (lysis buffer) at a ratio of *c*. 3 mg of lyophilised tissue to 100 µL of buffer using a TissueLyser bead mill (QIAGEN), operated at 30,000 revs/min for 3 min at room temperature with a 5 mm diameter stainless steel ball. The extract was then cleared by centrifugation at 14,000 *g* for 5 min, and the dipstick briefly dipped in the lysate, then the wash buffer (20 mM Tris−HCl, pH 8.0) and finally the RT-qPCR amplification mix, dipping three times in each solution. ASBVd cDNA was successfully amplified but a great variation in the Ct values was observed using the same leaf extract and replicate dipsticks (mean Ct value of 16.7, standard deviation of 5.1). It was hypothesized that this variability was due to factors such as minor differences in the area of filter paper that was immersed in the various solutions.

In order to reduce the variability in results associated with using the dipsticks, the alternative filter paper disk method of Zou et al. (2017) was tried. An 8-mm diameter disk of Whatman no. 1 filter paper was cut using a ring binder 2-hole punch, then placed at the bottom of an empty well of a microplate (Nunc). A 100 µL aliquot of lysate, prepared as described before, was then transferred into the well and the microplate incubated at room temperature for 15 min, after which the lysate was aspirated with a micropipette and discarded. The filter paper disks were then washed twice with 200 µL of wash buffer by flushing using a micropipette. RNAs were then eluted by adding 25 µL of nuclease-free water to the well, ensuring the disks were completely submerged and leaving for 2 h at room temperature. Using this method, the variation in Ct values was markedly reduced between replicates of the same sap extract that was dispensed evenly between different wells of the microplate (mean Ct value of 10.4, standard deviation of 0.47).

To improve the yield of ASBVd RNA, different lysis buffers were evaluated, principally to compare different salt concentrations (0, 0.1, 0.5. 1, 1.5 and 2 M) but also the type of salt, lithium chloride versus sodium chloride. As a starting material for the different treatments, a large amount of infected leaf was powdered by grinding in liquid nitrogen and approximately 10 mg amounts of the powdered tissue were added to microfuge tubes. The precise weights of the tissue samples were then measured and the required test lysis buffers added at a ratio of *c*. 1 mg of lyophilised tissue to 100 µL of buffer, using the same basic recipe to prepare the lysis buffers but varying the type or concentration of salt. The tubes were then vortexed, briefly centrifuged and 200 µl of cleared lysate was added to each microplate well containing a single filter paper disk. Each evaluation experiment was performed four times, each time using three biological replicates (different subsamples of tissue) per treatment and for RT-qPCR, each RNA extract was run in duplicate and the Ct values averaged.

To measure the significance of differences in Ct values between treatments, a non-linear regression analysis was done using the exponential model $Y = A + B(R^X)$, where Y is the response variable (Ct value), R is the exponential rate of change and A and B are the regression coefficients (A represents the horizontal asymptote and A + B, the y-intercept of the curve). All analyses were done using GenStat® Release 21.1. The overall exponential curve was significant ($p < 0.001$; Fig. 1), with there being a significant improvement ($p < 0.05$) in the model by having separate coefficients, A and B, for the different salt types. However, there was no significant improvement in the model by having separate R coefficients for salt type. In other words, the rate of decline in Ct value in response to increasing the concentration of the two salts was

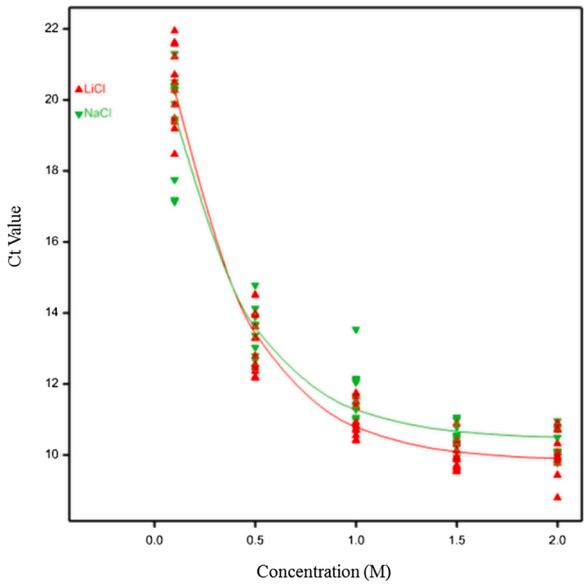

Fig. 1. Effect of the type and concentration of salt in the lysis buffer on yield of avocado sunblotch viroid RNA using the filter paper extraction method, as measured by reverse transcriptase qPCR. Green and red triangles are Ct data points for the sodium chloride and lithium chloride lysis buffers, respectively, and the coloured lines are the best-fit regression lines produced by the exponential model.

similar but there were significantly higher yields of ASBVd RNA at the higher salt concentrations using the LiCl lysis buffer compared to the NaCl lysis buffer, as reflected in the different horizontal asymptotes for the response curves (Fig. 1). The negative exponential model accounted for 95.6 % of the observed variation in Ct values. The effects of changes in salt concentrations in the lysis buffers appeared to be due to changes in adsorption of viroid RNA and not due to increases in the amount of non-specific amplification, as Ct values for healthy avocado leaf tissue remained consistently high (mean Ct values of *c*. 33) with all lysis buffers that were used. Based on the results of this experiment, a lysis buffer containing 1.5 M LiCl was used for all future experiments (the optimised filter paper disk method).

Other minor variables were tested to improve viroid RNA yields. Increasing the number of filter paper disks per microplate well from one to three increased the yield of viroid RNA, presumably due to the greater surface area of cellulose fibres available for RNA adsorption (Zou et al., 2017), and this modification was adopted hereafter. Although the tissue lysate turned a reddish-brown colour, RNA yields were not significantly improved by adding 0.01 % sodium sulphite to the tissue lysis buffer as an antioxidant, although the colour of the lysate was less intense (data not shown).

To provide an estimate of the limit of detection using the optimized filter disk extraction method, a ten-fold dilution series was created, whereby a lysate from an infected leaf was successively diluted in lysate from a healthy leaf. ASBVd was successfully detected at a dilution of 1 in 100,000 (Fig. 2).

Finally, the optimised filter paper disk method was validated using field samples. A row of trees in an old avocado farm (>50 years old) in South-East Queensland, which contained a single symptomatic tree (variegated leaves and scarred fruit), was surveyed and eight leaves from each tree collected for testing by RT-qPCR. In the laboratory, the leaves from each tree were stacked and biopsied six times using an 8 mm diameter biopsy punch (Kai Medical). Three replicate batch samples were then processed using the optimised filter paper disk method, using a ratio of *c*. 10 mg of fresh leaf tissue to 100 µl of lysis buffer. For comparative purposes, the remaining three replicate batch samples were processed using an RNeasy kit (QIAGEN) as per the manufacturer's

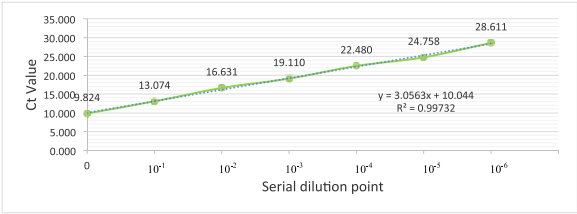

Fig. 2. Determination of the analytical sensitivity of detection of avocado sunblotch viroid (ASBVd), using a ten-fold dilution series of an infected leaf RNA extract prepared using the filter paper method. The infected leaf RNA extract was diluted in healthy leaf RNA extract. The Ct values for a healthy leaf RNA extract and a no template control were 34. 3 and 40, respectively.

instructions. Each RNA extract was tested in duplicate by RT-qPCR and the Ct values averaged.

A single infected tree was identified from the field survey using both RNA extraction methods and this was the tree that was symptomatic. The filter paper method gave a comparatively stronger positive result (lower Ct value) than the RNeasy kit for both the diagnostic sample and the positive control sample (Fig. 3). Healthy trees gave similarly high Ct values.

Experiments were also undertaken to validate the filter paper disk method in an independent laboratory at the Agricultural Research Council-Tropical and Subtropical Crops (ARC-TSC) in Mbombela, South Africa, where avocado leaves are routinely processed for testing using the cellulose column chromatography extraction method of Luttig and Manicom (1999). In this experiment, which was conducted entirely at the ARC-TSC, 21 avoted in duplicate by RT-qPCR and the Ct values averaged.

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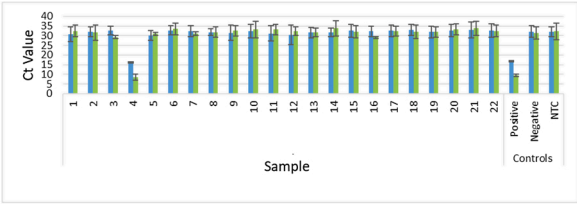

Fig. 3. Comparison of the reverse transcription qPCR assay results obtained using the filter paper disk (green bars) and RNeasy kit (blue bars) methods to extract avocado sunblotch viroid RNA. Each sample represents a different tree within a row of an orchard. The positive control is ASBVd isolate SB-1, the negative control is healthy avocado cv. Hass and NTC is the no template control. Bars represent the standard deviation of three replicate samples from the same batch of leaves.

Table 2
Comparison of cellulose column chromatography and the optimised filter disk method for preparing avocado sunblotch viroid RNA template for reverse transcription qPCR. All samples were collected from previously identified infected trees.

Sample no.[1]	Cellulose Column[2]	Filter Paper[2]	Sample no.	Cellulose column	Filter paper
1	9.0	8.0	14	14.5	14.7
2	18.8	17.0	15	13.2	12.0
3	10.0	6.9	16	10.5	10.2
4	6.0	3.4	17	14.0	12.4
5	23.9	24.2	18	10.7	10.6
6	11.1	9.6	19	10.2	16.6
7	22.2	20.6	20	23.8	22.5
8	24.2	26.0	21	26.0	23.1
9	11.7	11.0	22	23.7	23.9
10	23.9	23.3	23	22.9	25.7
11	8.9	7.3			
12	22.1	21.1			
13	22.6	25.7			
Neg[3]	–	–		–	–
Pos	14	13.2		12.2	13.3

[1] Samples 1–13 and samples 14–23 were tested in two independent experiments.
[2] Data provided are Ct values, rounded to one decimal point. Ct values less than 25.5 were considered positive.
[3] No Ct value was recorded as fluorescence did not exceed the threshold of detection by cycle 30.

(Table 2).

The extraction protocol described in this paper provided a rapid and inexpensive method to process avocado leaves for screening for ASBVd by RT-qPCR. Yields of viroid RNA using the filter paper disk method were greater than that obtained using the commercial RNeasy kit based on binding to a silica solid phase in the presence of chaotropic salts. While there are considerable savings in the cost of materials using the filter paper disk method, arguably the greatest benefit is in workforce labour savings, particularly in countries such as Australia where labour costs are high. However, we found that the viroid RNA in the filter paper eluate was more labile compared to a highly purified extract obtained with a commercial spin column extraction kit and therefore use of the filter paper method was not appropriate if long-term storage and retesting was intended.

In their original paper, Zou et al. (2017) tested only one type of salt in the lysis buffer, sodium chloride, and only to a maximum concentration of 150 mM. We have demonstrated that very large improvements in yield of viroid RNA can be obtained by increasing the concentration of salt and to a lesser extent, changing the cation in the salt to lithium. Zou et al. (2017) hypothesised that the cation in sodium chloride dampened the negative surface charge of cellulose caused by acidic groups such as carboxyl groups, and this increased the binding of nucleic acids, which also have a net negative charge. Following this line of reasoning and the data presented in this paper, a more complete neutralization of the negative surface charge of the cellulose can be obtained by increasing the salt concentration to at least 1 M. Lithium cations also interact with the negative phosphate backbone of RNA and change the polarity of these molecules, resulting in precipitation of a 300 nucleotide-long RNA molecule out of an aqueous solution at room temperature when the concentration of LiCl is as low as 0.5 M (Anon., 2021). Li cations may make the viroid RNA molecules more 'sticky' to the filter paper and do this in a more effective manner than sodium cations. Supporting this hypothesis, the secondary and tertiary structures of some RNA molecules become more stable as the size of the interacting cation decreases and RNA aptamers may bind more strongly to their complementary ligand in the presence of Li^+ compared to other monovalent cations (Lambert et al., 2009).

Data availability

Data will be made available on request.

CRediT authorship contribution statement

Lara-Simone Pretorius: Conceptualization, Methodology, Investigation, Writing - original draft. **Kerri A. Chandra:** Formal analysis, Writing - original draft. **Anna E.C. Jooste:** Investigation, Writing - original draft. **Lebogang C. Motaung:** Investigation. **Louisamarie E. Parkinson:** Investigation, Funding acquisition. **Andrew D.W. Geering:** Conceptualization, Methodology, Writing - original draft, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Extreme resilience of avocado sunblotch viroid RNA in sampled avocado leaves and fruit

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Abstract

The main purpose of the Avocado Nursery Voluntary Accreditation Scheme (ANVAS) in Australia is to prevent the spread of pathogens such as avocado sunblotch viroid (ASBVd) in planting material by implementing rigorous testing regimes for these pathogens during the propagation stages. There can sometimes be significant delays in delivery of a plant sample to the pathology laboratory for testing and the storage conditions may be suboptimal during transport. To address these concerns, experiments have been done to investigate how time and storage temperature affect the ability to detect ASBVd in leaf or fruit tissues. Most importantly, ASBVd was shown to be remarkably resilient and easily detected in detached avocado leaves, even when stored at room temperature for 4 weeks and the leaves had become desiccated and necrotic.

Avocado sunblotch viroid (ASBVd; genus *Avsunviroid*) is a sub-cellular pathogen of avocado (*Persea americana*), which likely originated in Central America but has spread to many avocado-producing countries around the world (Kuhn et al. 2017; Geering 2018). ASBVd has a small, circular, single-stranded RNA genome of 239-250 nt, which adopts a rod-shaped secondary structure *in planta* and is unprotected by either capsid or host proteins (López-Carrasco and Flores 2017). The geographic distribution of ASBVd reflects those nations with the oldest avocado industries, which imported avocado varieties before viroid testing protocols were available (Geering 2018; Whitsell 1952). The oldest record of sunblotch disease in Australia dates back to 1967 (Trochoulias and Allen 1970) but it was likely present much earlier. The Australian avocado industry has greatly benefited from pioneering research done on ASBVd by the domestic scientific community, particularly work to characterize the viroid (Symons 1981), which allowed the development of highly sensitive molecular detection assays (Palukaitis et al. 1981; Allen and Dale 1981) that ultimately prevented the pathogen from becoming widespread in the industry. The avocado 'Virus-Tested Tree Registration Programme' began in Australia in December 1980 and was amalgamated with the Avocado Nursery Voluntary Accreditation Scheme (ANVAS) during the 1990s (Geering 2018). ANVAS continues to this day and nuclear and multiplication blocks of avocado trees used for propagation are routinely screened for ASBVd as part of this clean planting material scheme.

Avocados are commercially grown in every Australian State and ANVAS-registered nurseries are located in most production regions. Samples for ASBVd testing may be submitted to the central testing laboratory in Brisbane from as far afield as Walkamin on the Atherton Tableland to Mildura on the Murray River. Postage and courier problems encountered during the COVID pandemic lockdowns in 2020-21 prompted us to do a series of experiments to examine the resilience of ASBVd in leaf and fruit samples, to validate the protocol in the event of delayed delivery times. Leaves were collected on 7 July 2021 while fruits were collected on 19 August 2021 from a 'Hass' tree in South-east Queensland that was known to be infected (Pretorius et al. 2022) and comparable samples taken from uninfected trees in the same block. The samples were either stored at room temperature (23 ± 2°C) or in the cold room (4°C) in zip-lock plastic bags for a maximum of four weeks and sub-sampled at weekly intervals until the end of the experiment.

For each timepoint, the leaves were processed using the standard protocol used for ANVAS, which entails placing eight leaves in a stack and simultaneously taking cores from three different spots on each leaf lamina using an 8-mm biopsy punch (Kai Biomedical). These leaf cores were then combined to create a batch sample. Similarly, shallow skin cores (avoiding the white flesh) were collected from four different locations on the fruit (back, front and two sides) and these cores combined to create a single sample. All samples were immediately freeze-dried in 2mL SafeLock (Eppendorf) tubes and stored at -80°C until testing. At each time point, three replicates of the leaf or fruit samples were prepared. Photographs were also taken on a weekly basis on the day of processing to show the state of the tissue at the corresponding timepoint.

Viroid RNA was obtained using the filter disc extraction method as described by Pretorius et al. (2022) with one adjustment. As the fruit skin cores were greater in volume than the leaf tissue, 1000 µL of lysis buffer was used instead of the standard 800 µL aliquot prescribed for the leaves. The reverse transcription (RT)-qPCR assay was also done as described by Pretorius et al. (2022) using the TaqMan Fast Virus 1-Step Master Mix (Thermo Fisher Scientific) and a QuantStudio 5 Real-Time PCR system (Thermo Fisher Scientific).

Ct values for the RT-qPCR assay were exported to Excel and analysed by averaging the technical and biological replicates of each timepoint. The kernel density estimate method of van Brunschot et al. (2014) was used to derive a probability distribution of Ct values for uninfected plants and the 99th percentile of this distribution was designated the positivity threshold.

The appearance of both the leaves and fruit remained relatively unchanged when stored at 4°C for the entire 4 weeks. However, leaf tissue stored at room temperature began to become necrotic at the 2-week timepoint and the fruit was nearly fully ripe at the 1-week timepoint (Fig. 1). By 4 weeks, the leaves stored at room temperature were shrivelled, brittle and brown and the fruit, dry and hard. Nevertheless, the mean Ct value for the leaves was only slightly higher at the 4-week timepoint (Ct = 17.6) compared to week 0, the day of collection (Ct = 14.6), and both test results were classified as strongly positive. In contrast, ASBVd could readily be detected in the skin of the fruit at the 1-week timepoint but at later sampling dates, when the skin turned fully purplish-brown and fungal rots became visible,

the Ct values no longer exceeded the positivity threshold of detection. Storage at 4°C delayed ripening of the fruit and death of the leaf tissue and accordingly, strongly positive diagnostic results were obtained at each timepoint for this storage treatment.

The most remarkable result of this set of experiments was the stability of ASBVd RNA in the detached leaves, even when this tissue had become necrotic and desiccated. Peach latent mosaic viroid (PLMVd), a related viroid from the family *Avsunviroidae* (Di Serio et al. 2018), also displays considerable longevity in plant leaves that have been stored under suboptimal conditions, as it was detected in 50-year-old herbarium specimens that had been dried in a plant press and kept at ambient temperature and humidity (Guy 2013). The PLMVd RNA in these specimens was still easily detectable even though endogenous plant mRNA (RuBisCO) had long since degraded. The RNAs of both ASBVd and PLMVd are circular and single-stranded but have strong secondary structures (internal base-pairing causing rod-shaped or branched confirmations), which would help protect the RNA from digestion by ribonucleases (Edy et al. 1976). Furthermore, both viroids replicate within the chloroplast (Lima et al. 1994) and compartmentalization within this organelle may offer some protection from degradation. In conclusion, delays in delivery of avocado leaf samples to the testing laboratory are unlikely to invalidate the diagnostic results.

It was also demonstrated that ASBVd was present in a high concentration in the skin of the fruit while it remained green and this would provide opportunities to test fruit after arrival at a port to confirm its infection status. The question arises as to whether ASBVd could be transmitted after handling infected fruit but we consider this scenario very unlikely because the abrasive-rubbing technique of mechanical inoculation has previously been shown to be ineffective for transmitting the viroid (Desjardins et al. 1980).

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Figure 1. Appearance of the avocado leaves and fruits from the day of collection (week 0) to 4-weeks (week 4) after collection, when stored at either room temperature or in the cold room (4°C). The leaves that are pictured are representative of the eight leaves tested at the nominated timepoint while all three fruits that were tested are shown. The avocado sunblotch viroid infection status of the plant tissue samples is also provided.

Figure 2. Avocado sunblotch viroid (ASBVd) test results for (A) leaves and (B) fruits stored at either room temperature or in the cold room (4°C) for varying periods of time up to a maximum of 4 weeks (week 4). The red and green columns correspond to the ASBVd-infected and uninfected samples, respectively. Ct is the cycle threshold and the orange line is the positivity threshold of detection, below which the samples are regarded to have tested positive. NTC is the no template control. Error bars represent the standard deviation of the replicates.

PHYTOPATHOLOGY MANUSCRIPT

Surveillance for Avocado Sunblotch Viroid Utilizing the European Honeybee (*Apis mellifera* L.)

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ABSTRACT

Avocado is one of the world's fastest growing tropical fruit industries and the pathogen avocado sunblotch viroid (ASBVd) is a major threat to both production and access to international export markets. ASBVd is seed transmissible, with infection possible via either the male (pollen) or female gametes. Surveillance for ASBVd across commercial orchards is a major logistical task, particularly when aiming to meet the stringent standards of evidence required for a declaration of pest-freedom. Like many fruit crops, insect pollination is important for high avocado yields and honey bee (*Apis mellifera*) hives are typically moved into orchards for paid pollination services. Exploiting the foraging behaviour of honey bees can provide a complementary strategy for traditional surveillance methods. High-throughput sequencing (HTS) of bee samples for plant viruses shows promise but this surveillance method has not yet been tested for viroids or in a targeted plant biosecurity context. Here we tested samples of bees and pollen collected from pollination hives in two ASBVd orchard locations, one in Australia where only four trees in a block were known to be infected, and a second in South Africa where the estimated incidence of infection was 10%. Using real-time RT-PCR and HTS (total RNA-seq and small RNA-seq), we demonstrated that ASBVd can be confidently detected in bees and pollen samples from hives within 100 m of infected trees. The potential for using this approach in ASBVd surveillance for improved orchard management and supporting market access is discussed.

INTRODUCTION

Avocado sunblotch viroid (ASBVd; genus *Avsunviroid*) has a circular, single-stranded RNA genome of 238–250 nt, which makes it one of the smallest pathogens in the world. The most recognizable symptoms caused by ASBVd are the sunken, yellow to purplish longitudinal scars or broad spots that appear on the surface of the fruit and are most pronounced at the pedicel end (Kuhn et al. 2017). Foliar symptoms are rarer, but some infected trees may produce clusters of leaves that are variegated or have bleached petioles and midribs with adjacent patches of bleached tissue (Semancik and Szychowski 1994). Infected trees may also be stunted, have a thinner canopy and a distinctively decumbent or sprawling growth habit. Importantly, many infected trees can also be entirely asymptomatic. The yield of symptomatic 'Hass' cultivar trees, expressed as total fruit

weight, may be reduced by as much as 83%, and even when the trees are asymptomatically infected, there is still a significant yield penalty (Saucedo-Carabez et al. 2014).

There are no natural arthropod vectors of ASBVd and transmission occurs mainly by mechanical or seed transmission and potentially by natural root grafting (Kuhn et al. 2017). Experimentally the viroid can be transmitted from avocado to avocado using the razor-slash technique (Desjardins et al. 1980) and by extrapolation, it is probably transmitted on sap-contaminated pruning or grafting blades. Transmission rates of 86%–100% have been observed in seed from asymptomatic carrier trees but the rates are about twenty-fold less in seed from symptomatic trees (Wallace and Drake 1962). Pollen transmission does occur but this only results in infection of the seed (1%–4% infection) but not the pollen-recipient tree (Desjardins et al. 1979; Desjardins et al. 1984).

The main point of intervention to manage ASBVd is at the nursery stage, and it is very important that trees from which seed or budwood is sourced are tested and demonstrated to be free of the pathogen. In Australia, new avocado plants that are certified to be clean of ASBVd are produced by nurseries participating in the Avocado Nursery Accreditation Scheme (ANVAS) (Geering 2018). A similar certification scheme, the Avocado Plant Improvement Scheme (APIS), managed jointly by the South African Avocado Growers' Association (SAAGA) and the Avocado Nurserymen's Association (ANA), is available for managing ASBVd in avocado propagation material from certified nurseries in South Africa.

An indirect economic impact of ASBVd is the impediments placed on international trade of fresh fruit, as the seed remains viable and therefore presents a pathway for spread of the viroid. This problem is best exemplified by a trade dispute between Costa Rica and Mexico, which began in early 2015 and concerned bans imposed by Costa Rica on the importation of fresh avocados for consumption from Mexico because of the perceived risk of introducing ASBVd. This trade dispute was resolved in Mexico's favour in April 2022 by a panel of the World Trade Organization (Horlick et al. 2022) but for the seven interim years, fruit imports from Mexico had been blocked.

Among the major tropical fruits, avocado production has grown at the fastest rate worldwide in recent years. By 2030, global avocado production is projected to reach 12 Mt,

which represents a threefold jump in production since 2010 (FAO 2021). This increase in production is being driven by ever-increasing consumer demand, particularly in developed nations of the world, where the avocado is viewed as a nutrient-rich food (FAO 2021). With increasing volumes of fruit that are being traded, quarantine conditions imposed on the shipments will come into even sharper focus, particularly those pertaining to ASBVd.

To facilitate trade by providing evidence that an orchard is a pest free place of production (ISPM 10, International Standards for Phytosanitary Measures), there is a need for cheaper and more effective surveillance methods for ASBVd. Surveillance for ASBVd poses special challenges, particularly as symptoms of infection are often not apparent, particularly when the tree is not bearing fruit. An avocado tree can reach a height of 5 m–18 m at maturity, making it difficult to thoroughly inspect a tree for symptoms and collect leaves for laboratory testing. Finally, it is commonplace for an orchard to contain thousands of trees distributed over tens of hectares, making surveillance a major logistical task.

The avocado tree is insect pollinated and the European honeybee (*Apis mellifera*) is a major provider of pollination services to this plant (Dymond et al. 2021; Vithanage 1990). In both Australia and South Africa, it is common commercial practice to pay beekeepers to place hives in avocado orchards at the time of flowering to increase the rate of fruit set. This, combined with the fact that ASBVd is pollen-transmitted, suggests that bees could be exploited for surveillance of ASBVd. Viroids in general are very stable molecules and can persist outside of a plant cell, such as in water solutions, for periods of 7 weeks (Mehle et al. 2014), making them ideal subjects for environmental monitoring. Honey bees have been used effectively for biomonitoring of contaminants and agrochemicals but their potential in plant pathogen surveillance remains under-utilized (Cunningham et al. 2022; Roberts et al. 2018; Tremblay et al. 2019), despite having a known role in transmission of several pollen-transmitted plant viruses (Bristow and Martin 1999; Childress and Ramsdell 1987; Darzi et al. 2018; Liu et al. 2014). High throughput sequencing (HTS) of bee samples has been previously shown to be a powerful surveillance method for plant viruses, providing evidence of their occurrence well before they were detected *in planta* (Roberts et al. 2018). However, to our knowledge, this surveillance method has not yet been tested for viroids. Furthermore, bee-assisted surveillance for plant viruses has never been done with a specific

plant biosecurity purpose in mind, with those viruses detected being a matter of serendipity rather than a consequence of experimental design.

In this paper, we describe experiments done to test the hypothesis that bees can be used for surveillance for ASBVd. Two orchard locations were chosen for the study, one in Australia where only four trees were known to be infected with ASBVd, and a second in South Africa where the estimated incidence of infection was 10%.

MATERIALS AND METHODS

Field collection of hive pollen and bees – Queensland, Australia

Samples of stored pollen and adult worker bees were collected from managed bee hives situated across an avocado orchard in South-east Queensland, which was previously identified to contain four ASBVd-infected trees (Pretorius et al. 2022). Sampling occurred on 28 September 2020 during avocado flowering while hives were placed in the orchard for pollination. Managed hives were placed at approximately 100 m, 200 m, 300 m and 400 m from a group of four ASBVd-infected trees (Figure 1). Between two and six randomly selected hives were sampled for approximately 100 worker bees and stored pollen from 10 random cells per hive. Pollen samples were collected from hives using a spatula to scoop pollen from cells into 2 ml tubes. Worker bees were collected by opening the hives and rolling an open 50 ml tube over a frame of bees. In addition, approximately 30 foraging bees (with and without visible pollen loads) were collected with a handheld insect vacuum directly from flowers of an infected tree. Samples were transported on ice and stored at -20°C until further use.

Figure 1. Hive locations in relation to four ASBVd-infected trees in an orchard block in South East Queensland. Hives QLD3 and QLD4 were located at the corners of other orchard blocks with younger avocado trees, which nevertheless were at flowering stage at the time the hives were sampled.

Field collection of hive pollen and bees – South Africa

Samples of stored pollen and adult worker bees were collected in October 2020 from managed bee hives at two avocado orchards in KwaZulu-Natal province of South Africa. Hive sites SA1, SA2 and SA3 were at one farm and SA4 was at the second farm, 37 km away. Bee and pollen samples were collected as described above for the Australian orchard. The distance from infected trees to hives varied between 100 m to 1.7 km (Figure 2). In October 2021, single hives were placed under 10 infected trees at one orchard (same orchard as SA1-SA3 in 2020) (Figure 3) and sampled for bees and pollen as previous. In addition, anthers were carefully removed from the flowers of trees using forceps and transferred to small Petri dishes, sealed with Parafilm and cold stored until use.

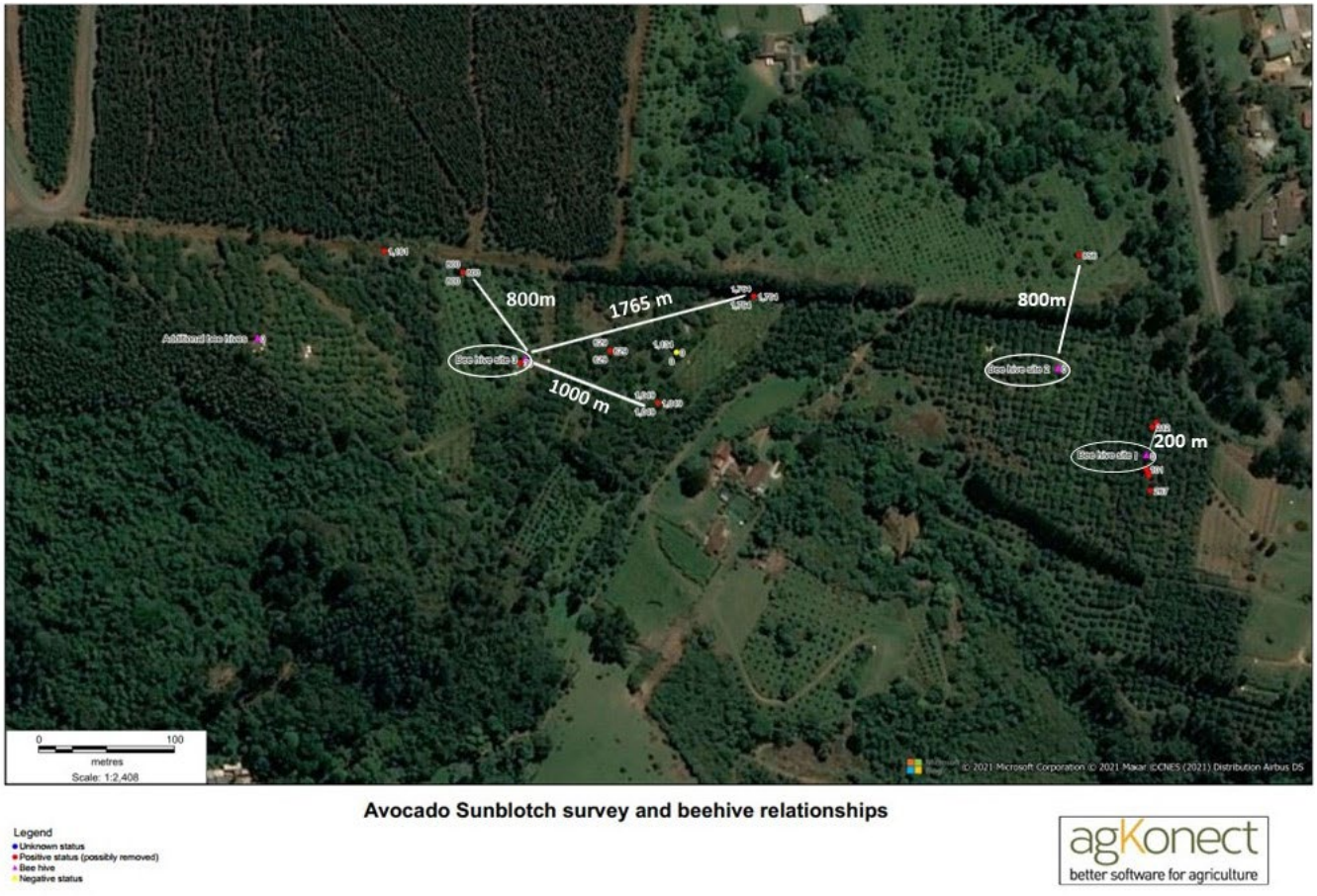

Figure 2. Three hive positions in relation to infected trees in 2020 from a South African avocado orchard

Figure 3. The spatial position of positive trees (red dots) in relation to bee hives that were placed underneath infected trees (yellow dots). ASBVd infection in the neighbouring farm indicated with blue dots.

RNA extraction and real-time RT-PCR

The Queensland (QLD) bees were extracted using a filter paper method of Pretorius et al. (2022) and these samples were used for real-time RT-PCR analysis only.

For HTS, 50 QLD bees per hive were macerated using a Stomacher 80 (Seward, UK) and extraction bag in 10 ml phosphate buffered saline (PBS). A 1.5 ml aliquot was collected and centrifuged (17,000 *g*, 3 min) with the supernatant passed through a 0.22 µm syringe filter (Sartorius, Germany) before proceeding with RNA extraction using the Maxwell RSC simplyRNA Tissue Kit (Promega, USA).

Individual foragers were added to a 2 ml Beadbug™ tube (Benchmark Scientific Inc., USA) with 1.5 mm zirconium beads and 500 ul of homogenisation buffer (Promega, USA). Foragers with pollen loads were first separated with tweezers and the pollen loads/hind legs

placed in separate tubes. Foragers and pollen loads were macerated in a FastPrep™ instrument for 45 sec at 6 m/s then centrifuged (22,000 *g*, 2 min) before using 350 µl supernatant for RNA extractions with the Maxwell® RSC simplyRNA Tissue Kit.

Pollen from each hive was first mixed in 5 ml PBS before a 1 ml aliquot was collected and centrifuged at 22,000 *g* for 5 min to pellet. Supernatant was removed and 500 µl Homogenisation buffer (Promega, USA) was added and transferred to a 2 ml Beadbug™ tube (Benchmark Scientific Inc., USA) with 1.5 mm zirconium beads and macerated in a FastPrep™ for 45 sec at 6 m/s twice. Samples were centrifuged at 22,000 *g* for 2 min, then 350 µl of supernatant used for RNA extraction with the Maxwell® RSC simplyRNA Tissue Kit.

South African leaf, bee and pollen samples were extracted using a dsRNA method (Luttig and Manicom 1999) from 400 mg of starting material. Pollen samples were weighed and mixed with the extraction buffer using the required weight:buffer ratio. Whole bee samples were macerated and used for dsRNA extraction.

Real-time PCR and HTS detection for ASBVd

Bee and pollen samples were was tested for ASBVd by real-time RT-PCR in Australia as described by Pretorius et al. (2022) and in South Africa using a qPCRBIO SyGreen 1-Step kits (PCR Biosystems, UK) and primers (5'- AGAGAAGGAGGAGTCGTGGTGAAC -3'; 5'-TTCCCATCTTTCCCTGAAGAGAC -3') (ref?) to amplify a 99 bp fragment using a Rotor-Gene Q instrument (QIAGEN, Germany). C_T values were analysed using GraphPad Prism 9.

Equal volumes of extracted RNA from each hive were pooled for each site and sample type to create four QLD bee, four QLD pollen, four SA pollen and one QLD forager sample for HTS. Each pooled RNA sample was submitted to Azenta Life Sciences (Suzhou, China) for library preparation and total RNA sequencing (NEBNext® Ultra II™ RNA Library Prep Kit for Illumina®) with Ribo-Zero™ rRNA removal and small RNA sequencing (NEBNext® Small RNA Library Prep Set for Illumina®). The SA dsRNA samples only underwent small RNA sequencing. Libraries were sequenced on the NovaSeq platform generating 150 bp paired-end reads for RNA sequencing and 50 bp single-end reads for small RNA sequencing.

High Throughput Sequencing (HTS) Analysis

Sequence analysis was carried out with CLC Genomics Workbench v20 (CLC Bio, Aarhus, Denmark) with raw data first quality trimmed and adapter sequences removed. Small RNA sequencing data were also size selected for 21–22 nt reads, relating to virus-produced small interfering RNAs (Vivek et al. 2020). Trimmed reads were mapped to the NCBI viral reference genome database (downloaded May 2021) using a length fraction of 0.5 and similarity fraction of 0.8. Consensus viral sequences were manually inspected for genome coverage and similarity to mapped reference genomes using BLASTn. Sequence alignments, annotation and phylogenetic analysis of viral genomes were done in Geneious v2020.0.5 (Biomatters Ltd., Auckland, New Zealand).

Trimmed reads from the RNA sequencing of QLD pollen samples were also *de novo* assembled using Megahit v1.2.9 (Li et al. 2015) and contigs were blasted against the NCBI nr database (BLAST+ v2.12.0; default parameters). Contigs larger than 500 nucleotides and with at least 90% similarity to a plant reference sequence were compiled to identify the diversity and relative abundance of plant species represented in the pollen samples.

RESULTS

ASBVd bee surveillance – QLD orchard

The QLD orchard in which the study was undertaken only contained four ASBVd infected trees, all near neighbours either along or across rows, among a total population of 343 trees in the block. ASBVd was detected by real-time PCR in all foraging bees collected directly from the flowers of an infected tree (Figure 4). ASBVd-levels in the pollen loads of foraging bees were significantly higher than bee bodies ($p = 0.003$) and were similarly high to levels in the flowers ($p=0.952$). Detection of ASBVd was much lower in pollination hives (Figure 4). Only pollen from hives within 100 m of infected trees gave a positive detection of ASBVd based on a conservative threshold of $C_T < 30$ and was significant compared to hives at further distances ($p < 0.05$).

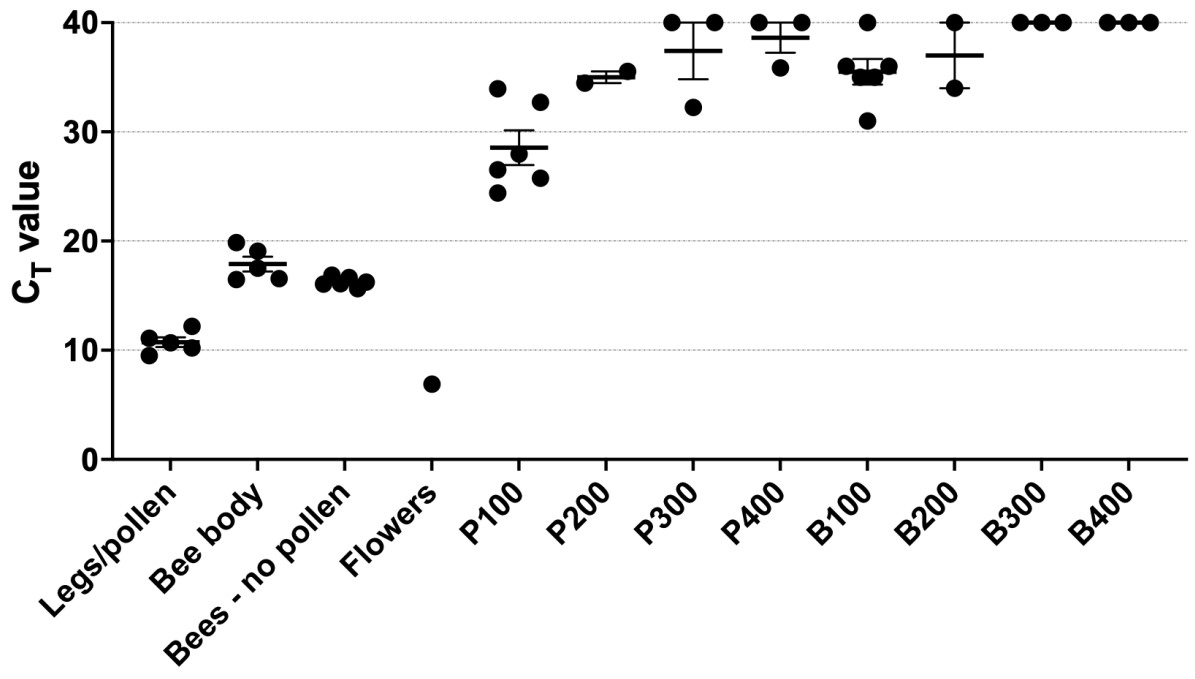

Figure 4. Detection of avocado sunblotch viroid by real-time PCR from foraging bees and pollination hives in an Australian avocado orchard. Bees with pollen loads were dissected to test the legs/pollen loads separately to the bee body. Pollen (P) and bees (B) were collected from hives distanced at 100 m, 200 m, 300 m and 400 m from four infected trees.

ASBVd bee surveillance – South African orchards

ASBVd was detected consistently in pollen and bees from pollination hives at four South African orchard sites in 2020. Detection was similar in pollen and bees at each site, except for SA4 where there was no ASBVd detection in any bee sample (Figure 5). The prevalence of ASBVd-infected trees at SA4 was unknown but was presumed low because the site belongs to a certified nursery that undergoes regular indexing of orchards. ASBVd levels in hive pollen at SA1 were significantly higher than other sites ($p=0.0112 – 0.0001$), reflecting the higher prevalence of ASBVd-infected trees within 100-200 m from these hives (Figure 2).

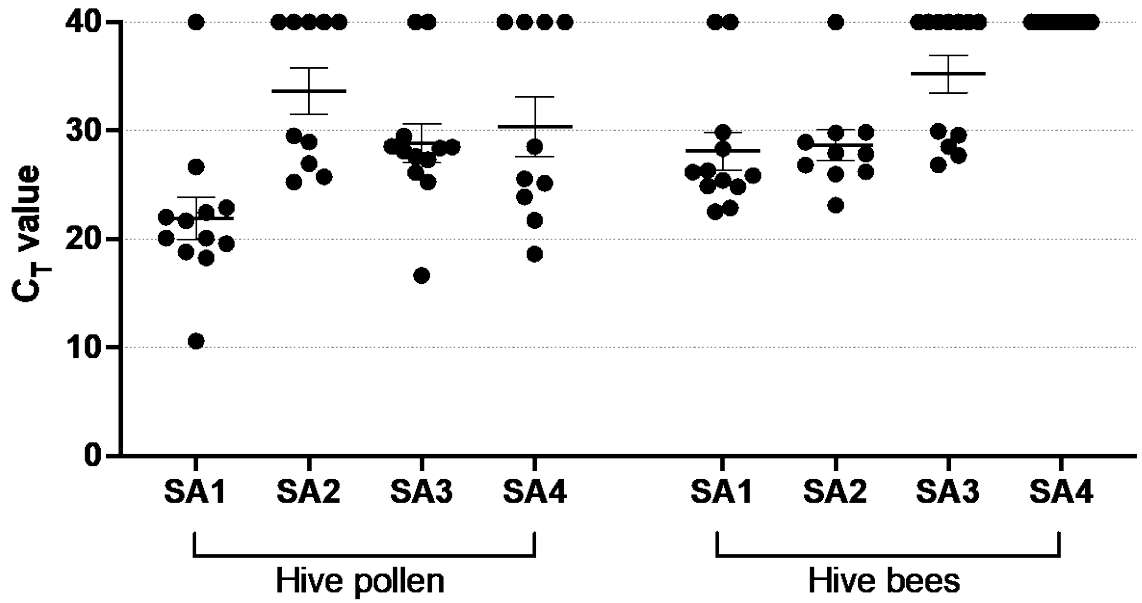

Figure 5. Detection of ASBVd by real-time RT-PCR from pollen and bees collected from South African pollination hives in 2020.

Pollination hives positioned underneath infected trees in 2021 were also tested and showed a significant difference between pollen and bee samples ($p = 0.017$, Figure 6). The position of the hives that were placed under infected trees are shown in Figure 3. The spatial distribution of positive plants increased in the 2021 season, as shown. All but one pollen sample was ASBVd-positive whereas only three corresponding bee samples were ASBVd-positive. Testing of flowers from each tree confirmed high ASBVd levels in all trees, except for one sample testing negative, and all trees had adjacent ASBVd-positive trees (Supplementary material 1). Pollen and bee samples were also tested from these hives three months later with three hives still returning positive detections of ASBVd.

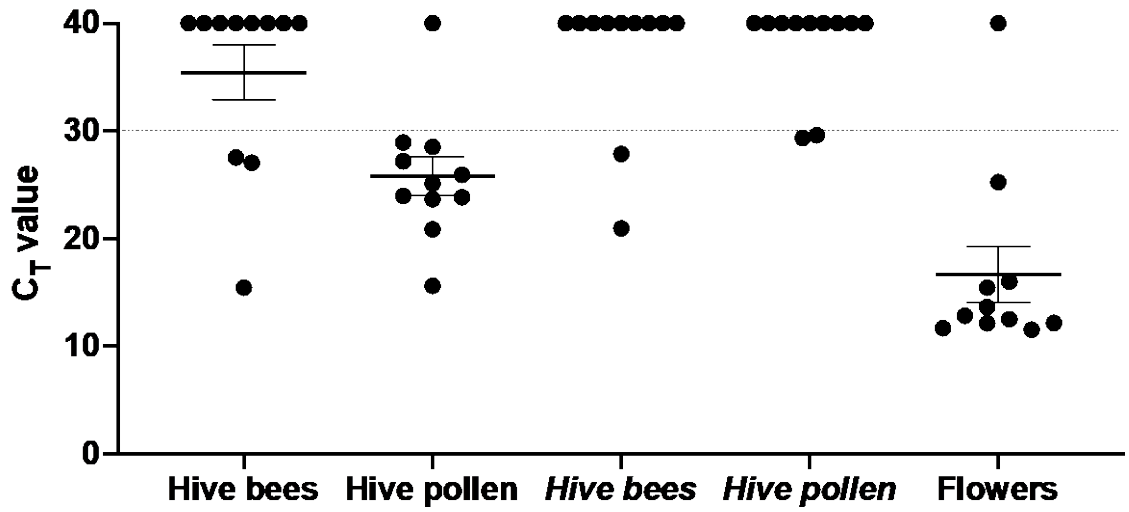

Figure 6. Detection of ASBVd by real-time PCR from pollen and bees collected from South African pollination hives in 2021 positioned underneath infected trees and resampled three months later (italics).

High-throughput sequencing of hive samples

Pollen and bee samples were also analysed by HTS for detection of ASBVd and other viruses. Using both total RNA sequencing and small RNA sequencing approaches, ASBVd was detectable in hive pollen but was not reliably found in bee samples (Table 1). Detection was consistent with the real-time PCR results, with ASBVd confidently detected in foraging bees collected from infected plants, in pollen from the closest pollination hives (QLD1), and in all South African pollen samples. Small RNA sequencing recovered a higher number of reads for ASBVd than total RNA sequencing, including a single read in the QLD1 bee sample that was negative in real-time PCR testing.

Table 1. Detection of ASBVd in reads per kilobase million (RPKM) from HTS of pollination hive samples in Australian and South Africa.

Sample name	Sample type	HTS type	ASBVd RPKM
QLD foragers	Foraging bees	RNA-seq	0.6
QLD foragers	Foraging bees	small RNA-seq	289
QLD1	Hive bees	RNA-seq	0
QLD2	Hive bees	RNA-seq	0
QLD3	Hive bees	RNA-seq	0
QLD4	Hive bees	RNA-seq	0
QLD1	Hive bees	small RNA-seq	0.3
QLD1	Hive pollen	RNA-seq	0.5
QLD2	Hive pollen	RNA-seq	0
QLD3	Hive pollen	RNA-seq	0
QLD4	Hive pollen	RNA-seq	0
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
SA1	Hive pollen	small RNA-seq	832
SA2	Hive pollen	small RNA-seq	352
SA3	Hive pollen	small RNA-seq	14.5
SA4	Hive pollen	small RNA-seq	125

HTS of bee and pollen samples also identified several other plant and bee virus genomes with pollen having a larger number of viruses detected (Table 2). Three viruses were most common among the Australian pollen and bee samples: Persea americana alphaendornavirus 1 (PaEV1), Persea americana chrysovirus (PaCV) and pelargonium zonate spot virus (PZSV). These viruses and pear blister canker viroid (PBCVd) were also the few viruses detected by small RNA sequencing.

Tomato ringspot virus (ToRSV) was a notable detection. This virus is not considered present in Australia but has been detected previously by the authors through similar bee surveillance activities in Queensland and Western Australia (Roberts et al. 2018). Peanut stunt virus, solanum nigrum ilarvirus 1 and blueberry latent virus also are new to Australia.

The South African pollen samples, which underwent dsRNA extraction before small RNA sequencing, had higher recovery of virus reads (3,577–20,182 reads) than the QLD pollen samples (< 2400 reads) but were similarly dominated by ASBVd, PaEV1 and PaCV. The latter viruses, which were also prevalent in the QLD orchard appear to have a global distribution in commercial avocado orchards. Other viruses detected at lower abundance were ageratum latent virus (AgLV), alfalfa mosaic virus (AMV) and tobacco streak virus (TSV).

1 Table 2. Plant and bee virus genomes detected by HTS of hive samples and foragers during avocado pollination in Queensland.

Plant viruses/viroids	Family	% max identity	% genome coverage	Total RNA sequencing			Small RNA sequencing		
				Pollen	Bee	Forager	Pollen	Bee	Forager
Persea am. alphaendornavirus 1	*Endornaviridae*	99	99	+	+	+	+	+	+
Persea am. chrysovirus	*Chrysoviridae*	99	98	+	+	+	+	+	+
Pelargonium zonate spot virus	*Bromoviridae*	99	98	+	+	+	+	+	+
Turnip rosette virus	*Sobemovirus*	94	98	+	+	+			
Tomato ringspot virus	*Secoviridae*	97	63	+	+				
Solanum nigrum ilarvirus	*Bromoviridae*	99	16	+					
Blueberry latent virus	*Amalgaviridae*	99	29	+					
Peanut stunt virus	*Bromoviridae*	99	78	+					
Ribgrass mosaic virus	*Tobamovirus*	98	83	+	+	+			
White clover cryptic virus 2	*Partitiviridae*	96	48	+					
White clover cryptic virus 1	*Partitiviridae*	99	42		+				
White clover mosaic virus	*Alphaflexiviridae*	96	33	+	+				
Turnip mosaic virus	*Potyviridae*	91	37	+					
Broad bean wilt virus 1	*Secoviridae*	95	40	+					
Cucumber mosaic virus	*Bromoviridae*	99	11	+	+				
Pear blister canker viroid	*Pospiviroidae*	99	100	+			+		
Strawberry necrotic shock virus	*Bromoviridae*	81	3	+					
Alfalfa mosaic virus	*Bromoviridae*	98	83	+					
Tomato mosaic virus	*Virgaviridae*	99	8	+					
Lettuce necrotic yellows virus	*Cytorhabdoviridae*	98	15	+					
Prunus necrotic ringspot virus	*Bromoviridae*	98	34	+					
Turnip yellows virus	*Solemoviridae*	99	25	+					
Bee viruses									
Lake Sinai viruses	*Sinaiviridae*	99	100	+	+	+		+	+
Black queen cell virus	*Dicistroviridae*	99	91	+	+	+		+	+
Sacbrood virus	*Iflaviridae*	99	99	+	+	+		+	+
Israeli acute paralysis virus	*Dicistroviridae*	99	34	+	+	+		+	+

2

HTS of pollen samples also provided information on the diversity of plant species visited by foraging bees. Based on the number of assembled contigs a large plant diversity was identified but dominated by a small number of species (Table 3). *Eucalyptus grandis* was the predominant pollen source in all hive locations, alongside other natives *Syzgium oleosum* and *Rhodamnia argentea*. *Citrus* spp., *Raphanus sativus* and *Brassica* spp. were the most common crop plants represented. *Persea americana* (avocado) had relatively low presence in the pollen samples and was only detected in QLD1 hives and QLD4 hives, with lower presence in QLD1 where ASBVd was detected.

Table 3. Total contigs recovered from RNA sequencing for the 20 most common plant species present in hive pollen at four locations across the Queensland orchard.

Plant species	**QLD1**	**QLD2**	**QLD3**	**QLD4**
Eucalyptus grandis	4,143	3,638	3,672	2,927
Syzygium oleosum	701	220	557	562
Citrus spp.	440	227	638	8
Raphanus sativus	738	138	127	99
Rhodamnia 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
Vaccinium macrocarpon	21	0	21	0
Ailuropoda melanoleuca	0	24	14	0
Malus domestica	13	0	1	17
Persea americana	**2**	**0**	**0**	**20**
Plantago spp.	1	5	4	8
Hibiscus syriacus	0	7	6	0
Durio zibethinus	0	7	5	0
Eucalyptus smithii	4	7	0	0
Lactuca sativa	9	0	1	0

DISCUSSION

This study demonstrates that bee-assisted surveillance is a useful tool to test for the presence or absence of ASBVd within an orchard. At the low prevalence orchard in Queensland, ASBVd was detectable in a pollen sample but not from bees from the

pollination hives, and the failure to detect the viroid in the bees probably reflects the limited retention time of pollinia on the mouthparts and feet of the bee (Morse 1982) and the low probability that a bee had recently visited an infected tree within this maximum retention time. By contrast, in the higher prevalence orchards of South Africa, ASBVd was detectable in both the pollen and bee samples from the pollination hives. ASBVd was also detectable in pollen samples in South Africa about three months after the end of flowering, attesting to the extreme resilience of the viroid's genomic RNA. In experiments done in Australia, ASBVd is readily detectable by RT-qPCR assay in detached leaves from an infected tree that have been stored at room temperature for 4 weeks, with no significant decline in viroid titre even when the leaves have browned and become desiccated (Pretorius and Geering, unpublished).

Apart from ASBVd, pear blister canker viroid (PBCVd; genus *Apscaviroid*) was detected in a pollen sample from a hive in Queensland, emphasising the utility of bees for viroid surveillance. The source of this viroid isolate was most likely *Pyrus* x *bretschneideri* (Asian white pear or nashi; syn. *Pyrus serotina*), which was represented in pollen samples from three of the four hives. Nashi is a recorded host of PBCVd in Australia (Joyce et al. 2006) but this detection does extend its known geographic distribution from Victoria to Queensland.

Two avocado-infecting viruses, namely PaEV1 and PaCV, were also detected in bee and pollen samples from both Australia and South Africa. While these detections represent first records of these viruses in both countries, they have no biosecurity significance as they are both considered cryptic viruses (Villanueva et al. 2012). Members of the *Endornaviridae* such as PaEV1 lack cell-to-cell movement proteins, rarely have a phenotypic effect on the plant and are only transmitted in a vertical manner through the gametes (Valverde et al. 2019). Trisegmented chrysoviruses such as PaCV also asymptomatically infect plants and are likely to be only capable of being transmitted through the gametes (Ghabrial et al. 2018). It is probable that avocado cv. Hass is uniformly infected with these viruses, hence their high copy numbers in the bee and pollen samples from the hives even though avocado pollen was only present in relatively small amounts compared to other tree species such as *Eucalyptus grandis*, which is indigenous to the area. These two cryptic viruses do serve a useful purpose in surveillance as they provide unambiguous evidence that the bees have

been foraging on avocado flowers and therefore serve as a type of endogenous plant gene control.

As revealed in a previous study (Roberts et al. 2018), bee-assisted surveillance has the capacity to detect a broad diversity of plant viruses that are present in the environment, whether in introduced or native vegetation. Tomato ringspot virus (ToRSV) was again detected in both pollen and bee samples from the hives in Queensland, matching the results of Roberts et al. (2018) from hives in northern Queensland, providing even more weight to the argument that this virus still occurs in Australia (Geering and Thomas 2022). Although there are old records of ToRSV from South Australia, the Australian Government has determined that this pathogen has failed to establish and is no longer present in Australia (IPPC Report no. AUS-58/2).

Using the ASBVd study system we gained important insights for how best to implement honey bee surveillance at the orchard level. Our data from Australia and South Africa showed that hives positions within 100 m of infected trees contained detectable levels of ASBVd. This is consistent with field observations reporting that honey bees are more abundant within 100m of hives during avocado pollination (McGregor 1976). Hive stocking rates of 2-3 hives/ha are recommended for effective pollination (Vithanage 1990), which would theoretically put all trees within 100 m of a hive and deliver orchard-wide surveillance.

Pollen was also shown to be a better sample matrix for ASBVd, especially when at low prevalence, and for broader plant virus detection using HTS. This is likely the case for many plant pathogens even if they are not considered to be pollen transmissible. However, pathogens present at low levels in a hive could be variably distributed and not sampled when collecting relatively small amounts of stored pollen as we did in this study. Trapping pollen as foragers return to the hive is another method that has been used in pollen analysis studies (Milla et al. 2022; Smart et al. 2017; Tremblay et al. 2019). This approach restricts sampling to the trapping period (typically several days) and provides a biomonitoring snapshot from the current foraging activity. This could be used over several weeks to monitor for pathogens across the pollination period and could be a better method for detecting less persistent pathogens. An important benefit of sampling stored pollen is the

opportunity for pathogens to homogenise and accumulate in food stores through internal hive activities and is well suited for persistent pathogens like ASBVd. Further examination of different pollen sampling strategies will help to determine and optimise the sensitivity of bee surveillance in different systems.

The three different molecular detection methods used in this study also gave valuable insights. Our real-time RT-PCR assay gave robust detection of ASBVd in both pollen and bee samples and was an efficient approach for targeting a single pathogen, especially when combined with a fast RNA extraction protocol (Pretorius et al. 2022). It was also a cost-effective method for obtaining individual hive data to gain insight into the variability in ASBVd presence in hives. However, there are clear benefits for taking a HTS approach to obtain a holistic view of the pathogen landscape. A number of recent studies have highlighted the potential for HTS in plant biosecurity for surveillance and diagnostics of plant material (Gauthier et al. 2022; Maree et al. 2018; Massart et al. 2017; Whattam et al. 2021). Using HTS of hive samples allows surveillance activities to have cross-industry benefits by identifying priority pathogens of different crops as well as the honey bees themselves. We also used a small RNA sequencing approach based on previous studies finding a greater recovery of viroids (Pecman et al. 2017). This was also the case for our data with a greater detection sensitivity of ASBVd with small RNA sequencing. However, this approach was not as effective as total RNA sequencing for detecting the full range of viruses in these samples, as small RNA sequencing is based on the plants immune response to actively replicating viruses/viroids (Ding and Voinnet 2007). While the best approach will depend on the target plant-pathogen system, taking a combined strategy as we have used here is likely to be the most informative overall.

In summary, biomonitoring with honey bees, particularly in combination with HTS, is a powerful complementary strategy to existing plant biosecurity efforts. Each honey bee hive placed in an orchard or field crop for pollination delivers thousands of forager bees that are collecting pollen and nectar from multiple plants and returning this environmental sample back to the hive. Through sheer weight of numbers, bees are undoubtedly more thorough at sampling the orchard than a team of a few people.

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Rational design of a sampling protocol for detection of a subcellular plant pathogen to demonstrate area freedom in commercial orchards

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Abstract

Avocado sunblotch viroid (ASBVd) is a subcellular pathogen of avocado that reduces yield from a tree, diminishes the appearance of the fruit by causing unsightly scarring and impedes trade because of quarantine conditions that are imposed to prevent spread of the pathogen via seed-borne inoculum. For countries where ASBVd is officially reported, permission to export fruit to another country may only be granted if an orchard can be

demonstrated to be a pest free production site. The survey requirements to demonstrate pest freedom are negotiable and usually defined in export protocols that have been mutually agreed upon by the trading partners. In this paper, we introduce a flexible protocol for use in optimizing sampling strategies to establish pest free status from ASBVd in avocado orchards. The protocol, which is supported by an interactive app, integrates statistical considerations of multistage sampling of trees in orchards with a RT-qPCR assay allowing for detection of infection in pooled samples of leaves taken from multiple trees. While this study was motivated by a need to design a survey protocol for ASBVd, the theoretical framework and the accompanying app have broader applicability to range of plant pathogens in which hierarchical sampling of a target population is coupled with pooling of material prior to diagnosis.

Introduction

Worldwide, the avocado industry is growing at an unprecedented rate, faster than any other tropical fruit. Modelling suggests that by 2030, global production will have tripled compared with 2010 levels and the avocado (*Persea americana*) will become the most traded tropical fruit, overtaking both pineapple and mango in quantity terms (OECD/FAO, 2021). With this growing trade, closer scrutiny will be placed on the risks of spreading pests and pathogens in fresh fruit. Avocado sunblotch viroid (ASBVd) is a major trade concern for many countries as the pathogen is seed transmitted at rates of 86–100% in the case of symptomless carrier strains of the pathogen (Wallace and Drake, 1962).

Free trade is governed by bilateral or multilateral agreements that typically are subject to sanitary or phytosanitary conditions to protect human, animal or plant life and health. The principles guiding application of biosecurity measures within a free trade environment are codified in 'The World Trade Organization (WTO) Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement)', which aims to minimize the arbitrariness of decisions and to encourage consistent decision-making. The WTO itself does not develop the international standards but leaves this to scientists in the field and government experts in plant protection (WTO web site, accessed 13 Aug 2021). Responsibility to develop international phytosanitary standards is delegated to the 'Commission on Phytosanitary Measures', which is the governing body of the 'International Plant Protection Convention (IPPC)'. These standards are known as 'International Standards for Phytosanitary Measures' (ISPMs), and as of January 2022, there were 44 adopted ISPMs (https://www.ippc.int/en/core-activities/standards-setting/ispms/).

A common thread of all the ISPMs is that any decision on phytosanitary measures must be justified by scientific evidence. It is recognised that when plants or plant products are imported into a country, the risk of introduction of pests (including pathogens) cannot be entirely removed and therefore a policy of 'managed risk' should be applied (clause 1.3, ISPM 1). There is provision for an exporting country to declare an area as being pest free or a place of low pest prevalence (clause 2.3, ISPM 1) but this claim must be supported by survey data (clause 1.1, ISPM 10). The scale of a pest-free area can vary in size, from an entire nation to an individual farm and when referring to the latter, the term 'pest-free production site' is used. Several criteria need to be satisfied for a farm to be designated a pest free production site including geographic isolation of the farm, lack of natural or artificial infection pathways by which the pathogen could be introduced onto the farm, and the availability of sufficiently sensitive methods for detection of the pest (clause 2.1, ISPM 10). Systems need to be established to establish pest freedom in the first place and then maintain this status (clause 2.2, ISPM 10) but no guidance is provided as to what these systems should be, other than they are normally set by the national plant protection organization.

Avocado sunblotch viroid has many biological characteristics that are conducive to the creation of pest free production sites, as recently reviewed by Kuhn et al. (2017) and Saucedo Carabez et al. (2019). The viroid has no arthropod vector and in nature its host range is restricted to avocado. While pollen transmission is recorded at a low rate, it only results in infection of the seed embryo and not the maternal tree (Desjardins et al., 1979; Desjardins et al., 1984). There are few spatiotemporal analyses of ASBVd epidemics but in the only notable study, the infection status of avocado trees in the National Germplasm Repository-Miami, Florida was monitored for many years, and field spread shown to be slow

(Schnell et al., 2011). Across the entire germplasm collection, the incidence of infected trees only increased by 4.7% over a 9-year period (Schnell et al., 2011). In the field containing the oldest trees, 12 of the 14 newly infected trees were adjacent to previously infected trees, suggesting transmission by natural root grafting (Schnell et al., 2011).

Reverse transcription-(RT) qPCR is now the preferred diagnostic method for ASBVd in many countries around the world (Kuhn et al., 2017). However, there are variations in the RT-qPCR assay format and sampling protocol used for ASBVd diagnosis between countries. In New Zealand, the diagnostic standard specifies that when testing asymptomatic trees, a total of 10 leaves should be collected from the four compass points of the tree at the height of a standing person, ideally taking single leaves from separate branches (MAF, 2009). In Florida, six leaves are sampled from each tree, four from around the base and two from the top of the tree (Kuhn et al., 2019). The sampling protocol in South Africa specifies that 20 to 24 leaves should be collected from all the main branches of a tree, when testing individual asymptomatic trees, and eight leaves per tree when pooling three trees in one sample. In these sampling protocols, the premise for collecting multiple leaves from a tree is a presumption of an uneven distribution of the viroid within the tree, particularly between different branches (Allen and Dale, 1981; Schnell et al., 1997).

Surveying to demonstrate pest freedom in an avocado orchard is not a trivial task. In Australia, an orchard may contain 30,000 trees, which at maturity are *c*. 10 × 5 m in dimensions. The expense of surveying dramatically increases if there is a requirement to collect leaves from any layer other than at ground height, with additional safety issues

associated with using ladders or mechanical lifters. Batch-testing methods must be employed to test the thousands of leaves that may be collected.

The objective of this study was to develop a tool to optimize, with respect to cost, a sampling protocol to demonstrate pest freedom from ASBVd at the production site level, given a set of constraints required by regulatory authorities. These constraints are expressed in terms of detecting the viroid with a given level of confidence in an orchard with a specified prevalence, maximum size and efficacy of detection of the viroid, where the latter is referred to as 'method sensitivity' (Lázaro, 2020). Optimization is achieved by calculating the minimal sample size needed to satisfy the regulatory constraints. The method sensitivity is conditional on the laboratory diagnostic protocol that is used and needs to be estimated afresh for different diagnostic protocols (Lazaro 2020). Formulae exist to obtain the optimal allocation when a pathogen is either uniformly distributed or occurs in a predictable pattern within a plant, the pattern of infection across the field is completely random and samples from different plants are tested individually (Hester et al. 2020). For example, the optimal number of trees to be tested can be determined using a simple stratified sampling optimization for disease freedom (Cameron and Baldock 1998). However, if any one of these conditions are not met, then the formula for estimating the risk of no detection inevitably changes.

In this paper, we describe experiments that were done to determine the method sensitivity for detection of ASBVd in an orchard by RT-qPCR. The Methods section of this paper formalizes the definition of the regulatory constraints for optimal sampling and describes the experiments that were conducted to support optimal sampling to test for freedom of

infection. The experimental results and the protocol for optimal allocation, in terms of sample distribution and pooling of leaf samples, are summarized in the Results section along with general recommendations for the protocol. Finally, a user-friendly software application is presented to allow selection of the optimal allocation of samples to demonstrate pest freedom.

MATERIALS AND METHODS

Field sites and PCR diagnostic protocols

Experiments were undertaken in South Africa, where there is ready access to ASBVd-infected trees and in Australia, the initial target for the sampling protocol but where the pathogen is extremely rare and known to be present in only four avocado 'Hass' trees at a single orchard in South-east Queensland (location protected for privacy reasons). A 'Hass' orchard in the eastern region of the Mpumalanga province was used in experiments done in South Africa, where an estimated 7% of the trees are infected with ASBVd, all asymptomatically.

The laboratories in Australia and South Africa are the reference testing laboratories for ASBVd in their respective countries and utilize TaqMan® and SYBR Green™ detection methods, respectively. The two methods described below are both highly sensitive and provide comparative results (Ct values) when the filter disc extraction method is used for RNA extraction and parallel tests are done using the same starting material (Pretorius et al., 2022).

In Australia, an 8-mm biopsy punch was used to obtain leaf discs that were placed in 2 mL Safe-Lock tubes (Eppendorf). Leaves were freeze-dried overnight and then stored at -80°C until testing. RNA was extracted using the filter disc extraction method and then qRT-PCR done as described by Pretorius et al. (2022), using the TaqMan Fast Virus 1-Step Master Mix (Thermo Fisher Scientific). The Ct value was obtained within 40 cycles, and thereafter, the cycling process was stopped, and the value reported as undetermined.

In South Africa, a similar method to that described above was used to obtain the leaf discs and double-stranded RNA (dsRNA) was extracted using the cellulose column-chromatography method of Luttig and Manicom (1999). A one step qRT-PCR assay for ASBVd was done using a qPCRBIO SyGreen 1-Step Go Lo-Rox kit (PCRBIOSYSTEMS, UK) according to the manufacturer's instructions and primers (5'-AGAGAAGGAGGAGTCGTGGTGAAC -3'; 5'- TTCCCATCTTTCCCTGAAGAGAC -3') were used, each present at a final concentration of 400 nM in a 12.5 µl reaction volume. Reverse transcription was done at 50°C for 10 min, followed by polymerase activation at 95°C for 2 min. The cycling conditions included reverse transcription at 50°C for 10 min, followed by polymerase activation at 95°C for 2 min. The PCR step included 35 cycles with a denaturation step at 95°C for 5 sec and an annealing step at 56°C for 30 sec, followed by a melt analysis. All reactions were run on a Rotor-Gene Q machine (QIAGEN) and the accompanying software (v. 2.3.1) used for the analyses.

Experiments 1 and 2: Leaf, branch (and octant) and tree effects on viroid titer

Experiment 1 was designed to investigate patterns of ASBVd infection across the tree. Leaf samples were collected on 24 August 2020 from two trees in the Australian orchard that

were asymptomatically infected. As negative controls, leaves were also collected from two trees that were classified as uninfected after repeated testing by qRT-PCR prior to this experiment. Each tree was divided into eight octants to ensure even sampling of the tree, covering the top and bottom of the tree and all four sides. A total of 96 leaves were collected per infected, all of which were asymptomatic, and uninfected tree. Comprising 12 leaves per octant and taking note of which of the main branches each leaf was collected from. Bags were labelled accordingly and leaves from the same octant and branch were placed in the same bag. Samples were transported to the laboratory in a cooler box containing ice-bricks. The Ct values obtained from the qRT-PCR analyses were summarized in a data frame containing the following variables: tree, branch, quadrant, leaf, biological replicate index, technical replicate index and Ct measurement [Supplementary Material Fig A1].

Tree status and tree and branch effects were treated as fixed effects and leaf and position of leaf disc were taken as random effects. The octant and disc effects were analyzed as both nested and crossed effects. A crossed (i.e., interaction) effect would indicate systematic differences in the viroid at different positions on the tree or within a leaf; for example, by preferential accumulation in upper octants or at the tip of the leaf.

Experiment 2 was designed to test for systematic variation in titer of ASBVd within a leaf. samples were collected from a third tree in Australia, which was almost entirely asymptomatic except for a small branch with a cluster of variegated leaves. Five each of symptomatic and asymptomatic leaves were collected and each leaf was sampled using the biopsy punch at eleven consistent locations. RNA extracts were tested by RT-qPCR in duplicate and Ct values for each leaf disc averaged [Fig 1; Fig SM2].

Potential patterning (privileged areas) of infection within trees was tested by comparing Ct values from leaves on different branches and octants in a sample of healthy and infected trees. General mixed models were used with likelihood ratio tests (LRTs) (Crainiceanu, 2009) to estimate the variances in ASBVd titer between trees, between branches within a tree, between leaves within a branch and between leaf discs from a single leaf. The statistical form of the model is given in Section B of the Supplementary Material.

Experiment 3: Modelling the dilution effect of batch testing on detection of ASBVd

To economize, it is necessary to pool leaves from different trees into batches for RT-qPCR testing. Experiment 3 was designed to estimate the dilution effect of batch size by measuring the Ct value for leaf tissue composites from batches in which a single infected leaf was mixed with an increasing number of healthy leaves. A 400 mg quantity of tissue was punched from an infected leaf with predetermined Ct value of 10.55. The Ct value of the infected leaf was determined using the filter disc extraction method (Pretorius et al., 2022) and mixed with 0, 9, 19, and 39 to 199 by increments of punched material from healthy leaves. A second infected leaf with Ct value in the same order, 11.34, as the original leaf was used for leaf dilutions from increments of 109 healthy leaves to 199.

The relationship between the average of the Ct measurements of a batch of n leaves, the average of the Ct measurements of the single infected leaf in the batch and the number of leaves in the batch is given by Equation 1 (see Section C in Supplementary Materials for details):

$$\overline{Ct}_{batch} - \overline{Ct}_i = A \times log_{10}(n) + \varepsilon\,, \qquad \text{(Eqn 1)}$$

where $\overline{Ct}_{batch}$ and $\overline{Ct}_i$ denote the average Ct measurements of the batch and an infected leaf i respectively; A corresponds to the increase of Ct for a dilution factor of 10, and ε is a measure of residual error, of standard deviation, σ_ε, which includes the intrinsic variability of the biological material and the technical variability of the assay (repeated measurements from the same biological material being denoted as technical replicates, see Blainey, 2014). More specifically, the variance of the average of a Ct measurement over a number, *j*, *of* technical replicates is the sum of terms that account for biological variations and technical measurements, with the latter proportional to the inverse of the number of technical replicates : $\sigma_\varepsilon^2 = \sigma_{bio.\ rep.}^2 + \sigma_{tech.\ rep.}^2/j$ (for details, see Supplementary Materials section C).

RESULTS

The aims of Experiments 1 and 2 were to study variation in viroid titer within a tree and to determine if a particular part of the tree were preferable for sampling to maximize the probability of detection of the viroid.

Experiment 1: leaf, branch (or octant) and tree effects on viroid titer

Experiment 1 revealed significant variation in viroid titer between the samples but there was no evidence for preferential accumulation of the viroid at a particular position(s) in the tree (see sections B.1 and B.2 of Supplementary Materials for detailed results and associated statistical analyses). For example, the height or orientation of the tree octant could not be used to predict viroid titer. Recommendations on sample selection can be drawn from these results. If four or more leaves are sampled from a tree, then ideally the leaves should be collected from different branches or octants. However, if fewer leaves are collected, there is

no justification for sampling from the top of the tree if it is costly or poses an unacceptable health risk to the worker.

Experiment 2: Variation in viroid titer across the leaf

No preferential areas of viroid accumulation across the leaf were observed (see sections B.1 and B.3 of Supplementary Materials for detailed results and associated statistical analyses). The distribution of the viroid in a symptomatic leaf (variegated and distorted) is shown in Fig. 1 and the range of Ct values across the 11 sampling locations was 14.8 – 18.8. The titer of viroid in the strongly variegated portion of the leaf (discs 7 and 8, Ct = 15.9 and 16.1, respectively) was only marginally higher than the adjacent greener portion of the leaf (discs 1 and 2, Ct = 17.8 and 17.3, respectively).

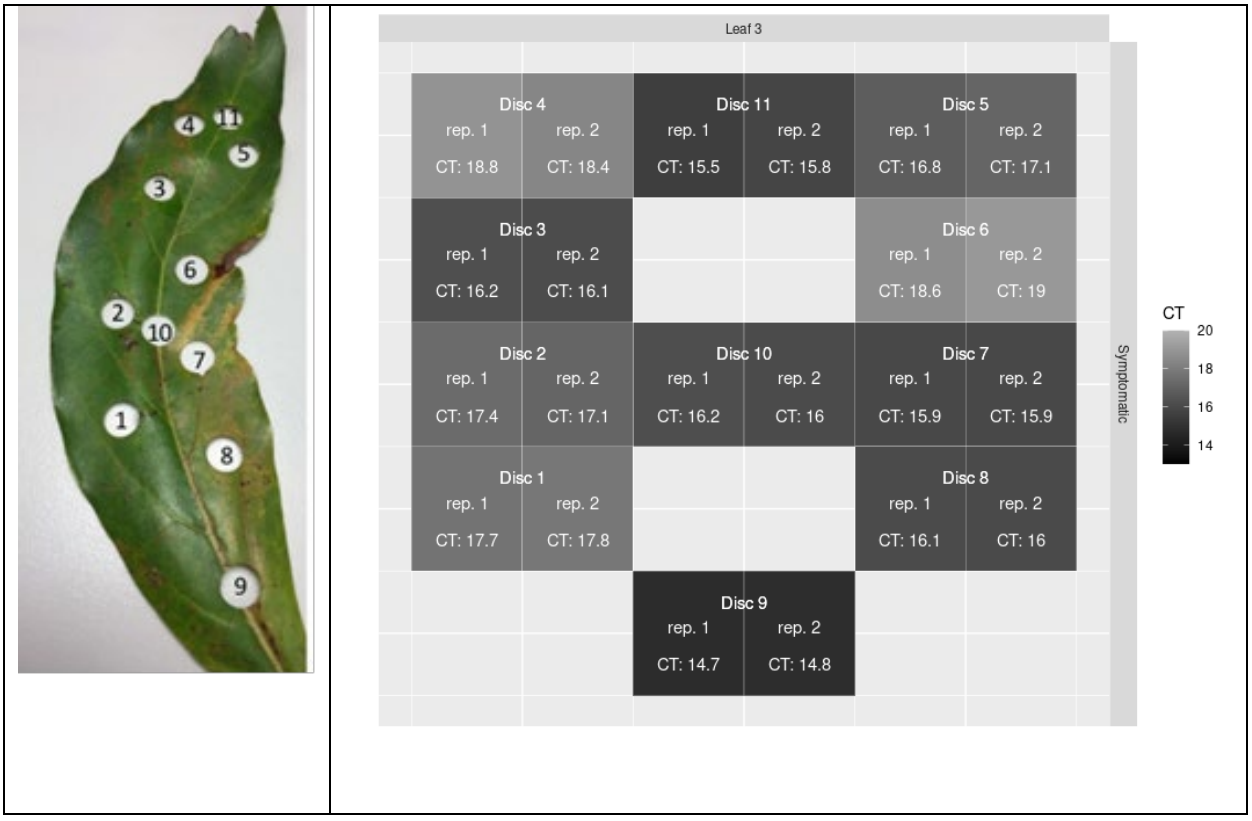

Figure 1 Image of leaf disc locations and heatmap representation of Ct values from duplicate RT-qPCR assays of each disc for a specific leaf.

Experiment 3: Modelling the dilution effect of batch testing

The simple linear model (Eqn 1) was successfully fitted to a metric for the average CT difference between batch and infected leaf, and the logarithm of the sample size (Figure 2). This, in turn, allowed computation of the slope parameter, *A*, and a simple measure of the two components of the variability of the assay (i.e., taking account variability due to intrinsic biological variation and variability associated with technical replication) for use in designing an optimal sampling strategy.

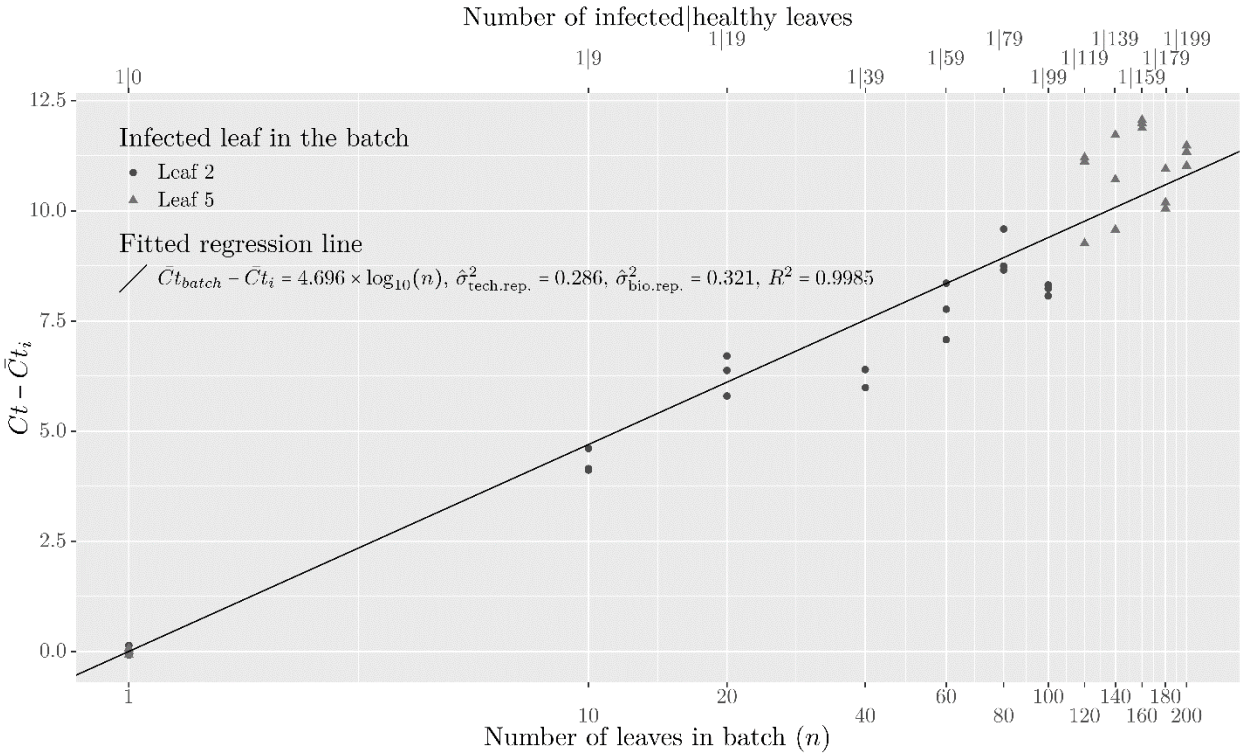

Figure 2: Relationship between number of leaves in a mixed batch of leaves and increase in Ct where batches comprise a single ASBVd-infected leaf pooled with healthy avocado leaves.

The estimates with three significant digits for parameters A, $\sigma^2_{tech.\ rep.}$ and $\sigma^2_{bio.\ rep.}$ are $\hat{A} = 4.70$, $\hat{\sigma}^2_{bio.\ rep} = 0.321$ and $\hat{\sigma}^2_{tech.\ rep} = 0.286$. Noting, the number of technical replicates was $j = 3$, we then estimate $\hat{\sigma}_\varepsilon = \left(0.321 + \frac{0.286}{3}\right)^{1/2} = 0.645$.

Design of an optimal survey protocol

Computation of an optimal sampling design to establish pest free status for ASBVd in an orchard depends upon regulatory constraints, the specifications of the diagnostic assay and orchard characteristics (Table 1). Regulatory constraints reflect international biosecurity standards in trade agreements, which are expressed in terms of thresholds of *unit level detection limit* $(1 - \beta)$, *field prevalence* (r) and *level of confidence* (α), as described by Sequeira and Griffin (2014). For ASBVd infecting an avocado tree, the unit level limit of detection of infected leaves per tree $(1 - \beta)$ is the percentage of leaves on a tree with a defined amount or copy number of the viroid. We define a target threshold for the Ct value (Ct_{target}) that is lower than the *detection Ct* (denoted Ct_{detect}), which corresponds to the minimal Ct value that can be obtained for leaves that do not contain the viroid. The prevalence corresponds to the percentage of trees in an orchard with a unit level of infection above the unit level detection limit. The confidence level is the probability of detecting the viroid in a field with a given prevalence. Default values for the key parameters are summarized in Table 1. The default value for $\beta = 0.67$ is similar to that used by the Ministry of Agriculture in New Zealand (MAF, 2009). The default values for the PCR parameters that we use reflect the estimates that were obtained from Experiment 3. They are used here for

illustrative purposes and can be adjusted by regulatory authorities. The accompanying App allows flexibility in setting all the parameters (Bonnéry, 2022a).

Table 1: Index of notation for parameter values, with default values, and variables used in computing an optimal sampling design for disease-free status.

Category	Parameter/ variable	Default value	Definition
High level regulatory constraints	α	0.95	Confidence level (for false negative)
	r	0.005	Prevalence (infected trees in target orchard)
	β	0.67	Target percentage of healthy leaves (e.g., with Ct above Ct_{target}) for infected trees
Additional regulatory constraint	Ct_{target}	15	Ct threshold used to define β
	α_b	0.99	Batch level accepted risk
PCR characteristics	j	1	Number of technical replicates
	A	4.7	Effect of a dilution by 10 on the Ct value
	Ct_{detect}	25	Threshold used for detection
	$\widehat{\sigma}^2_{\varepsilon}$	0.8	Variance of the Ct measurement at the batch level. This variance accounts for variation related to differences between biological samples and technical measurements $\widehat{\sigma}^2_{\varepsilon} = \widehat{\sigma}^2_{bio.rep.} + \widehat{\sigma}^2_{tech.rep.}/j$
Orchard characteristics	N	6000	Population size
Sampling characteristics	n_0	-	Number of batches
	n_1	-	Number of donor trees in one batch

(to be determined by optimisation)	n_2	-	Number of leaves per donor tree
Cost function	$Cost(n_0, n_1, n_2)$	n_0	Cost associated with a specific allocation. By default, the costs are equated with the number of batches

Calculating optimal strategies

We consider a population of trees of size N, such that at least $N \times r$ trees each have a proportion higher than $1 - \beta$ of leaves with a Ct value smaller than Ct_{detect}. When (i) n_0 batches are being tested, (ii) each batch contains exactly n_2 leaves from each of n_1 different trees, (iii) leaves from the same tree are used for at most one batch, (iv) the residuals can be treated as independent Gaussian variables with zero mean and equal variances, (v) the sampling design is simple random sampling or systematic sampling, then the upper boundary for the probability of not detecting ASBVd (i.e., the Type II risk) is approximately given by:

$$\text{Maj}Risk(N, r, n_0, n_1, n_2, \beta)$$

$$= \left(\sum_{t=0}^{n_1} \left(\binom{n_1}{t} r^t (1 \right. \right.$$

$$\left. - r)^{n_1 - t} \sum_{l=0}^{t \times n_2} \left(\binom{n_2 \times t}{l} \beta^{t \times n_2 - L} (1 - \beta)^L \left(1 \right. \right. \right.$$

$$\left. \left. \left. - \Phi \left(\frac{Ct_{detect} - Ct_{target} - A \times (log_{10}(n_1 \times n_2) - log_{10}(\mathrm{l}))}{\sigma_\varepsilon} \right) \right) \right) \right) \Bigg)^{n_0},$$

where Φ is the cumulative normal distribution function (see Section D Supplementary Material: for details).

Cost function

The cost function is the total cost of the operation and is a function of n_0, n_1 and n_2 denoted by $Cost(n_0, n_1, n_2)$. The cost function depends upon resourcing and it is reasonable to assume the cost function is an additive weighted function of n_0, n_1, n_2.

Optimal allocation

Optimal sampling parameters are defined as the ones that minimize $Cost(n_0, n_1, n_2)$ under the constraints that the approximated risk function (computed with estimates $\widehat{A}$ and $\widehat{\sigma}_\varepsilon$ for A and σ_ε) is less than α, and that the number of leaves per batch is such that the probability of detecting a single infected leaf in a batch is above a batch level confidence parameter, α_b. Details of the computation of the optimal sample allocation are given in Section E, Supplementary Material.

We investigated optimal designs including pooling of leaf samples amongst trees in a multistage framework (Table 2). The sampling mechanism is characterized by three parameters: the total number of batches (denoted n_0), the number of trees per batch (denoted n_1) and the number of leaves sampled from each tree (denoted n_2), with a simple cost function in which cost is dominated by the number of batches (i.e. $Cost(n_0, n_1, n_2) = n_0$). The optimal allocation under an assumption of the cost being a function only of the number of batches ($Cost(n_0, n_1, n_2) = n_0$) was computed for a set of baseline parameters (first row in Table 2), then re-computed after changing each parameter (Table 2).

When the cost is only a function of the number of batches, the optimization results indicate preference for saturating the number of leaves per batch ($n_1 \times n_2$). The maximal number of leaves per batch is a function of the RT-qPCR parameters, as well as α_b and Ct_{target} and is given in Eqn SM4 in Supplementary Methods. Then the optimization results indicate that the trade-off between n_1 and n_2 favours n_1 over n_2: i.e., sampling a large number of trees with low numbers of leaves per tree is preferred, and often only a single leaf from each tree is required. Table 2 shows that the optimal number of leaves per tree is ordinarily 1, except in the case where the targeted percentage of infected leaves in the tree is comparatively small (e.g., β = 0.9, corresponding to 10% leaves infected in an infected tree, in Table 2). Sampling one or a few leaves per tree carries a risk of failing to detect an infected tree, albeit with low probability $1 - \alpha_b = 0.01$ (Table 2) and our analysis indicates that it is better to sample more trees for the same cost than to sample more leaves and fewer trees in testing for freedom from infection in an orchard. This holds when the proportion of infected leaves on a tree is high, but optimal designs indicate sampling more leaves per tree when the proportion decreases (*cf*. (1- β) = 0.1 in row 3 in Table 2). Reducing the critical value for prevalence in the target population, switches the balance towards sampling more batches with fewer trees per batch Table 2). Some parameters, when changed, impact the maximal number of leaves per batch, which may need to be compensated for to reach the required level of confidence by increasing the number of batches (*cf*. changing Ct_{detect}, Ct_{target} and $\hat{\sigma}_\varepsilon$ in Table2). Other parameters do not impact the maximal number of leaves per tree but impact directly the required level of confidence or the prevalence (see Supplementary Material and SM Eqn 4 for further details).

We chose a default value of N = 6,000 trees for a target orchard (Tables 1 and 2). Increasing N to 12,000 trees does not affect the optimal sampling protocol in terms of n_0, n_1, and n_2, (*cf.* row 6 with row 1 in Table 2). Although this result at first appears counter intuitive, it reflects an asymptotic influence of orchard size in which drawing from a population of 6,000 trees with a fixed rate (r) of infected trees is similar to drawing from an infinite population of trees with the same rate of infected trees in the sense that the probability distribution of the number of infected trees in the sample is approximately the same. Setting criteria in relation to the field size N and the prevalence r is under the control of a regulatory authority. The regulatory authority may choose to assess the presence in an extensive large area that includes a large number of trees or to constrain areas for detection to a maximum size, say 6000 trees for separate assessment.

An interactive app available at Bonnéry (2022a) allows all the key parameters including cost functions to be changed (see also selective screen shots in Supplementary Material).

Table 2: Optimal sample sizes for a selection of inputs

Inputs									Optimal sample designs				
Regulations: Confidence level (α), percentage of healthy leaves in infected trees (β), prevalence (r), target Ct				No. trees in orchard	PCR testing: Detection threshold (ct_{detect}) standard deviation of Ct measurements ($\hat{\sigma}_\varepsilon$), dilution assay ($\hat{A}$) effect, and batch level confidence (α_b)				Optimal number of batches (n_0^*), trees per batch (n_1^*) and leaves per tree (n_2^*)			Total number of leaves per batch ($n_1^* \times n_2^*$) at the optimum and limit L of leaves per batch.	
α	β	r	Ct_{targ}	N	Ct_{detec}	$\hat{\sigma}_\varepsilon$	$\hat{A}$	α_b	n_0^*	n_1^*	n_2^*	$n_1^* \times n_2^*$	L
0.95	0.67	0.005	15	6000	25	0.8	4.7	0.99	35	53	1	53	53
*0.99**	0.67	0.005	15	6000	25	0.8	4.7	0.99	54	53	1	53	53
0.95	*0.90**	0.005	15	6000	25	0.8	4.7	0.99	131	17	3	51	53
0.95	0.67	*0.001**	15	6000	25	0.8	4.7	0.99	219	25	2	50	53
0.95	0.90	0.001	*20**	6000	25	0.8	4.7	0.99	456	4	1	4	4
0.95	0.67	0.001	15	*12000**	25	0.8	4.7	0.99	35	53	1	53	53
0.95	0.67	0.005	15	6000	*19**	0.8	4.7	0.99	908	2	1	2	2
0.95	0.67	0.005	15	6000	25	*1.6**	4.7	0.99	88	21	1	21	21

0.95	0.67	0.005	15	6000	25	0.8	*6.2**	0.99	92	20	1	20	20
0.95	0.67	0.005	15	6000	25	0.8	4.7	*0.999**	47	39	1	39	39
0.95	0.67	0.005	15	6000	25	0.8	4.7	*0.95**	28	68	1	68	70

*Indicates parameter has been changed compared with base line (first row).

Optimal sample sizes for a selection of different cost functions show marked changes in the balance of sampling effort depending upon the weighting given in cost functions to batches, trees and leaves (Table 3). The baseline (Table 3, 1st row of results) is the same as for Table 2, 1st row of results. When the cost of sampling trees dominates (Table 3, row 2 of results), and the costs related to PCR and collecting leaves are negligible, the optimal strategy reflects a trade-off between n_1 and n_2 with a large number (19) of leaves collected per tree but only one tree per batch. The outcome is a very large number (n_1 x n_2) of leaves to collect (Table 3).

If the cost were dominated by the number of leaves collected (Table 3, third row of results), there is no gain in pooling leaves together, then each leaf is tested individually and the number of PCR tests is impractically large. When the cost is a combination of the PCR, trees and leaves sampling costs, (Table 3, 4th and 5th row of results), the optimal allocation reflects a trade-off between the different costs.

The accompanying app allows custom cost functions to be defined, (*cf*. Table 3, sixth row of results), which may reflect extra constraints, as, for example, putting an infinite cost on allocations with more than 10 trees per batch, which is equivalent to putting a hard constraint on the number of trees per batch.

We note that the total number of leaves sampled often approaches a minimum of 1,815, unless the costs involve the total number of trees but not the number of leaves. This is

consistent with the results from Table 2 that minimizing n_0 favours the number of trees per batch rather than the number of leaves per tree.

Table 3: Optimal sample sizes for different cost functions

Specific Inputs		Outputs Optimal number of batches (n_0^*), trees per batch (n_1^*) and leaves per tree (n_2^*)			Practical outputs At the optimum, total number of selected trees ($n_0^* \times n_1^*$), total number of selected leaves ($n_0^* \times n_1^* \times n_2^*$) and cost.	
Cost function	$Cost(n_0, n_1, n_2) = \cdots$	n_0^*	n_1^*	n_2^*	$n_0^* \times n_1^*$	$n_0^* \times n_1^* \times n_2^*$
Number of batches	n_0	35	53	1	1855	1855
Number of trees	$n_0 \times n_1$	598	1	19	598	11362
Number of leaves	$n_0 \times n_1 \times n_2$	1815	1	1	1815	1815
Combination	$n_0 + (n_0 \times n_1)$	104	6	8	660	3960
Combination	$n_0 + 2 \times (n_0 \times n_1 \times n_2)$	55	33	1	1815	1815
Custom	$n_0 + (Inf \times (n_1 \times n_2) > 10)))$	182	10	1	182	1820

Optimal allocation is given for default parameters as specified in Table 1

DISCUSSION

We have developed a flexible protocol for use in optimizing sampling strategies to establish pest free status from ASBVd in avocado orchards. The protocol, which is supported by an interactive app (Bonnéry, 2022a), integrates statistical considerations of multistage sampling of trees in orchards with a RT-qPCR assay allowing for detection of infection in pooled samples of leaves taken from multiple trees. While the approach was designed with ASBVd in mind, we note that it has broad applicability for a wider range of plant pathogens in which hierarchical sampling of a target population is coupled with pooling of material

prior to assay. For example, in sampling for regional pest-free status of an agricultural or horticultural crop, samples may be collected within fields and aggregated amongst fields to minimise the costs of running the diagnostic assay.

Our analyses for ASBVd take account of high-level regulatory constraints as well as practical constraints associated with the assay used to detect a positive response (Table 1). Regulatory constraints would normally be set by a government or other regulatory authority. The constraints include an arbitrary low-limit of prevalence (r) against which an orchard is declared pest free with a confidence level (α) for a false negative assertion. It is also necessary to set a regulatory constraint for the target percentage of infected leaves in an infected tree (1 - β). The default value for β = 0.67 used in our analyses was chosen to match the value set by the New Zealand Ministry of Agriculture and Forestry (MAF, 2009), which is the only biosecurity regulatory authority to have addressed the question as to what constitutes acceptable evidence of pest freedom with regards to ASBVd (Pugh and Thomson, 2009). Critical parameters related to the RT-qPCR assay include not only the usual Ct threshold ($Ct_{threshold}$) used for detection but an additional Ct threshold (Ct_{target}) related to β.

We used a simple experiment to demonstrate a linear relationship for the dilution effect of mixing healthy with an infected leaf on Ct value. The slope and variance from the relationship were used in the calculation of optimal designs. The experiments were designed as proof of concept using small numbers of infected plants. We recommend recalibration of experiment 3 using larger numbers of leaves to improve the estimates for $\hat{A}$ and $\hat{\sigma}$.

Analysis of the variability in Ct measurements indicated that the distribution of ASBVd varied between branches and octants within infected trees but not in a consistent way to

identify viroid 'hotspots' that could be preferentially sampled in order to maximize the chances of detecting the viroid in an infected tree. Similar results applied to detection of the viroid within infected leaves. The practical consequence is that to detect presence in a field, for a given number of collected leaves, the number of donor trees should be maximized in preference to sampling more than one leaf per tree (Table 2). From a practical perspective, it is not feasible to sample mature avocado trees in commercial orchards containing thousands of trees from any layer other than at ground height. However, for small populations of high-value avocado trees, such as those in germplasm collections or in multiplication blocks used for propagation, multiple leaves from different parts of the tree should be sampled, as is normal protocol in many countries such as Australia and South Africa.

The approach we followed here in defining two thresholds ($(1-\beta)$ and Ct_{target}) is analogous to the general situation described in official European Food Safety Authority guidelines (Lázaro et. al, 2020). Lázaro et. al, (2020) list four parameters that govern the sampling phase from which sample size can be computed: the confidence level, the field level prevalence, the field size, and a term referred to as 'method sensitivity' whereby the risk manager needs to assess the sensitivity of the assay method in detecting the target pathogen. Our parameters for the qRT-PCR A and σ_ε are used to characterize the method sensitivity and need to be estimated beforehand. In addition to setting a criterion for field prevalence, non-uniform distribution of ASBVd within a tree requires extra criteria to be set for prevalence within plants, which are defined by the thresholds $(1-\beta)$ and $Ct_{\{target\}}$.

In the absence of auxiliary information on the trees (such as root stock, variety, symptoms of infection or presence of infected trees in a vicinity), we recommend a simple protocol

involving systematic sampling of trees to ensure even coverage of the target orchard followed by simple random sampling (Cochran 1977) of units at each subsequent stage. The simulation tool we provide allows comparison of the risk of false negatives at the orchard level for simple random or systematic sampling. Use of the app shows that systematic sampling is optimal.

Prior to doing any diagnostic assays for ASBVd, trees in the orchard should be inspected for symptoms and when recognized, the diseased trees should be individually tested along with neighboring trees either in the same or adjacent row, since there is evidence for transmission of the viroid by natural root grafting (Schnell et al. 2011). The appearance of symptoms such as leaf bleaching or variegation may indicate that the infection is only at an early phase, in which case the symptomatic leaves should be sampled as they are more likely to contain a higher viroid titer (Semancik and Szychowski. 1994). However, according to the longitudinal study of Semancik and Szychowski. (1994), this initial acute phase of infection is followed by a chronic phase, when foliar symptoms disappear, and the viroid becomes uniformly distributed around the tree at a high titer. The trees in our study were typical of the chronic phase of infection, at which point it is likely that leaves could be collected from any point on the tree with an equal chance of detecting the viroid. The optimal design protocol should be applied to orchards without any signs of sunblotch disease. Ultimately, the surest way to guarantee that an orchard is free of ASBVd is to use certified planting material that has been propagated using seed and budwood from mother trees that have been tested and shown to be free of ASBVd. With basic orchard hygiene, such as using dedicated pruning equipment for that particular orchard, it would be extremely unlikely that

the viroid would be freshly introduced into the orchard and there should not be a need to do anymore testing for duration of the trees' life.

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Supplementary Material

A. Supplementary figures

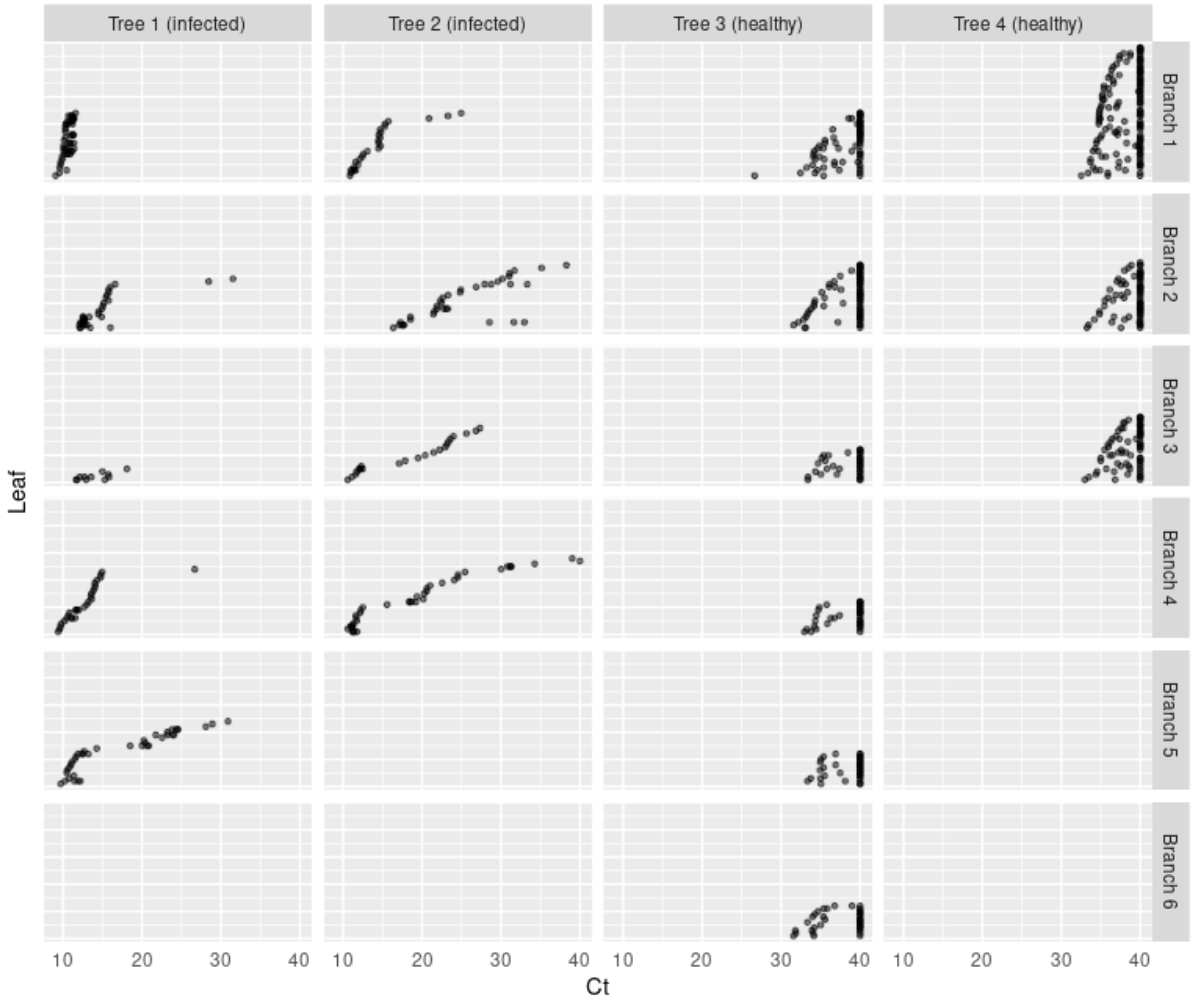

Figure SM1 Ct measurements by tree, branch, leaf, biological replicate index.

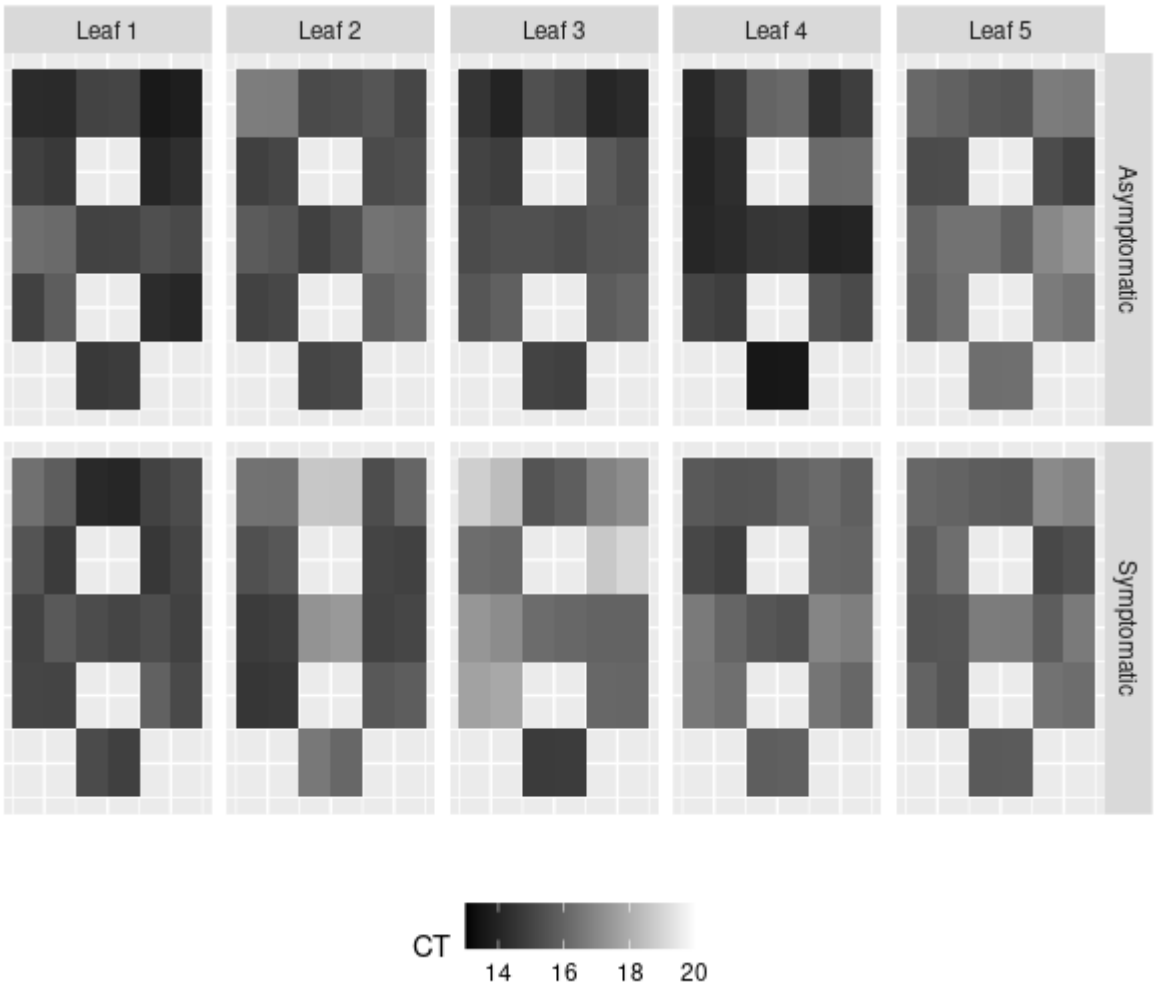

Figure A2. Heatmaps for five asymptomatic and five symptomatic leaves.

B. Hierarchical modelling for Ct measurements

B.1. General model

Equation (Eqn SM1) with a corresponding model and in Equation (Eqn SM2) where leaf samples were collected from octants instead of randomly selected branches.

$$Ct_{tree\ status,tree,branch,leaf,disc,technical\ replicate} = \mu_{tree\ status} + \varepsilon_{tree} + \varepsilon_{tree.branch} + \varepsilon_{leaf} + \varepsilon_{leaf,disc} + \varepsilon_{disc} + \varepsilon_{technical\ replicate} \quad \text{(Eqn SM1)}.$$

$$Ct_{tree\ status,tree,octant,leaf,disc,technical\ replicate} = \mu_{tree\ status} + \varepsilon_{tree} + \varepsilon_{tree,octant} + \varepsilon_{octant} + \varepsilon_{leaf} + \varepsilon_{leaf,disc} + \varepsilon_{disc} + \varepsilon_{technical\ replicate} \quad \text{(Eqn SM2).}$$

Under the models in Equations (SM1) and (SM2), Ct measurements for leaves of an infected or healthy tree (ASBVd tree status) are the sums of a theoretical average Ct measurement and variations due to the tree branch (or octant), the leaf, the sampling location in the leaf and random noise associated with the RT-qPCR assay.

B.2. Experiment 1

We tested the (nested or crossed) leaf, branch, (nested or crossed) octant, and tree effects. Before running the mixed model analysis, the assumption of homogeneity of variance of the Ct values was tested and variance shown to be equal across the samples (Bartlett test, p. value: 0.47). There was evidence of a fixed effect of the branch (LRT, p-value<0.0001), branch as well as nested octant (LRT, p-value<0.0001), of a leaf random effect (LRT, p-value<0.0001) and biological replicate (LRT, p-value<0.0001). The assumption of a crossed versus a nested fixed effect of octant (LRT, p-value<0.0001) was rejected.

B.3. Experiment 2

The statistical aim of Experiment 2 was to test the effect of the position of the leaf disc, to estimate the variance between leaf discs of the mixed effect and to test the crossed effect (i.e. across leaves) of leaf disc between different leaves. There was statistical evidence of a leaf mixed effect (LRT, p-value<0.0001), of a leaf disc location nested effect versus no leaf disc location effect (LRT, p-value<0.0001), and of a leaf disc location nested versus crossed effect (LRT, p-value<0.0001).

C. Relationship between Ct and dilution

We assumed that the relationship between the concentration c of the viroid RNA of the tested material and the Ct measurement is of the form $c = \kappa \times a^{-Ct} + \eta$, where a and κ are positive parameters, and η is a measure of residual error. The reciprocal relationship between Ct and c is then $Ct = (\log(\kappa) - \log(c))/\log(a) + \varepsilon$, where $\varepsilon = \log\left(1+\eta/(\kappa \times a^{-Ct})\right)/\log(a)$, or equivalently

$Ct = K - A \times \log_{10}(c) + \varepsilon$,

where $K = \log(\kappa)/\log(a)$ and $A = 1/\log_{10}(a)$.

When mixing an infected leaf i of concentration c_i with leaves $h_2, \dots, h_n$ of respective concentrations $c_{h_2}, \dots, c_{h_n} \ll c_i$, that are negligeable with respect to c_i, in a batch, the concentration of the batch is $\left(c_i + c_{h_2} + \cdots + c_{h_n}\right)/n \approx c_i/n$.

A k −th repeated Ct measurements $(k = 1, \dots, j)$ of the batch will be equal to $Ct_{batch,k} = K - A \times \log_{10}(c_i) + A \times \log_{10}(n) + \varepsilon_{batch,k}$, and a k −th repeated Ct measurement of the infected leaf Ct will be equal to $Ct_{i,j} = K - A \times \log_{10}(c_i) + \varepsilon_{i,k}$.

The relationship between the average of the batch technical replicate Ct measures and the average of the infected leaf technical replicates Ct measures will be:

$$\overline{Ct_{batch}} = \overline{Ct_i} + A \times \log_{10}(n) + (\overline{\varepsilon_{batch}} - \overline{\varepsilon_i}). \text{ (Eqn SM3)}$$

The variance of the residual term $\varepsilon = (\overline{\varepsilon_{batch}} - \overline{\varepsilon_i})$ is the sum of the variance due to the possible biological variations in batches of n leaves containing material from leaf i, and of the variance due to variations in repeated measures on the same biological material, this

variance being inversely proportional to the number J of technical replicates: $\sigma_\varepsilon^2 = \sigma_{bio.rep.}^2 + \sigma_{tech.rep.}^2/\mathrm{j}$.

D. Details of the computation of the risk function

Under the normality assumption of the residuals and the general model of the Ct measurements, an estimation of the probability that the batch Ct will be below the detection Ct when the batch contains l leaves with an expected Ct equal to Ct_{target} is $\tau(n,l) = P\left(Ct_{target} + A(\times \log_{10}(n) - \log_{10}(l)) + \varepsilon < Ct_{detect}\right) =$

$\Phi\left(\frac{Ct_{detect} - Ct_{target} - A\times(log_{10}(n) - log_{10}(\mathrm{l})}{\sigma_\varepsilon}\right)$ if L > 0, 0 otherwise.

The probability to select T infected trees when drawing n trees and sampling without replacement from a population containing m=$\lceil r \times N \rceil$ infected trees and *N-m* healthy trees is $h(N,\max(\lceil r \times N \rceil, 1), n_0 \times n_1, t)$ (where $\lceil . \rceil$ is the ceiling function and $h(N,m,n,t) = \left(\binom{N}{n}\right)^{-1}\binom{m}{t}\binom{N-m}{n-t}$ if $n + m - M \leq k \leq m$, 0 otherwise) is the probability to draw t infected trees without replacement in n draws from a population containing m infected trees and N-m non infected trees.

Let T be an integer between 0 and $n_0 \times n_1$. Let $(x_0, \ldots, x_T)$ be a sequence of integers between 0 and n_1 such that $\sum_{i=0}^{T} i \times x_i = T$ and $\sum_{i=0}^{T} x_i = n_0$. If T is the number of infected trees selected in the sample and x_i be the number of batches that contain leaves from exactly I infected trees and $n_1 - I$ healthy trees, then conditionally on T, the probability to observe the distribution $(x_0, \ldots, x_T)$ is exactly $\left(\frac{\prod_{i=0}^{T}\left(\frac{\mathrm{n_1}!}{i!(n_1-i)!}\right)^{x_\mathrm{i}}}{\frac{(n_0\times n_1)!}{T!(n_0\times n_1 - T)!}}\right) \times \left(\frac{\mathrm{n_0}!}{\prod_{\mathrm{i}=0}\mathrm{x_i}!}\right)$.

When a batch contains leaves from exactly i infected trees and $(n_1 - i)$ healthy trees, the probability to have l infected leaves in the batch and to have a negative result for the batch is $\binom{n_2 \times i}{l}\beta^{n_2 \times i - l}(1-\beta)^l\big(1-\tau(n_1 \times n_2, l)\big)$. Overall, when a batch contains leaves from exactly i infected trees and $(n_1 - i)$ healthy trees, the probability to have a negative result for the batch is $\sum_{l=0}^{n_2 \times i}\binom{n_2 \times i}{l}\beta^{n_2 \times i - l}(1-\beta)^l\big(1-\tau(n_1 \times n_2, l)\big)$.

Overall, the risk to have a negative result for all the batches is lower than:

$$\sum_{t=0}^{n_0 \times n_1}\left(\left(\sum_{\substack{(x_0,\ldots,x_T)\in 0,\ldots,{n_1}^{T+1} \\ \sum_{i=0}^{T} x_i = n_0 \\ \sum_{i=0}^{T} i \times x_i = T}}\left(\frac{\prod_{i=0}^{T}\left(\frac{\mathrm{n_1}!}{i!\,(n_1-i)!}\right)^{x_i}}{\frac{(n_0 \times n_1)!}{T!\,(n_0 \times n_1 - T)!}}\right) \times \left(\frac{\mathrm{n_0}!}{\prod_{\mathrm{i}=0} \mathrm{x_i}\,!}\right)\right.\right.$$

$$\left.\times\left(\prod_{\mathrm{i}=0}^{\mathrm{T}}\left(\sum_{l=0}^{n_2 \times i}\binom{n_2 \times i}{l}\beta^{i \times n_2 - l}(1-\beta)^l\left(1\right.\right.\right.\right.$$

$$\left.\left.\left.\left.-\Phi\left(\frac{Ct_{detect} - Ct_{target} - A \times (log_{10}(n_1 \times n_2) - log_{10}(\mathrm{l}))}{\sigma_\varepsilon}\right)\right)\right)^{\mathrm{x}_i}\right)\right)$$

$$\left.\times\, h(N, \max(\lceil r \times N \rceil, 1), n_0 \times n_1, t)\right) \qquad (Eqn\ SM4).$$

The computation of this probability is intractable for large values of $n_0 \times n_1$. And two approximations can be made. When the probability to get a positive result batch that contains material from a single infected leaf $\alpha_b = \Phi\left(\frac{Ct_{detect} - Ct_{target} - A \times (log_{10}(n_1 \times n_2) - log_{10}(\mathrm{l}))}{\sigma_\varepsilon}\right)$ tends to 1, then $1 - \Phi\left(\frac{Ct_{detect} - Ct_{target} - A \times (log_{10}(n_1 \times n_2) - log_{10}(\mathrm{l}))}{\sigma_\varepsilon}\right)$ tends to 0 for L>0. So when α_b is close to 1, the overall risk of not detecting is close to $\sum_{t=0}^{n_0 \times n_1}\left(\beta^t \times h(N, \max(\lceil r \times N \rceil, 1), n_0 \times n_1, t)\right) = E[exp(t\, log(\beta))]$, which is the moment generating function of the hypergeometric of parameters N (population size), $\max(\lceil r \times N \rceil, 1)$ (success states), and $n_0 \times n_1$ (sample size), applied to log(β), and also the probability that none of the n_0 batches contain material from an infected leaf. To account for the fact that there is a risk of not detecting the viroid in a batch that contains at least one infected leaf, another approximation of the intractable risk (5), valid when N, $r \times N$, and $n_0 \times n_1$ are large is based on the approximation of the without replacement sampling by a with-replacement sampling. Under this approximation the risk is:

$$MajRisk(N, r, n_0, n_1, n_2, \beta)$$

$$= \left(\sum_{t=0}^{n_1} \left(\binom{n_1}{t} r^t (1 - r)^{n_1 - t} \sum_{l=0}^{t \times n_2} \left(\binom{n_2 \times t}{l} \beta^{t \times n_2 - l} (1 - \beta)^l \left(1 - \Phi\left(\frac{Ct_{detect} - Ct_{target} - A \times (log_{10}(n_1 \times n_2) - log_{10}(\mathrm{l}))}{\sigma_\varepsilon} \right) \right) \right) \right) \right)^{n_0}.$$

This second approximation is preferred as it allows to take into account the risk of not detecting the viroid when present in a batch.

The interactive application allows 2500 independent simulations of an epidemic to be run for selected parameter sets (cf Table 1, main text). The application allows for three different selection methods, cluster sampling, simple random sampling and systematic sampling. Extensive simulations indicate that systematic sampling performs better than simple random sampling in reducing the risk of failure to detect the pathogen when it is present Table SM1). Systematic sampling is especially better than simple random sampling when the epidemic is concentrated in a small cluster, (reproduced in the simulations by setting the number of sources of infections to 1) since simple random sampling may lead to samples being less regularly spaced throughout the target population (De Jong, 1995). The approximated risk for simple random sampling (used in Table 3 main text) can be treated as an upper bound for the risk in systematic sampling.Table SM1: Risk of no detection and sampling design

Sampling design	No. sources of infections	Risk		
		Approximation for simple random sampling	Expected relationship between approximated and simulated risk	Estimated via 2500 independent simulations. Estimated half width of confidence interval is following the estimate.
Simple random sampling	1	0.044	$\approx$	0.044 ± 0.080
	10		$\approx$	0.046 ± 0.082
Systematic sampling	1		$>$	0.042 ± 0.078
	10		$>$	0.040 ± 0.076

The risk of no detection approximation and estimations for different sample schemes were obtained for the default parameters as listed in Table 1 (main text), for a field size of $N = 32000$, and a sample allocation of $n_0 = 35, n_1 = 53$, and $n_2 = 1$ all other parameters being the default parameters as in Table 1.

E. Details on the optimization

Optimization involves a class of multistage stratified sampling designs associated with a pooling of samples process that does not disperse leaves from the same tree into different batches. The first stage corresponds to the selection of trees. The second stage corresponds to stratified selection of leaves, with constant allocation per stratum, and simple random sampling within strata, where strata correspond to the division of the tree in octants or main branches.

To reduce the sample processing costs while obtaining the desirable confidence level for detection of the pathogen, leaves from multiple trees need to be tested in batches. The number of leaves per batch must not exceed the limit for which the Ct of a batch that contains a leaf below the target Ct will be higher than the detection Ct.

The estimated optimal allocation is defined as

$$(n_0^*, n_1^*, n_2^*) = \operatorname{argmin}\left\{\mathrm{Cost}(n_0, n_1, n_2) \middle| \, n_0, n_1, n_2 \in \right.$$

$$\left. \mathbb{N}: \begin{cases} MajRisk(N, r, n_0, n_1, n_2, \beta) < 1 - \alpha, \\ \Phi\left((\widehat{\sigma}_\varepsilon)^{-1}\left(\mathrm{Ct}_{\mathrm{detect}} - \mathrm{Ct}_{\mathrm{target}} - \hat{A} \times \log_{10}(n_1 \times n_2)\right)\right) > \alpha_b \end{cases} \right\}.$$

Note that $\Phi\left((\widehat{\sigma}_\varepsilon)^{-1}\left(\mathrm{Ct}_{\mathrm{detect}} - \mathrm{Ct}_{\mathrm{target}} - \hat{A} \times \log_{10}(n_1 \times n_2)\right)\right) > \alpha_b$ is equivalent to

$$n_1 \times n_2 < L = \exp\left(\hat{A}^{-1} \times \left(\mathrm{Ct}_{\mathrm{target}} - \mathrm{Ct}_{\mathrm{detect}} + \hat{\sigma}_\varepsilon \times \Phi^{-1}(\alpha_b) \right) \times \log(10) \right) \quad \text{(Eqn SM5)}$$

In the case of non-unique optimal allocations, allocation with the smallest numbers of leaves per tree, or if equal with minimal number of trees per batch is returned by the algorithm.

F. Screenshots of the interactive web app

An interactive web-based app has been developed to illustrate the detection problem, to simulate the epidemic, sampling and detection processes (Figure SM3) and to allow computation of the optimal allocation (Figure SM4). The web app can be run via R through the ASBVdDetection package (Bonnéry, 2022b), or directly accessed (Bonnéry, 2022c).

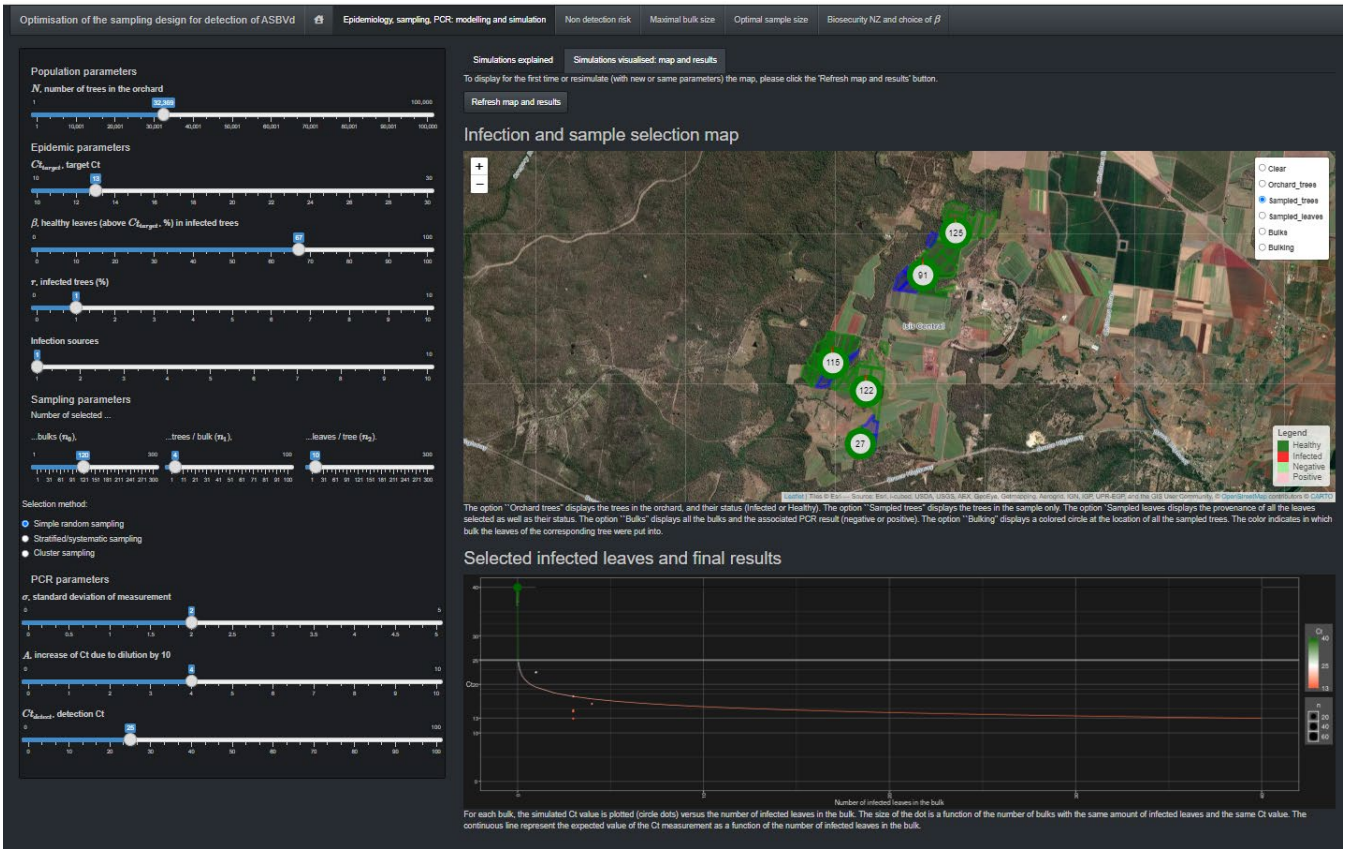

Figure SM3: Screenshot of the interactive webpage (Bonnéry, D.B. 2022a), second tabulation. This page allows the user to view the results of one simulation of an infection on an orchard, of the sampling and detection process. The process can be simulated a large number of times to check the validity of the formulae used for the risk (tabulation 3).

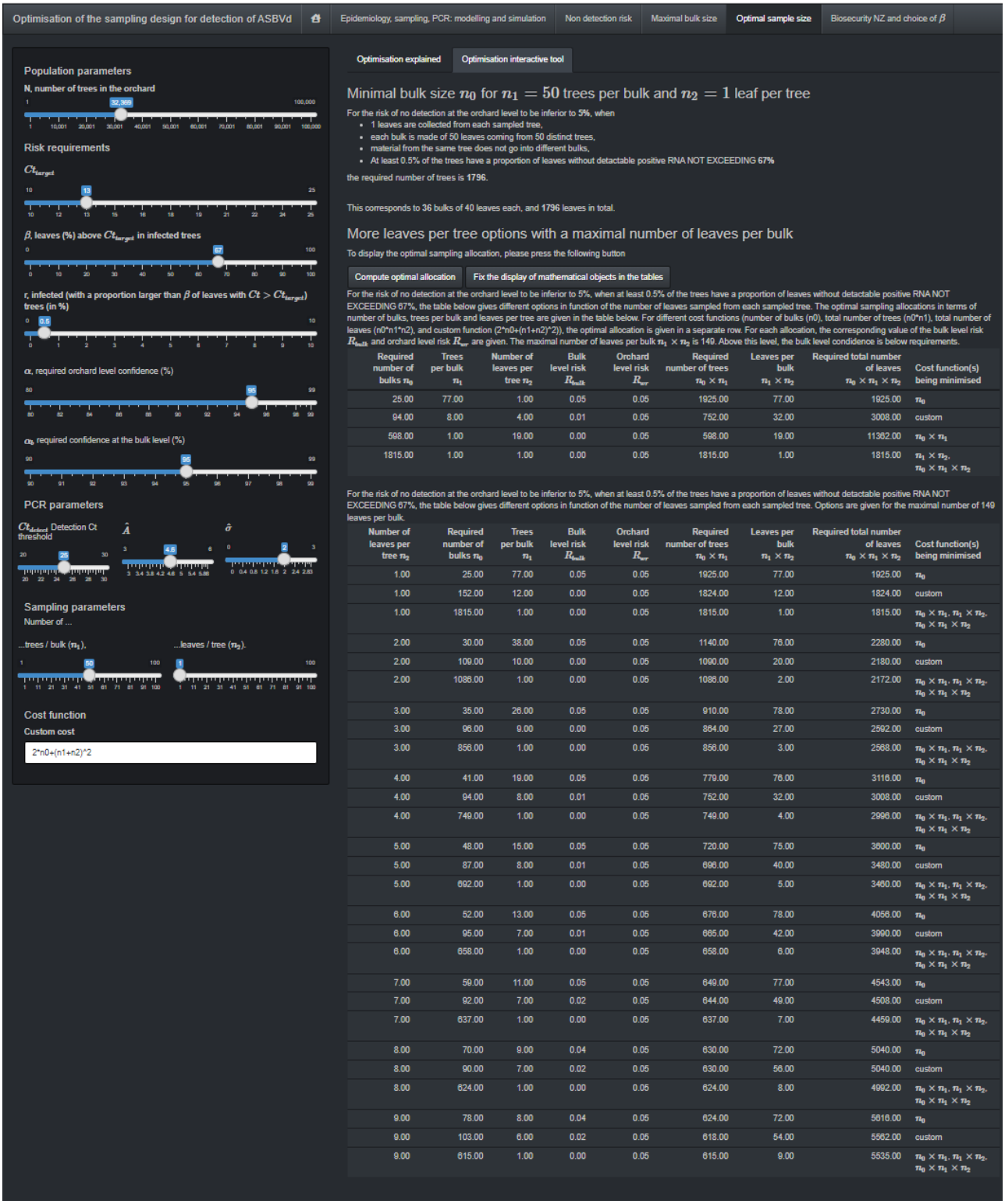

Figure SM4: screenshot of the interactive webpage (Bonnéry, D.B. 2022a), fifth tabulation.

This page allows to compute the optimal allocation, for different cost functions (number of

sample trees, number of sampled leaves per tree, number of sampled leaves as well as any custom function of n_0, n_1 and n_2.

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Survey Report for Atherton Tableland production area

Travel Dates: 14 December 2021- 16 December 2021

14 Dec 21		**Notes**
Time	Activity	
11:20	Arrive at Cairns Airport	
13:00	Arrived at accommodation (1 Hour 23 Minutes from airport)	
14:30	Pam and Peter Lavers 0412489725	100 Henry Hannam Dr, Walkamin
15 Dec 21		
9:30	Alan Poggioli 0407 596933	167 Mehmet Rd, Tolga
13:30	Howe Farming Denis 0427933791	Met with Orchard Manager: James Kennedy 1687 Chewko Rd, Walkamin
16 Dec 21		
Time	Activity	
9:30	Colin Foyster 0427933791	Met with Orchard Manager: Santiago 262 Mutchilba Rd, Mutchilba
14:30	Costa Group Nicola MacKay 0448 275 643	Older trees located at main farm Homestead: 238 Beantree R, Tolga
15:30	Leave for Airport	
18:10	Flight Departs	
20:25	Flight Arrives	

14 December 2021

Met with Pam and Peter Lavers-discussed ASBVd history in Australia as well as their concerns with the current ANVAS accreditation scheme.

Ended meeting with nursery tour.

Notes:

- System breakdown between nursery and orchard indicating that growers are not keeping records of where their planting material is from.
- This prevents the retrospective approach of declaring pest freedom from ASBVd as even though nursery are ANVAS approved, growers may mix planting material from various nurseries in one block without detailed planting records.
- Meeting will be schedule with John Tyas to discuss issue further and come up with possible solution.

15 December 2021

Met with Alan Poggioli from the Golden Triangle Nursery

Introduced ourselves as well as the project and its objects. Briefly discussed ASBVd origin, history in Australia and as an economically significant pathogen throughout the world.

Spent a few hours walk through old blocks to scan for ASBVd symptomatic fruit- none found, trees very old but nursery very well maintained. Trees had set a large amount of fruit.

Alan was happy to help with surveys by having his staff collect and send us leaves. We suggested we would first determine the best survey strategy with our statistician as it prossibly isn't necessary to test every tree.

Notes:

- Alan was unfamiliar with the disease though had not seen any symptomatic fruit in either is old blocks or newly planted ones.
- Purchased oldest block from Mike Colins and Ronnie Butler
- Oldest block planted between 1979-1981
    - 1400 trees
    - Wurtz and Feute rootstock top graphed with Hass
    - No ASBVd symptoms noticed
    - Trees still bearing decent yield
    - Trees dying of Phellinus
- Youngest block planted in 2005
    - Fleming planting material
- Also buys planting material from Turkinje
- Suggested contacting Mark Delai as he had old trees, though he did not have his contact details

Howe Farming

Met James Kennedy from Howe Farming who drove us to two sites (Block A1 and J1)

Introduced ourselves as well as the project and its objects. Briefly discussed ASBVd origin, history in Australia and as an economically significant pathogen throughout the world.

Orchards poorly maintained; grass not cut and dead weeds not removed. Trees planted in narrow rows. Trees had set a fair amount of fruit.

Notes:

- James was unfamiliar with the disease
- James mentioned possibly seeing symptomatic fruit in block A1
- Walked through both blocks, though were unable to identify any symptomatic fruit or leaves.
- Collected leaves from two trees from block J1 which James had noted as having abnormal growth patterns, specifically curling branches. Though as the orchard was recently pruned the abnormal branches were removed.
- Tree negatived for ASBVd
- Block A1:
    - Located near the main farm area at 1687 Chewko Rd, Walkamin

    - Trees ~25 years old
    - 16,000 trees
    - Most trees were Shepard on Feute, though there were a few Hass trees as well
- Block J1:
- Bee surveys not possible as no beehives are placed in blocks during flowering

16 December 2021

Aussie Orchards

Met with Santiago who is the Orchard Manger from Aussie Orchards Mutchilba site

Introduced ourselves as well as the project and its objects. Briefly discussed ASBVd origin, history in Australia and as an economically significant pathogen throughout the world.

From phone discussion Colin Foyster mentioned there were two sites (three blocks) where ASBVd was previously found. Block 2 all trees including the ASBVd positives were removed- ground remains fallow. Blocks 14 and 15 bulldozed in ~2014 and replanted in ~2016.

Walked through Block 1 of Mutchilba site and Mareeba site. No ASBVd symptoms seen.

Orchards fairly maintain though all trees had low number of fruits set.

Notes:

- Santiago was unfamiliar with the disease but had not seen symptoms
- Block 1 (Mutchilba):
    - Trees ~25 years old
    - 1200-1500 trees
    - Shepard
    - Unknown rootstock
- Block 2
    - Were Shepard
    - Rootstock unknown
    - ~ 25 years old
    - Bulldozed and not replanted
- Block 14 & 15
    - Age unknown but though to be older that 25 years
    - Variety and rootstock unknown
- Bee surveys not possible as no beehives are placed in blocks during flowering

Costa Group

Met with Nicola MacKay at Homestead Orchard of Costa Group.

Introduced ourselves as well as the project and its objects. Briefly discussed ASBVd origin, history in Australia and as an economically significant pathogen throughout the world.

Nicola was familiar with the disease though had not seen symptoms in any blocks. The Homestead Farm was previously owned by Lancaster's and therefore not aware of the ASBVd positive tree(s) previously removed.

Oldest trees located in Blocks A, B and C.

Drove through blocks with Nicola. No symptomatic fruit was seen.

Orchards very well maintained, and all trees had a high number of fruits set.

Notes:

- Block A, B and C
    - ~25-30 years old
    - Hass
    - Unknown rootstock
- Planting material purchased from Flemings, Andersons and Turkinje
- Bee surveillance possibly as they do pay for pollination services

Action Items:

- Meeting with Liz to discuss the registry of blocks planted with ANVAS accredited plants to be declared viroid free
    - Meeting with John Tyas to discuss same issue and how to go about organising the registry
- Bee surveillance may be possibly during flowering for each of the sites visited
    - Costa's places beehives in orchards during flowering
    - Howe Farming does not use pollinations services
    - Contact Alan Poggioli and Santiago to determine if bee surveillance would be possible in their orchards
- Discuss with Daniel what the best survey strategy for all sites visited, though as Alan Poggioli had oldest trees his block would be a priority
- Create ASBVd flyer to be distributed to growers to displayed in packhouses to make those handling the fruit and managing orchards more familiar with disease and symptoms
    - If they know what to look for, they may find it 😊

Survey Report for Tristates production area

Meeting Schedule

Day 1: 6th April 2022					
Time	Activity	Travel Time	Address	Mobile	Notes
8:45	Flight departs				
11:10	Arrive at Adelaide Airport		Sir Donald Bradman Dr, Adelaide Airport SA 5950	EUROPCAR Car Hire (08) 8150 3090	INTERMEDIATE SUV 2WD
		2 hr 51 min (260 km) via National Highway A20	Renmark Hotel	(08) 8586 6755	
Day 2: 7th April 2022					
8:00-10:00	Costa Farms	15 min (17.4 km) via Murtho Rd	1319 Murtho Road, Murtho, SA, 5340	Andrew Harty 0448 420 109 Mat 0447 517 310	andrew.harty@costagroup.com.au Meeting with Matthew Maunder (Mat)
12:30-14:30	Gill Farms	1 hr 53 min (178 km) via National Highway A20 and Millewa Rd/C254	1596 Kulkyne WayIraak VIC 3494	Hardeep Gill 0429 785 654	hardeep@gillfarmsmildura.com
15:30-17:00	Cottrell Farms	10 min (12.7 km) via Kulkyne Way/C253 and Brownport Rd	Lot 6 Mondall Rd, Iraak, 3494	Andrew Donaldson 0427 291 310	a.donaldson@cottrellfarms.com.au
Day 3: 8th April 2022					
8:30/9:00-12:00	Churinga orchards	57 min (84.2 km)	Dunstan rd	Troy Lehmann	churingaorchards@bigpond.com

		via National Highway A20	Waikerie, SA, 5330	0408 858 204	
13:00	Kym Thiel Craig Thiel	3 min (2.6 km) via Dunstan Rd and Noble Rd	22 Dearman Rd Golden Heights, SA, 5322	Kym Thiel 0437 939 119 Craig Thiel 0429 430 968	Meeting with Kym's brother-Craig kym.thiel@outlook.com
15:30	Leave for Airport	2 hr 2 min (181 km) via National Highway A20			
19:05	Flight Departs				
21:55	Flight Arrives				

Avocado Sunblotch Symptom College shown to growers

7 April 22

Costas

Block Info

- Size: ~40 hectares with
- Number of Trees: ~480 trees
- Planted: 1972
- Double planted ~20 years ago
- Main cultivar Hass with a few Reed (for pollination)
- Rootstock: Zutano
- No major pest problems
- No symptoms seen

Meet Matthew Maunder from Costas. Andrew explained projected and showed Mat fruit symptom photos of ASBVd to determine if he had ever seen the anything similar. Costas buy Avocados from Andersons, Flemings and Chisletts.

Mat said he would show packers the symptomatic fruit images, so they are able to identify any infected fruit.

Offered to collect leaves for testing.

Gills Farms

Block Info

- Size: 10 hectares
- Unsure about the number of trees
- Planted: 1970
- Various Cultivars: Hass, Zutano, Sharwil and Fuerte
- Rootstock: Unsure
- Previous owner Craig Urand (Mildura Farms- where previous positive was found)
- Main Pest: phytophthora- Phos-acid sprays and injections
- Use pollinators
- No symptoms seen

Andrew explained project and that the last record of ASBVd was found in the Iraak area ~15 years ago on the Mildura Farms.

Hardeep explained that the Mildura Farm was divided between himself, Ray Marr, Crus Farms and Cottrell Farms.

He said it would be difficult to see any scarring on the current yield as fruit had a wind damage- though no symptoms have been seen. Hardeep agreed to show packers symptomatic fruit images to allow them to identify any infected fruit.

New trees are bought from Victorian Citrus Farms (Hass and Gwen).

Gill Farms' grow many various crops including grapes, almonds, and citrus. Avocadoes are not their main priority.

Cottrell Farms

Block Info:

- Size: Unsure
- Number of Trees: ~100
- Planted: 1970
- Various Cultivars: Hass, Sharwil and Fuerte
- Rootstock: Zutano
- No main pests
- No symptoms seen

Andrew explained the project and show Andrew Donaldson symptom photos. Andrew agreed to collect leaves from all trees for testing. Cottrell Farms is mainly citrus, though they also grow grapes and avocados. Andrew Donaldson agreed to show symptomatic fruit images to packers, so they can identify any infected fruit.

Bobby Sigh

Adjust property of the Cottrell's also part of the Mildura Farms though only 3 rows. Trees are same age as those mentioned above.

Bobby has not seen any symptoms in his trees.

Andrew Donaldson said he would include the few trees Bobby had in the samples he sends for testing.

<u>8 April 22</u>

Kym Theil Block 2 blocks

Block Info:

- Size: ~20 acres
- Number of trees: unsure
- Main cultivar: Hass
- Age: ~25-27 years old
- Rootstock: Zutano
- No major pests
- Uses pollinators
- No symptoms seen

Kym Theil was the only grower available in the Waikerie area though he took us to all blocks that were roughly the age we were interested in.

Andrew explained the project to Kym and showed him ASBVd fruit symptoms photos. Kym's Business is about 70% avocados and 30% citrus. He buys new trees from Flemings and Andersons. Kym agreed to show packers the symptoms images so they can identify any infected fruit.

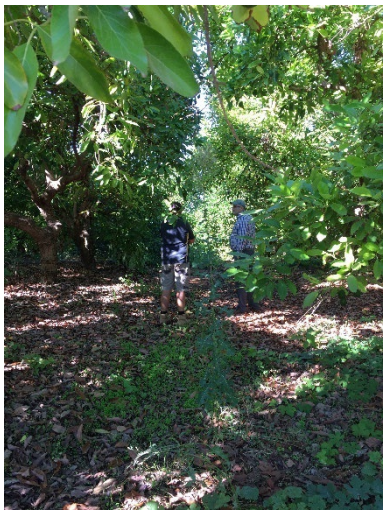
David Hartwig

Block Info:

- Size: 6 Acres
- Number of trees: Unsure
- Age: ~30yrs
- Cultivar: Hass
- Rootstock: Unsure

David Hartwig has only recently bought the property- it would be his second harvest this year and therefore is unsure of tree number as well as rootstock. He has agreed to look out for any symptomatic fruit in the next picking season.

Troy Lehmann



- Size: 5 Acres
- Number of trees: 275
- Age: ~25yrs
- Cultivar: Hass
- Rootstock: Zutano

Justin Ward

Block Info:

- Size: Unsure
- Number of trees: ~550 Trees
- Age: ~25-27yrs
- Cultivar: Hass
- Rootstock: Zutano

Avocado sunblotch viroid surveys as part of the Avocado Nursery Accreditation Scheme

Introduction

Since January 2020, the Avocado Nursery Accreditation Scheme (ANVAS) has been aligned to the Nursery Industry Accreditation Scheme Australia (NIASA) program, with nurseries having to follow NIASA Best Management Practices (BMP) Guidelines and be subject to the auditing requirements of this organization. As part of the BMP Guidelines, testing for ASBVd must be done in one of two ways. Avocado trees are tested as either unplanted propagation material, called in-line testing, or planted multiplication blocks. The NIASA protocol for in-line testing of propagated plants states that the number of plants to be tested is based on the nursery's estimated annual output of trees (Table 1). Plants are only tested once the trees begins to harden off, requiring only one leaf per plant to be sampled, which can be batch-tested in multiples of up to 100 leaves.

Table 1: Number of trees to be tested based on the annual avocado tree production

Estimated Annual Avocado Tree Production	Number of Trees to be Tested
< 30, 000	1 in 20
>= 30,000	1 in 50
>= 60,000	1 in 100
>= 90,000	1 in 150
>= 120,000	1 in 200
>= 150,000	1 in 250

Multiplication blocks are defined as trees "used to provide seed, scion or budwood" as stated in the NIASA Best Management Practises Guidelines, 9th Edition 2021. All trees within a multiplication block must be tested for ASBVd every five years. The block is defined by the grower and is considered one unit, separate from other blocks either through geographical or artificial boundaries. Growers are required to keep multiplication block records which included: name of the nursery and owner, property address (where the block is located), unique identifier/name of the block being tested, cultivar and rootstock if unknown, GPS co-ordinates and satellite image of block as well as the number of trees.

Project AV18007 was established to complete surveys for ASBVd in Victoria (VIC), New South Whales (NSW) and Queensland (QLD) with the aim of declaring pest-freedom. ASBVd has never been found in Western or South Australia, and as such both have pre-existing pest-freedom (Geering, 2018). Declaring pest-freedom in these eastern States would ultimately help in facilitating trade with countries like New-Zealand where ASBVd is absent as well as better accessing the risks of importing fruit from countries where ASBVd is prevalent. Due to the enormity of surveying all Australian orchards in the eastern part of the country, one of the project's main objectives was to create a scientifically robust, practical, and cost-effective sampling strategy of production blocks. The project offered free testing of multiplication blocks, helping nurseries obtain NIASA accreditation by demonstrating that their current sources of seed and budwood were not infected with ASBVd.

Methods

Sample Collection

The number of leaves needed to test multiplication block trees is not specified in the NIASA BMP Guidelines, as such a standard operating procedure (Supplementary File 1) was created by the AV18007 project team. Detailing that eight hardened-off leaves are needed per tree which should be collected in an even distribution around the tree; four from the top section and four from the bottom section. Leaves should be stacked on top of each other (in no particular order), placed in a labelled Ziplock bag and shipped in an ice-packed cooled esky to the testing facility. It was also recommended that growers use gloves to collect the leaves and spray their gloved hands with a 10% bleach solution between trees to prevent cross contamination.

Sample Processing, RNA Extraction and qRT-PCR testing

The stacks of the eight leaves were processed using an 8 mm biopsy punch. The leaf discs were transferred to 2 mL Safe-Lock® Tubes (Eppendorf) and then either stored fresh at 4°C or freeze-dried then stored at –80°C for longer term storage until processing.

Total nucleic acid was extracted from the avocado leaves according to Pretorius et al. (2022).

The total nucleic acid was tested for the presents of ASBVd using the primers and probe published in Geering et al. (2006). The thermocycling parameters and reagents used for the qRT-PCR are also detailed in Pretorius et al. (2022).

Positivity Threshold

The threshold for detection (positivity threshold) was set using the "Determination of Thresholds for Positivity" method of van Brunschot et al. (2014). The Cycle threshold (Ct) results of 162 negative trees were used as the input values to calculate the positivity threshold using the R code in van Brunschot et al. (2014) paper. This value was then used to determine where tested trees were ASBVd positive or negative. Ct values were fell below the threshold were considered positive while those above the threshold were negative.

Results

This report details the testing results for nine ANVAS nurseries, four each in Queensland and Victoria and a single nursery in NSW (Tables 2 and 3). The names of these nurseries are censored to maintain commercial confidentiality. An example of a typical diagnostic report, reproduced with the permission of Turkinje Nursery, is provided at the end of this report. Eight of the nurseries maintain multiplication blocks, while a single nursery exclusively does in line testing. A single nursery has a hybrid model, combining in line testing with maintenance of a multiplication block. To date 5,287 multiplication blocks trees have been tested and no ASBVd-positive trees were identified. Furthermore, 3,033 nursery plants have been tested and again no ASBVd-positive plants were identified.

Table 2. In-line testing for avocado sunblotch viroid

Nursery	Date	Number of plants tested
Nursery 1, Qld	May 2020	185
	August 2020	247
	November 2020	314
	February 2021	414
	May 2021	355
	September 2021	319
	November 2021	280
	March 2022	239
	June 2022	164
Nursery 2, Qld	March 2021	293
	March 2022	156
	June 2022	67
Total		**3,033**

Table 3. Testing of trees in multiplication blocks

Nursery/ Farm Name	Type of Testing	Results	Testing Status	Number of Trees Tested
Nursery 3, Vic	Multiplication Blocks	All Negative	Completed	212
Nursery 4, Vic	Multiplication Blocks	All Negative	Completed	1,040
Nursery 5, Vic	Multiplication blocks	All Negative	Complete	743
Nursery 6, NSW	Multiplication blocks	All Negative	Incomplete	2,417
Nursery 2, Qld	Multiplication blocks	All Negative	Incomplete	324
Nursery 7, Qld	Multiplication blocks	All Negative	Complete	326
Nursery 8, Qld	Multiplication blocks	All Negative	Complete	75
Nursery 9, Vic	Trees >30years Old	All Negative	Complete	150
Total				**5,287**

Discussion

Diagnostic support has been provided to ANVAS, allowing the scheme to operate effectively and provide clean planting material to the avocado industry. Intervention at the nursery stage is the critical stage for sunblotch disease management, as it is by far the most likely stage when the tree could become infected with ASBVd. The negative diagnostic results that were obtained support the hypothesis that ASBVd is now exceedingly rare within the Australian avocado industry. By following the basic precaution of using ANVAS-certified planting material, it is likely that an orchard will remain ASBVd-free for the remainder of its production lifetime.

One very encouraging development since the launch of the ANVAS/NIASA alliance is the growth in participation of nurseries in the scheme, particularly those from Victoria, a place where ANVAS nurseries previously did not exist. All Victorian nurseries are located near Mildura in the Tristates production region. This region has the oldest avocado industry in all of Australia and is a region where ASBVd has been historically recorded. Prior to this project, routine testing of trees from the Tristates production region for ASBVd had not been done.

Overall, the diagnostic support work provided to ANVAS has operated without any major problems and received a high level of client satisfaction, as indicated by the comments below. Furthermore, this work was done with minimal staffing (less than half a full time equivalent of a research assistant), and this has only been feasible through improvements in sample throughput, particularly use of the filter paper method of viroid RNA extraction.

Client feedback

"It's a rave review, I have never had a more organized and professional person that was so helpful in achieving the desired result for both parties. You really did make the process so easy and smooth. Your continual contact was terrific and being able to easily communicate to work out sample numbers and when best suited you was truly amazing. I hope if I ever need assistance it is you Lara who I ask. You did a fantastic job and I can't thank you enough"

Sean Arkinstall, Victorian Citrus Farms, Red Cliffs, Vic, email sent 20 June 2022.

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"Firstly, I would like to thank you, Lara for making the task of sending the samples so easy and secure.

The planning, down to the timing of sending the samples has been made easy due to the excellent level of communication.

Our experiences with the QAAFI and in particular the Centre for Horticulture Science from the receptionist to the specialised team have been a pleasure to deal with. The reports are very comprehensive and easy to read along with any requests for repeat samples.

The Group 1 Viroid testing the department has been doing for us, has been invaluable, in giving us, as Nurserymen, confidence that we are supplying Nursery trees free of Avocado Sunblotch Viroid.

Chislett Farms Pty Ltd looks forward to an ongoing relationship with the QAAFI, in particular the avocado viroid testing program."

Susan Chislett, Chislett Farms, Kenley, Vic, email sent on 20 June 2022

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"As a production nursery with particular emphasis on avocado tree production we have had the benefit since 2020 of having our anti viroid testing carried out by the ASBVd Team, specifically by Dr Lara Pretorius who is our principal contact.

This testing is a mandatory requirement of our NIASA Avocado Stock Specification Accreditation and our continuing ANVAS accreditation with Avocados Australia, so it is of great importance to our nursery.

Our communications with Dr Lara Pretorius have been excellent throughout and we could not speak more highly of her. She responds promptly to any enquiry we have, is very accommodating with our testing timetable and provides us with high quality testing results in a format which we as lay people are able to follow clearly. In addition to her professional and communication skills, Lara is a delightful person to deal with and a credit to your organisation.

We were fortunate to have a personal visit from Lara and Associate Professor Andrew Geering earlier this year and we appreciated the opportunity to meet them both and gain a better overview of the ASBVd project and also the interest they took in our nursery.

Our overall experience with the ASBVd team has been a positive one, of great benefit to our nursery and we look forward to maintaining the association on future projects as the opportunity arises."

Pam & Peter Lavers, Turkinje Nursery, Walkamin, Qld, letter sent 20 June 2022

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"My experience with the team at QAAFI was easy and professional the whole way through. Testing samples were easily collected since communication flowed easily between the scientist and labour collecting, a mission that is not commonly achieved. The results obtained from the team were able to be interpreted with the same ease, as they were formatted brilliantly with clear communication of information. Experiences of personal communication with the team were timely and professional, when time-sensitive matters were an issue the team was able to prioritise and this effectivity enabled the business to continue where otherwise there would be lacking results and less economy in the business. I was greatly impressed with the quality of results and service given by the QAFFI team and hope to continue further interactions."

Graham Anderson, Anderson's Nursery, Durambah, NSW, email sent 14 June 2022.

Centre for Horticultural Science
20 June 2022

AVOCADO SUNBLOTCH VIROID TESTING REPORT FOR TURKINJIE NURSERY

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AVOCADO SUNBLOTCH VIROID TESTING REPORT FOR TURKINJE NURSERY

Avocado High Health Production Checklist

Name of Nursery Turkinje Nursery
Name of the Owner Peter and Pam Lavers
Property Address 100 Henry Hannam Drive
Walkamin
QLD
4872

Table 1: Details Turkinje Nursery samples sent for testing including codes to identify the sample, variety of avocado, rootstock and number of leaves sent for testing

Code	Variety	Rootstock	Number of leaves
83. HASS TF21 KA13	Hass	TERRANOVA FARMING SEEDLING 21	25
84. HASS LVS21 KA13	Hass	LAVERS SEEDLING 21	5
85. HASS TF21 KA13	Hass	TERRANOVA FARMING SEEDLING 21	6
86. SHEP TF21 LE	Shepard	TERRANOVA FARMING SEEDLING 21	12
87. HASS LVS21 LD2	Hass	LAVERS SEEDLING 21	6
88. HASS TF21 LD2	Hass	TERRANOVA FARMING SEEDLING 21	42
89. HASS TF21 KA13	Hass	TERRANOVA FARMING SEEDLING 21	48
90. HASS LC KA13	Hass	LAVERS CLONE	12
91. HASS ADC KA13	Hass	ANFIC DUSA CLONE	8

Background

Avocado sunblotch viroid (ASBVd) is one of the smallest pathogens in the world and can only be detected using molecular tests. The standard test for ASBVd in Australia is a quantitative reverse transcription PCR (qRT-PCR) assay. This test involves cycles of amplification of the target viroid nucleic acid molecule, and if the viroid is present in the test sample, fluorescence light is emitted at a known wavelength during each amplification cycle. The **Ct** (cycle threshold) is **defined** as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceeds the background level from known healthy trees). **Ct** levels are inversely proportional to the amount of target nucleic acid in the sample (i.e. the lower the **Ct** level, the greater the amount of viroid nucleic acid in the sample). Using the specified diagnostic assay, the typical Ct value for an infected sample is 8-15, whereas the background Ct value for a healthy tree is 25-40.
The NIASA protocol for in-line testing of propagated plants states that the number of plants to be tested is based on the nursery's estimated annual output of trees (Table2). Testing of plants for ASBVd is also done once trees begins to harden off and only requires one leaf per plant to be sampled, which can be batch-tested in multiples of up to 100 leaves. Nurseries that require this testing will usually send several batches of leaves for testing throughout the year as their trees reach the correct level of maturity. Table 1 details the code name used by Turkinje to identify their plants as well as the variety, rootstock and number of leaves/plants tested.
All trees were tested with the sampling, extraction and ASBVd testing procedures outlined below.

Table 2: Number of trees to be tested based on the annual avocado tree production

Estimated Annual Avocado Tree Production	Number of Trees to be Tested
< 30, 000	1 in 20
>= 30,000	1 in 50
>= 60,000	1 in 100
>= 90,000	1 in 150
>= 120,000	1 in 200
>= 150,000	1 in 250

Methods

Samples were collected by employees of Turkinje Nursery and placed in clearly labelled zip lock bags and sent via a courier to the Ecosciences Precinct, 41 Boggo Road, Dutton Park, QLD.
Total nucleic acids were extracted from the avocado leaves which were tested in batches of 6-12 leaves (Table 3) using the Whatman filter paper method of Zou et al. (2017) with the following modifications. Stacks of the eight leaves were sampled using an 8 mm biopsy punch and the leaf discs freeze-dried within 2 ml snap-lock microfuge tubes, then stored at –20°C until processing. A 700 µl aliquot of tissue lysis buffer, comprising 1.5 M LiCl, 20mM Tris, 5mM EDTA, 2% PVP. 0.05% SDS and 1mM DTT, was placed in the tubes together with a 5-mm diameter stainless steel ball bearing and the tissue lysed by shaking in a TissueLyser II (QIAGEN) for 3 minutes at a frequency of 30 revs/min. The extract was briefly centrifuged at 14,000 g for 5 min, and 200 µl of cleared lysate added to the well of a microplate containing three 6-mm filter discs. After a 15 min incubation at room temperature, the lysate was aspirated using a micropipette and the filter paper discs washed twice with 10 mM Tris-HCl pH 8. After the final wash, 25 µl of water was added to the wells and the microplate incubated at room temperature for 2 h.
qRT-PCR for ASBVd was done using the primers and probe of Geering et al. (2006) and 1 µl of eluate from the filter paper discs. The thermocycling conditions were 50 °C for 5 minutes followed by 95 °C for 20 seconds. Each reaction was multiplexed by the addition of an internal control PCR template (RT-qPCR Extraction Control Orange, Bioline) to notify the operator if technical errors occurred with the setup of the assay. The PCR stage had 2 steps; 95 °C for 3 seconds and 60 °C for 30 seconds, repeated for 40 cycles.
The threshold for detection was set using the “Determination of Thresholds for Positivity” method of van Brunschot et al. (2014).

Table 3: Leaves from each variety tested were divided into smaller batches allowing leaf disk to fit easily in 2 mL Eppendorf tubes for testing

Code	Batches	Total Number of Leaves
83. HASS TF21 KA13	1 of 13 1of 12	25
84. HASS LVS21 KA13	1 of 5	5
85. HASS TF21 KA13	1 of 6	6
86. SHEP TF21 LE	1 of 12	12
87. HASS LVS21 LD2	1 of 6	6
88. HASS TF21 LD2	3 of 14	42
89. HASS TF21 KA13	3 of 16	48
90. HASS LC KA13	1 of 12	12
91. HASS ADC KA13	1 of 8	8

Results

Test results for Turkinje Nursery are shown in figure 1 and supplementary file 1 specifying the Ct values. Mean Ct values are provided, along with standard deviations. The positivity threshold, designated by an orange line, is set at Ct = 25.4363. Ct values above this line are considered negative test results. All trees sampled tested negative for ASBVd, and all assays were valid, using the internal control PCR template (supplementary file 2, figure 1).

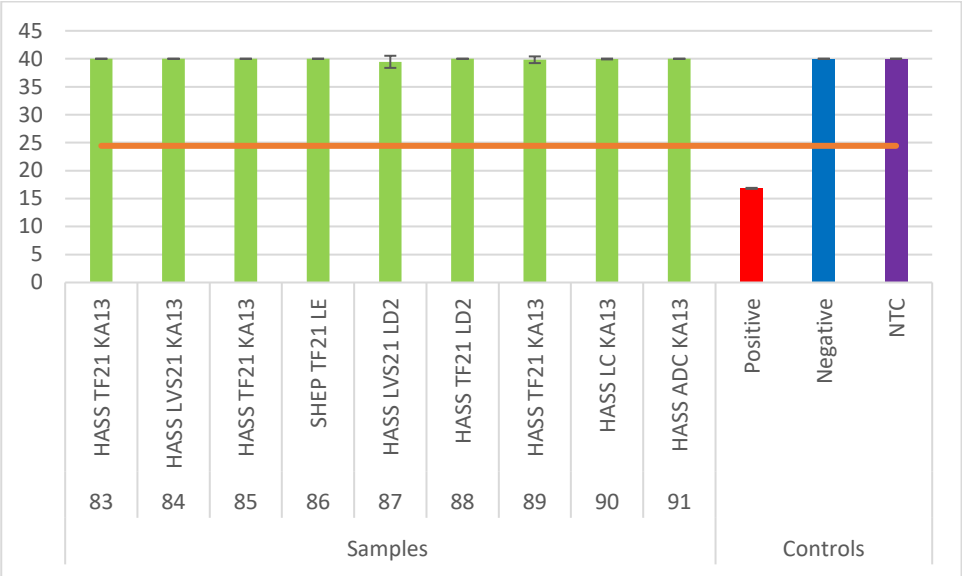

Figure 1: ASBVd test results for propagated plants from Turkinje Nursery. Green bars represent the mean Ct values for the different samples designated by a code. Each code also representing a different number of individual plants tested. The orange line is the positivity threshold while the red, blue, and purple bars are the Ct values for the positive, negative (healthy plant) and no template controls (NTC) (water), respectively. The error bars represent the standard deviation between batches tested.

Conclusions and recommendations

These results and testing procedures are the evidence needed to justify freedom from Group 1 viroids in the propagated plants from Turkinje Nursery. These results also conclude all propagated plants in the hardening off phase identified by the codes in Table 4 have tested negative for ASBVd.

Table 4: Codes of Samples Tested and Found to be ASBVd Negative

Code
83. HASS TF21 KA13
84. HASS LVS21 KA13
85. HASS TF21 KA13
86. SHEP TF21 LE
87. HASS LVS21 LD2
88. HASS TF21 LD2
89. HASS TF21 KA13
90. HASS LC KA13
91. HASS ADC KA13

References

Geering ADW, Steele V, Kopittke R (2006) Final report for Horticulture Australia Limited Project AV03009: Development of Avocado sunblotch viroid indexing protocols for the avocado nursery industry.
van Brunschot, SL, Bergervoet, JH, Pagendam, DE, de Weerdt, M, Geering, AD, Drenth, A, & van der Vlugt, RA (2014) Development of a multiplexed bead-based suspension array for the detection and discrimination of pospiviroid plant pathogens. PloS one, *9*(1).
Zou Y, Mason MG, Wang Y, Wee E, Turni C, Blackall PJ, et al. (2017) Nucleic acid purification from plants, animals and microbes in under 30 seconds. PLoS Biol 15(11): e2003916.

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CREATE CHANGE

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CRICOS Provider Number 00025B

20 April 2022

Queensland Alliance for Agriculture and Food Innovation (QAAFI)

To whom it may concern

Status of avocado sunblotch viroid on the Atherton Tableland

I am responsible for avocado sunblotch viroid testing as part of the Avocado Nursery Voluntary Accreditation Scheme. I have also undertaken the most recent surveys (year 2021) of avocado orchards on the Atherton Tableland for sunblotch disease as part of Hort Innovation Project AV18007.

There have been no detections of avocado sunblotch viroid (ASBVd) on the Atherton Tableland, Queensland since 1990. Although there were 12 symptomatic trees identified in the period 1983–1990, these trees were destroyed immediately after identification (Geering, 2018).

Geering ADW (2018) A review of the status of Avocado sunblotch viroid in Australia. Australasian Plant Pathology 47 (6):555-559. doi:10.1007/s13313-018-0592-6.

Please do not hesitate to contact me again if you need further information.

Yours sincerely

Andrew Geering
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President of the Australasian Plant Pathology Society

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14 July 2022

TO WHOM IT MAY CONCERN

I am project leader of the national avocado sunblotch viroid (ASBVd) surveillance project (Hort Innovation Project AV18007) and also provide pathogen testing services as part of the Avocado Nursery Voluntary Accreditation Scheme. Recently I have reviewed all historical records of this viroid in Australia, and the results of this review have been published in the following journal article:

Geering ADW (2018) A review of the status of Avocado sunblotch viroid in Australia. *Australasian Plant Pathology* **47**:555-559. doi:10.1007/s13313-018-0592-6

There are no documented records of ASBVd in the Childers avocado production area in central Queensland and in surveys done by my team and university colleagues, symptoms of infection have never been observed.

If you need any more information on the status of ASBVd in Australia, please do not hesitate to contact me again.

Yours sincerely

Andrew Geering
Associate Professor
President of the Australasian Plant Pathology Society
Vice President-elect of the International Society of Plant Pathology

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