# Investigation of the distribution and incidence of Avocado sunblotch viroid in Australia

Dr Andrew Geering
Department of Employment, Economic Development & Innovation

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**AV07001**

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# Investigation of the distribution and incidence of *Avocado sunblotch viroid* in Australia

By Andrew D.W. Geering *et al*.

Queensland Department of Employment, Economic Development and Innovation

# HAL Project Number AV07001

**Project Leader:** Dr Andrew D.W. Geering
Level 2C, Ecosciences Precinct
41 Boggo Road, Dutton Park QLD 4102.

**Phone:** (02) 32554389

**Email:** a.geering@uq.edu.au

**Other Key Personnel:** Ms Vish Steele and Dr Paul Campbell
Level 2C, Ecosciences Precinct
41 Boggo Road, Dutton Park QLD 4102

**Purpose:** This report describes the results of surveys for *Avocado sunblotch viroid* in Australia.

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## *Disclaimer*

# Contents

## Media Summary

Sunblotch disease affects both the yield of avocado trees and also the quality of the fruit. Sunblotch disease is caused by the smallest pathogen known to science, *Avocado sunblotch viroid* (ASBVd). This pathogen is so small that it is invisible even under the most powerful microscope and can only be detected using biochemical methods. Fortunately, ASBVd is not very contagious and can easily be controlled by avocado growers using clean planting material. To address this need, the Avocado Nursery Voluntary Accreditation Scheme was initiated in 1978. Nurseries participating in ANVAS are obliged to have avocado nuclear stock tested for ASBVd on a regular basis.

Prior to the commencement of this project, there had been no official record of ASBVd in Australia since 1989. Questions therefore arose as to whether this pathogen still occurred in Australia and if so, how common was it and was it a significant constraint to production? To answer these questions, we have undertaken broad-scale surveys in the subtropical production areas of northern NSW and south-eastern Queensland. Before these surveys could be undertaken, we had to develop semi-automated testing procedures to allow large numbers of avocado leaves to be processed in a short period of time with minimum labour.

To maximize chances of finding ASBVd, older trees were targeted during the surveys. Over 3,000 trees were tested and only one infected tree discovered, a tree growing on Mt Tamborine. This tree was infected with a very mild strain of the pathogen. Despite comprehensive testing of other trees on this farm and elsewhere on Mt Tamborine, no other infections were discovered. It is likely that the tree was infected when it was first planted over twenty years ago and had remained undetected the whole time.

Finally, to allow survey data to be collated in a central location, a web-based database has been created. Using the website, avocado growers will be able to create confidential user accounts, fill out online diagnostic sample submission forms and have test results reported back to them as soon as they become available. There is potential to expand this web site for all diagnostic services.

## Technical Summary

*Avocado sunblotch viroid* (ASBVd) is a potentially damaging pathogen but one that is easily controlled through implementation of a clean planting material scheme. ASBVd has no known insect vector and is primarily spread through the inadvertent use of infected rootstocks or scions during grafting procedures in the nursery. In Australia, the Avocado Nursery Voluntary Accreditation Scheme (ANVAS) has been operational since 1978 with a goal of ensuring that only ASBVd-indexed planting material is distributed to growers. The last authenticated record of ASBVd in Australia was in 1989. This project sought to address the question of whether ASBVd was still present in Australia, and if so, how common a problem it was.

To enable large-scale surveys to be done, a high throughput method of processing samples was developed using a laboratory automation work station. A one-step real-time reverse transcriptase (RT) PCR method was also developed in the hope of reducing the labour input and time needed to complete the assay. However, this assay was inferior in sensitivity for detecting ASBVd when compared with a pre-existing two-step assay and therefore was not utilized during the surveys.

A total of 3,263 trees from South East Queensland and Northern NSW were tested for ASBVd, including trees from every commercial property on Mt Tamborine. Only one infected tree, located at Mt Tamborine, was discovered and this tree was asymptomatic. The strain of ASBVd infecting this tree was characterized and sequence variants were cloned that were identical to those isolated from an asymptomatic cv. Lima tree in the National Avocado Germplasm Repository in Florida, USA. The infected tree has now been destroyed.

To collate survey data and also data arising from ANVAS testing, a web-based database has been constructed. It is intended that this database be hosted on the Avocados Australia Limited web server. When operating, growers will be able to establish their own a private account, allowing them to submit sample details online. In turn, results from the laboratory can be uploaded into the database and linked to the sample submission details.

## Introduction

*Avocado sunblotch viroid* (ASBVd), type member of the family *Avsunviroidae*, causes a serious disease of avocados that is typified by discoloured (white, yellow or pink) and depressed stem streaks, grooves on the older branches and lesions on the fruit (Semancik 2003). Symptoms on the leaves are generally uncommon but in the early stages of infection, bleached or yellow areas may form around the leaf veins, which can progress to generalised variegation in the later stages of infection. The yield of infected trees has been estimated to be depreciated by 30% for cv. Fuerte with sunblotch symptoms (Semancik 2003). Further economic losses are incurred through the downgrading of fruit because of disfigurement and from quarantine barriers that prevent the trade of fruit between some countries.

ASBVd has no known vector but is transmitted at high rates through seed and infections are also perpetuated through the inadvertent use of infected scions for grafting (Semancik 2003). Mechanical transmission on contaminated pruning blades is also possible although probably inefficient. It therefore follows that the main method of control of ASBVd is through the use of clean planting material. To prevent dissemination of ASBVd in Australia, the Avocado Nursery Voluntary Accreditation Scheme (ANVAS) was introduced in 1978, which obliges participating nurseries to source propagation material from nuclear stock that is tested annually for ASBVd (Whiley 2000).

Before ASBVd was characterised in the early 1980s, the only viroid-indexing method was by grafting green bark pieces onto seedlings of susceptible cultivars such as Hass and then monitoring symptom expression for periods of up to 2 years (Dale and Allen 1979; Utermohlen and Ohr 1981). More recently, a range of molecular detection tests have been developed, each progressively more rapid and sensitive, including analysis of RNA patterns on a polyacrylamide gel (Dale and Allen 1979; Utermohlen and Ohr 1981), dot-blot hybridisation using a radiolabelled cDNA or RNA probe (Palukaitis *et al.* 1981; Rosner *et al.* 1983) and RT-PCR (Luttig and Manicom 1999; Randles *et al.* 2003; Schnell *et al.* 1997; Schnell *et al.* 2001b; Semancik and Szychowski 1994).

Historically, ASBVd has been recorded in the field in the Tristates area (Coomealla, Red Cliffs and Paringi) and there are also records from unspecified locations in Queensland and the Northern Territory (Allen and Dale 1981; Palukaitis *et al.* 1981; Rakowski and Symons 1989; Symons 1981; Trochoulias and Allen 1970). ASBVd is also known from germplasm collections at Alstonville Research Station in northern NSW and the CSIRO Division of Horticultural Research, Merbein, Victoria. Some of the cultivars that ASBVd has been found in include Hass, Fuerte, Zutano AV35, Bacon and Mexicola. Since 1978, avocado nuclear stock has been tested for ASBVd as part of the Avocado Nursery Voluntary Accreditation Scheme (ANVAS).

Circumstantial evidence suggests that ANVAS has been successful at controlling sunblotch disease as disease symptoms have not been observed by farmers for a very long time. The last authenticated record of ASBVd in Australia was in 1989 but there have also been no concerted efforts to look for the pathogen in subsequent years. According to the International Plant Protection Convention, the pathogen-free status of an area must be established and then maintained by actual surveying.

The major objective of this project was to survey for ASBVd in Australia to determine if area-freedom status is, in the short term, an achievable goal for the avocado industry or alternatively, provide data on its distribution and incidence so that the economic impact can be determined. To enable large-scale surveys, a high throughput diagnostic assay was developed.

## Materials & Methods

*Viroids and plants*

The ASBVd isolates used in this project are listed in Table 1.

**Table 1.** Origin of *Avocado sunblotch viroid* (ASBVd) isolates used in this study

| ASBVd isolate | Cultivar | Source | Type of material |
|---|---|---|---|
| SB | Hass | John Randles, Adelaide | Frozen leaf |
| Q1337 | Unknown | Riccardo Flores, Spain | Lyophilised leaf |
| Q1342 | Lima Late | Riccardo Flores, Spain | Lyophilised leaf |
| Q1344 | Wilson Popenoe | Riccardo Flores, Spain | Lyophilised leaf |

*Survey design and sampling procedures*

The formula of Cannon and Roe (1982) (Cannon and Roe 1982) was used to calculate the number of trees in each property that needed be tested to detect 1% infection with a confidence limit of 99% (Table 2). Single leaves were collected from eight different branches on each avocado tree and stored at 4°C until testing.

**Table 2.** Minimum number of trees and number of leaves to be sampled

| **Number of trees on each area/property** | **Minimum number of trees to be inspected and sampled in each area/property** | **Minimum number of leaf samples to be collected for pest detection in each zone (a minimum of 8 per tree)** |
|---|---|---|
| 400-500 | 414* | 3312 |
| 501-600 | 442 | 3536 |
| 601-1000 | 506 | 4048 |
| 1001-2000 | 564 | 4512 |
| 2001-3000 | 585 | 4680 |
| 3001-4000 | 596 | 4768 |
| 4001-5000 | 603 | 4824 |
| 5001-10000 | 617 | 4936 |
| >10000 | 631 | 5048 |

* if the number of trees for an area/property is >400 but <414, all trees should be sampled; a total of 3312 leaf samples must still be collected

*Extraction of RNA or total nucleic acids from avocado leaves*

As starting material, eight avocado leaves from each tree were stacked on top of one another and discs of tissue collected using a sterile, 4-mm diameter dermal biopsy punch (KAI Medical). These discs of tissue were then macerated in 300 µl of lysis buffer (either Chloropure or QIAGEN RLT buffer) by shaking with a single 5 mm diameter stainless steel ball on a TissueLyser (QIAGEN) at a frequency of 30 vibrations/sec. The tubes were then briefly centrifuged at 13,000 *g* to clarify the extract.

When small numbers of samples were processed, an RNeasy Mini Kit (QIAGEN) was used as per the manufacturer's instructions. When large numbers of samples were processed, a Chloropure Kit (Agencourt) was used as per the manufacturer's instructions for total nucleic acid (TNA) extractions (Protocol 001075v001) and all pipetting and magnetic bead manipulations done using a Biomek 3000 Laboratory Automation Workstation (Beckman-Coulter). TNA aliquots from 12 different trees were then pooled for cDNA production. If samples tested positive, the TNA extracts from each tree contributing to the pooled sample were then retested individually.

Prior to adopting the Agencourt Chloropure Kit for use, comparisons were made with a MagAttract Kit (QIAGEN). For these comparisons, 0.03 mg samples of freeze-dried ASBVd-infected leaf tissue (isolate 2116) and 30 mg samples of fresh, healthy avocado cv. Hass tissue were weighed out for use with each method. The leaf material was then macerated as described above using a TissueLyser (QIAGEN). When using the MagAttract Kit, 200 µl of homogenized lysate was transferred to a 1.2 ml deep well processing plate (Beckman Coulter) and the purification done as per the manufacturer's protocol, except that an Agencourt Supermagnet was used to immobilise the beads when wash solutions were being aspirated. All purification steps using both kits were done manually.

*Conventional RT-PCR*

Conventional RT-PCR was done using a OneStep RT-PCR kit (QIAGEN) as per the manufacturer's instructions and using the primer pairs ASB-F1/R1 and AVGAPDH-F1/R1 listed in Table 3. Thermocycling conditions were one cycle at 50°C/30 min and 95°C/15 min, 40 cycles at 94°C/15 s, 55°C/30 s and 72/30 s and finally one cycle at 72°C/5 min.

*One-step real-time RT-PCR*

For one-step real-time RT-PCR, a QIAGEN QuantiTect Virus + ROX Vial Kit was used. The final optimised reaction mix (15 µl total volume) contained 1 × QuantiTect Virus NR Master Mix, 0.4 µM each of ASBTM-F1 and -R1 primers, 0.2

µM of ASBTM probe, 0.2 µM each of GAPTM-F1 and -R1 primers, 0.1 µM of GAPDHTM probe and 0.25 µl of RT mix: all primer and probe sequences are listed in Table 3. Thermocycling conditions were one cycle at 50 °C for 15 min, one cycle at 95°C for 2 min and 45 cycles of 95°C for 25 sec and 60 °C for 30 sec.

All amplifications were done using a Rotor-gene 6 (version 6.0, build 27) from Corbett Research.

*Two-step real-time RT-PCR*

First strand cDNA was synthesised using the reverse primers ASBTM-R1 and GAPTM-R1 (Table 3) and Superscript III (Invitrogen) as per the manufacturer's instructions. The cDNA was diluted 1:10 in water for use as template in real-time PCR.

The final optimised reaction mix (20 µl total volume) contained 1 × Eppendorf Real Master Mix, 0.4 µM each of GAPTM-F1 and GAPTM-R1 primers, 0.4 µM each of ASBTM-F1 and ASBTM-R1 primers, 0.2 µM each of ASBTM-probe and GAPTM-probe, and 5 µl of cDNA. Primer and probe sequences are listed in Table 3. Thermocycling conditions were one cycle at 95°C for 2 min, followed by 45 cycles at 95°C for 15 s and 60°C for 60 s.

All amplifications were done using a Rotor-gene 6 (version 6.0, build 27) from Corbett Research.

**Table 3.** Primer and probe sequences used in conventional and real-time RT-PCRs

| Target | Primer name | Primer sequence (5'→ 3') | Reference |
|---|---|---|---|
| ASBVd | ASB-F1 | GTGAGAGAAGGAGGAGT | Schnell *et al*. (1997) |
| | ASB-R1 | AAGTCGAAACTCAGAGTCGG | |
| | ASBTM-F1 | TTCCGACTCTGAGTTTCGACTT | unpubl. |
| | ASBTM-R1 | GTTCTTCCCATCTTTCCCTGA | |
| | ASBTM-probe | 6FAM-TGAGAGAAGGAGGAGTCGT-MGBNFQ | |
| GAPDH | AVGAPDH-F1 | TGCTGTCTTTGGTCTCAGGA | unpubl. |
| | AVGAPDH-R1 | GGCAGAACTTTTCCAACAGC | unpubl. |
| | GAPTM-F1 | TGGAGTGGACAGTGGTCATCAG | unpubl. |
| | GAPTM-R1 | GGCCAAGGTGATCAATGATAAATT | unpubl. |
| | GAPDHTM-probe | VIC-CCCTCAACAATGCC-MGBNFQ | unpubl. |

*Development of an ASBVd test result database*

The backend of a web-based database was written in MySQL (open source common web database language) and the web front end written in PHP (open source common web language).

## Results

*Development of a one-step real-time reverse transcriptase- (RT-) PCR for ASBVd and comparison with the two-step real-time RT-PCR*

A QIAGEN QuantiTect Virus + ROX Vial Kit was compared to an Invitrogen RNA UltraSense™ One-Step qRT-PCR Kit using the same RNA template and various concentrations of ASBVd primer and probe. The QIAGEN kit provided the greatest sensitivity of detection and reproducibility of results (data not shown) and therefore was selected as the kit of choice for development of a duplex real-time one-step RT-PCR. ASBVd and GAPDH primer and probe concentrations were then optimised using this kit (Table 4) and a final protocol developed, in which ASBVd primers and probe were used at the manufacturer recommended rate (0.2 and 0.4 μM final concentration of probe and of each primer, respectively) but those for GAPDH were used at half these rates.

**Table 4.** Effect of primer and probe concentration on the sensitivity of the one-step real-time reverse transcriptase PCR

| GAPDH[1] primer/probe conc. (μM) | $C_t$ (ASBVd) | $C_t$ (GAPDH) | ASBVd[2] primer/probe conc. (μM) | $C_t$ (ASBVd) | $C_t$ (GAPDH) |
|---|---|---|---|---|---|
| 0.8/0.4 | 23.5 ± 0.0882 | 32.5 ± 1.05 | 0.8/0.4 | 22.2 ± 0.0987 | 27.6 ± 0.233 |
| 0.4/0.2 | 22.3 ± 0.273 | 27.5 ± 0.349 | 0.4/0.2 | 18.1 ± 0.105 | 23.8 ± 0.0742 |
| 0.2/0.1 | 22.3 ± 0.145 | 26.5 ± 0.258 | 0.2/0.1 | 18.0 ± 0.158 | 24.3 ± 0.0240 |
| 0.1/0.05 | 21.3 ± 0.100 | 25.0 ± 0.240 | 0.1/0.05 | 17.7 ± 0.195 | 24.3 ± 0.172 |
| 0/0 | 22.1 ± 0.265 | Negative | 0/0 | Negative | 24.6 ± 0.234 |

The one-step and two-step real-time RT-PCRs were then compared for sensitivity of detection, starting with the same amount of leaf material. Unusually, the two-step assay provided superior sensitivity (lower $C_t$ values) over the one-step assay for ASBVd but inferior sensitivity for GAPDH (Table 5). As the primary purpose of the duplex assay was to detect ASBVd, the two-step assay was therefore chosen for use during the area-freedom surveys.

**Table 5.** Comparison of one- and two-step real-time RT-PCRs

| Sample | ASBVd assay ($C_t$ value) | | GAPDH assay ($C_t$ value) | |
|---|---|---|---|---|
| | One-step | Two-step | One-step | Two-step |
| SB | [1]16.7 ± 0.0967 | 12.7 ± 0.125 | 22.5 ± 0.0751 | 28.3 ± 0.312 |
| Q1337 | 21.2 ± 0.148 | 18.5 ± 0.373 | 27.2 ± 0.240 | 29.8 ± 0.263 |
| Q1342 | 19.1 ± 0.0961 | 14.0 ± 0.451 | 24.5 ± 0.0612 | 28.3 ± 0.188 |
| Q1344 | 19.4 ± 0.0285 | 16.9 ± 0.488 | 25.2 ± 0.0410 | 28.5 ± 0.351 |
| Healthy | Negative | Negative | 27.5 ± 0.309 | 29.3 ± 0.136 |
| Buffer | Negative | Negative | Negative | Negative |

[1]Mean and standard error of three replicates.

*Comparison of magnetic bead-based RNA extraction methods*

In order to reduce the labour input involved in processing samples during surveys, a method of RNA extraction was developed using a Biomek 3000 Laboratory Automation Workstation (Beckman-Coulter). Magnetic-bead based RNA extraction methods are designed for use on this type of equipment, and two commercially available kits, a Chloropure Kit (Agencourt) and a MagAttract Kit (QIAGEN), were compared. These comparisons were done using the same amount of starting material and the one-step real-time RT-PCR. Although there was no clear trend, the Chloropure Kit generally provided greater assay sensitivity than the MagAttract Kit (Table 6) and therefore was chosen for use during the surveys.

**Table 6.** Comparison of magnetic bead-based RNA extraction methods

| Sample | RNA dilution | Method of RNA preparation | $C_t$ value (ASBVd) | $C_t$ value (GAPDH) |
|---|---|---|---|---|
| ASBVd | 1/5 | Chloropure | 17.6 ± 0.235 | 25.103 ± 0.197 |
| | | MagAttract | 18.5 ± 0.227 | 22.7 ± 0.135 |
| | 1/10 | Chloropure | 17.7 ± 0.182 | 24.950 ± 0.261 |
| | | MagAttract | 20.5 ± 0.312 | 24.4 ± 0.218 |
| | 1/50 | Chloropure | 17.8 ± 0.210 | 25.0 ± 0.0578 |
| | | MagAttract | 22.0 ± 0.172 | 25.6 ± 0.119 |
| Healthy | 1/5 | Chloropure | Negative | 26.8 ± 0.100 |
| | | MagAttract | Negative | 23.2 ± 1.233 |

*Surveys for ASBVd*

During the period from June to August 2008, four avocado orchards at Mt Tamborine and Woombye in south-east Queensland and Duranbah in north-east NSW were surveyed for ASBVd (Table 7). These orchards were chosen as the trees were at least 20 years old and it was considered that the chance they would be infected with ASBVd would be greater than for younger trees. During these surveys, 2151 trees out of a total population of *c*. 4,450 were sampled and tested for ASBVd by real-time PCR. All trees tested negative for ASBVd except for one tree at Mt Tamborine, which was symptomless but nevertheless tested strongly positive by real-time RT-PCR.

The ASBVd-infected tree at Mt Tamborine that was identified during the surveys was retested on four more occasions by real-time RT-PCR (July and August 2008 and March and May 2009) and on all occasions, gave strongly positive values (Ct values ranging from 9.96-18.89). In the July survey, the tree was divided into four quadrants (upper and lower, left and right) and single leaves from two branches in each quadrant tested. The distribution of ASBVd around the tree was very uniform, with mean, maximum and minimum $C_t$ values of 11.74, 13.36 and 10.03, respectively. Twenty of the nearest neighbours of this infected tree were retested in May 2010 and all were negative for ASBVd.

The discovery of the ASBVd-infected tree at Mt Tamborine led to a change in milestones for the second year of the project, where instead of national surveys being undertaken, more comprehensive surveys of Mt Tamborine were done. In surveys of all remaining commercial properties on Mt Tamborine between November 2009 and March 2010, no more ASBVd-infected trees were identified (Table 7).

The sole infected tree on Mt Tamborine was cut down and the stump removed in July 2010.

**Table 7.** Surveys for *Avocado sunblotch viroid*

| Property | Date | Location | Cultivars | Total tree population | No. trees tested | No. positives |
|---|---|---|---|---|---|---|
| 1 | Jun-Aug. 2008 | Mt Tamborine, QLD | [1]Fuerte, Hass | *c*. 700 | 513 | 1 |
| 2 | | Duranbah, NSW | [2]Fuerte, Hass | *c*. 2000 | 564 | 0 |
| 3 | | Mt Tamborine, QLD | [1]Hass, Hazard, Reed, Sharwil | *c*. 1000 | 510 | 0 |
| 4 | | Woombye, QLD | [1]Fuerte, Hass, Pinkerton, Reed, Sharwil | *c*. 750 | 511 | 0 |
| 5 | Nov. 2009 | Mt Tamborine, QLD | Fuerte, Hass | 83 | 83 | 0 |
| 6 | Dec. 2009 | Mt Tamborine, QLD | Fuerte, Hass | 246 | 246 | 0 |
| 7 | Feb. 2010 | Mt Tamborine, QLD | Fuerte, Hass | 368 | 368 | 0 |
| 8 | | Mt Tamborine, QLD | Fuerte, Hass | 248 | 248 | 0 |
| 9 | Mar. 2010 | Mt Tamborine, QLD | Fuerte, Hass | 73 | 73 | 0 |
| 10 | | Mt Tamborine, QLD | Fuerte, Hass | 94 | 94 | 0 |
| **Totals** | | | | **6812** | **3263** | **1** |

[1]All trees >20 years old. [2]1915 trees *c*. 30 years old, 25 trees *c*. 50 years old.

*Characterisation of the ASBVd isolate from Mt Tamborine*

To identify the strain of ASBVd infecting the tree, a conventional RT-PCR was done and the amplicon cloned and sequenced. Four PCR clones representing the entire genome of the viroid were sequenced, of which three differed from each other. Sequence variant 1 (SV1, GenBank accession HQ700589) and SV2 (GenBank accession HQ700590) were identical in sequence to symptomless carrier isolates from cultivar Lima Late in the avocado germplasm collection at the National Germplasm Repository located in Miami, Florida (isolates CF21 [GenBank accession AF404035] and CF24 [GenBank accession AF404038], respectively). SV3 (GenBank accession HQ700591) was unique, although it was at least 98.7% identical to the two other two sequence variants obtained from the tree. When compared with SV1 and 2, SV3 had an additional A in the Right Terminal Loop (between positions 122 and 128) and one substitution in the stem area (U→C at position 145).

*Development of an electronic survey tool*

A web-based database has been constructed to house ASBVd-indexing results obtained from the Avocado Nursery Voluntary Accreditation Scheme (ANVAS), from area-freedom surveys such as those done in this project, and from general pathology enquiries from growers (Figs 1-3). Information can be entered into the database through a web page by any grower and by any diagnostic laboratory (not just the DEEDI plant health laboratory). It is anticipated that the back-end database will be located on the Avocados Australia Limited computer server. The website also provides fact sheets on avocado sunblotch disease and advice on how to submit samples for testing.

In the normal course of operation, a grower or nurseryman would complete a sample submission form online and the data would automatically be captured in the database. After clicking the “submit” button, the sample information is automatically entered into the database and a pdf file of the submission data is produced, which can be printed off to include in the postal package with the samples. Sample forms can also be downloaded and manually completed if so desired. The status of samples submitted through the web database can be tracked by the person submitting the samples, and pdfs of the results report downloaded. In the normal course of operation, a grower would create their own account, which would give them access to their own submission data and test results, but not that of anyone else.

The diagnostics laboratory will be able to read all sample submissions and enter test results when they become available, which will be viewable via the web or be able to be saved as a pdf file that can be emailed or posted to the relevant grower. Additionally, raw data files can also be uploaded into the database but these will not be viewable by the grower. The ASBVd-indexing database is integrated with the DEEDI diagnostics tracking database, and it will be possible to automatically update each database as new data is entered.

A separate administration area gives access to the real-time data files, and the ability to add submissions, reports and data-files. The data collected during the course

of this project has been entered into the database, result files generated and attached, and the sample definitions in the real-time RT-PCR data files relabeled to match the information in the database.

Currently the web pages are compatible with Firefox, Chrome, Safari and Internet Explorer version 7 and above. There are styling problems with Internet Explorer version 6 due to the lack of adherence of this web browser to web standards but these problems do not affect functionality, just appearance.

**Fig. 1.** Home screen of database

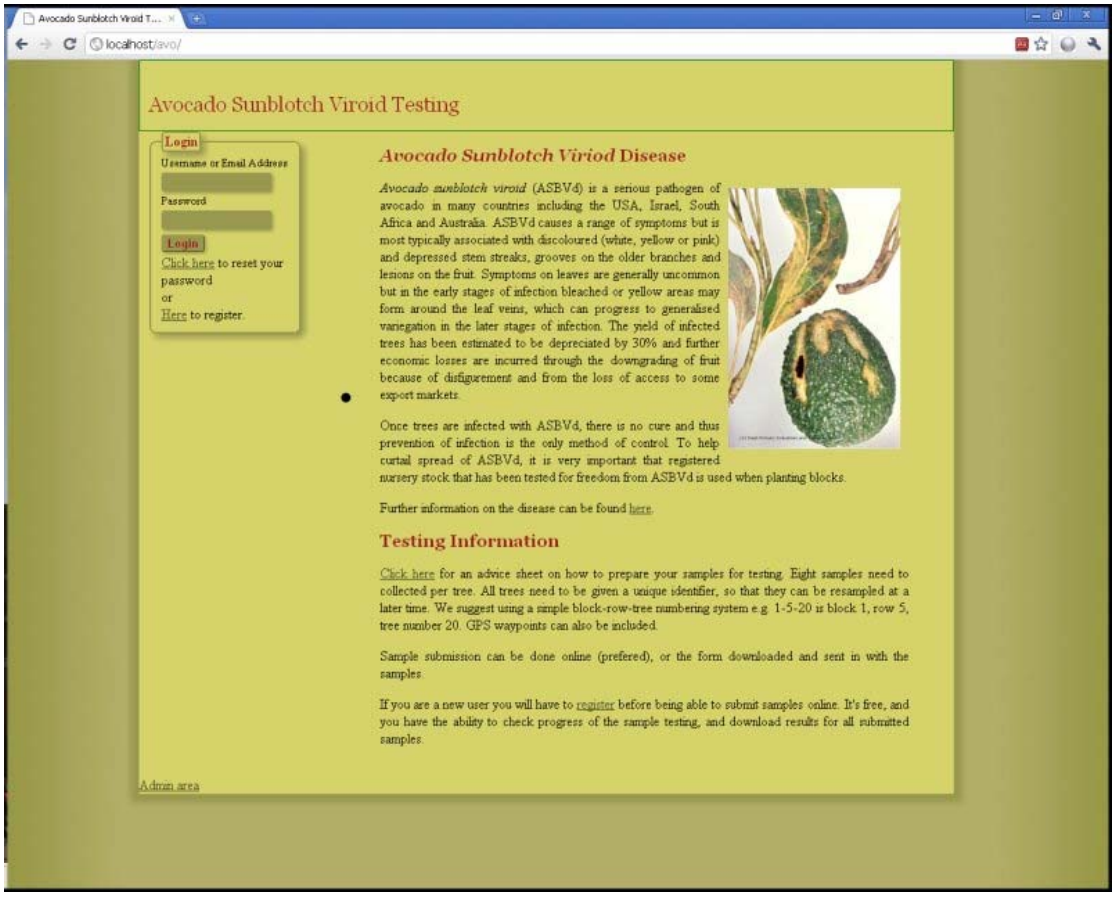



**Fig. 2.** Sample submission screen of database.

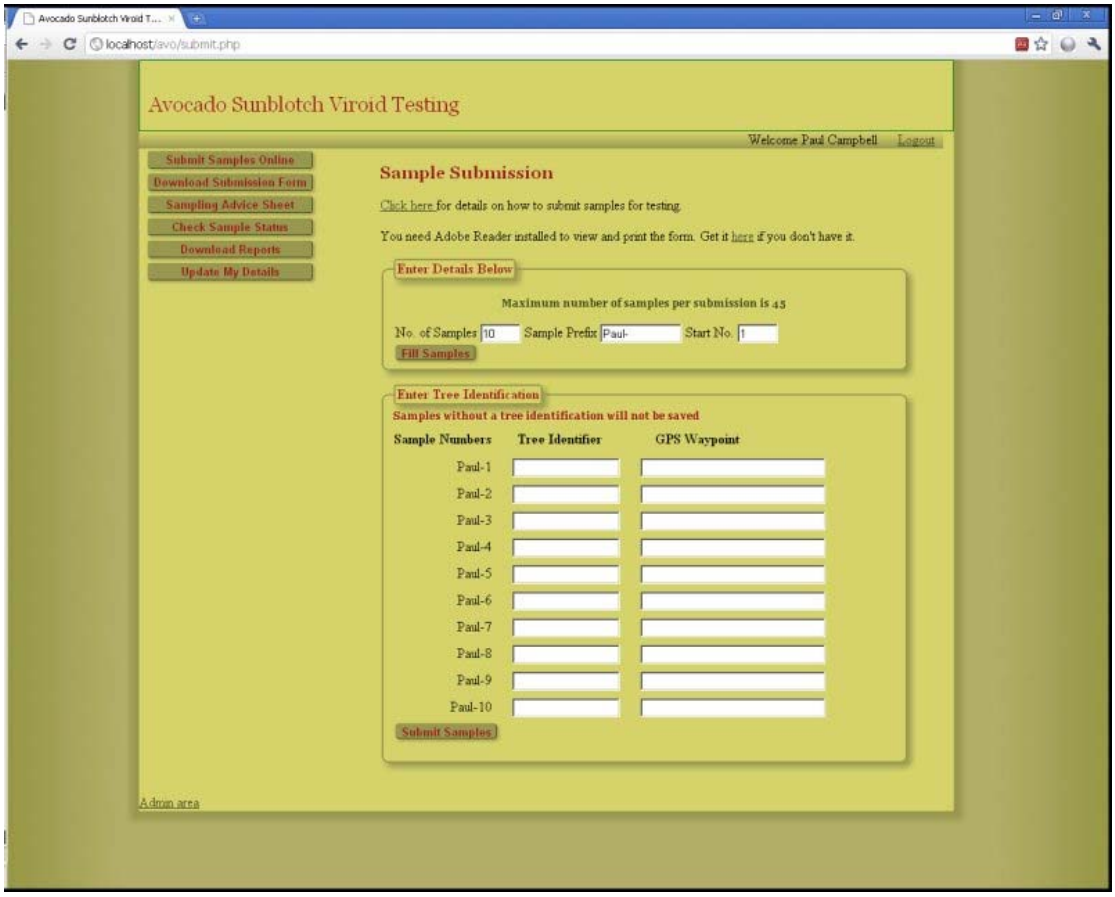
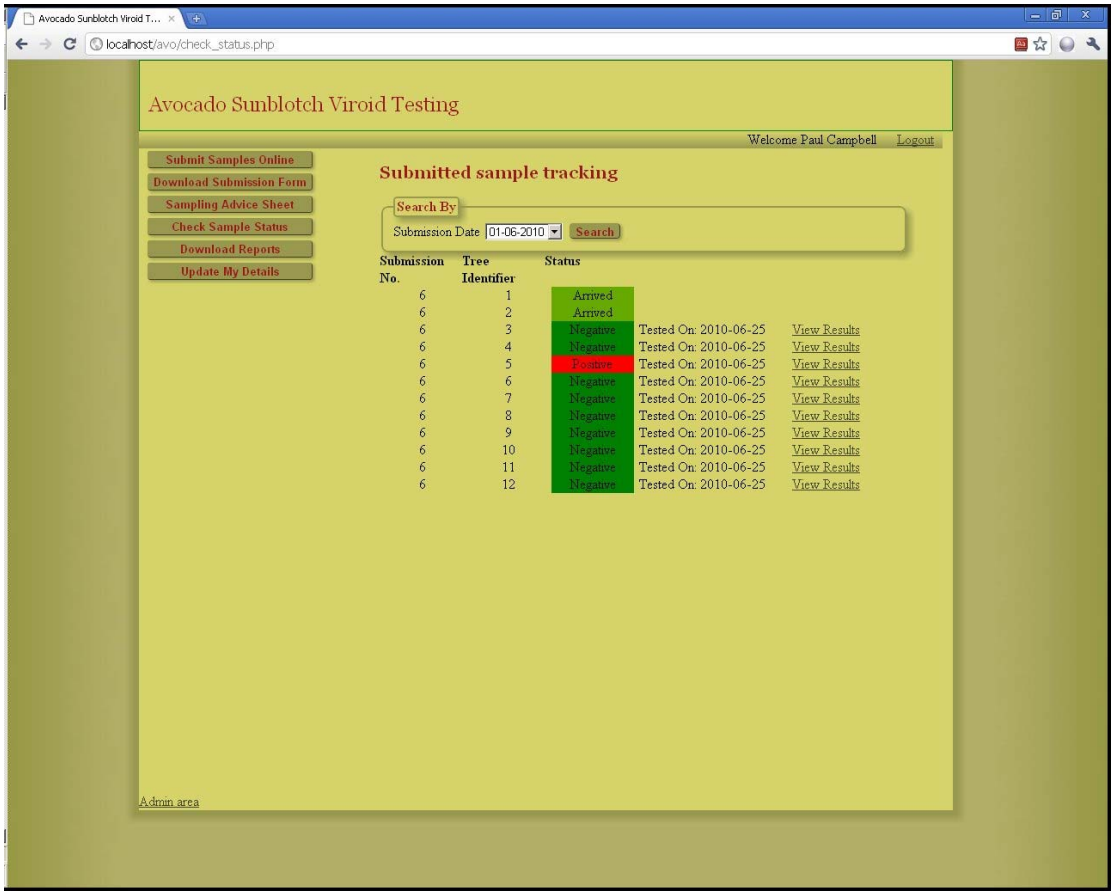

Avocado Sunblotch Viroid T...
localhost/avo/submit.php
Avocado Sunblotch Viroid Testing
Welcome Paul Campbell Logout
Submit Samples Online
Download Submission Form
Sampling Advice Sheet
Check Sample Status
Download Reports
Update My Details
Sample Submission
Click here for details on how to submit samples for testing.
You need Adobe Reader installed to view and print the form. Get it here if you don't have it.
Enter Details Below
Maximum number of samples per submission is 45
No. of Samples 10 Sample Prefix Paul- Start No. 1
Fill Samples
Enter Tree Identification
Samples without a tree identification will not be saved
Sample Numbers
Tree Identifier
GPS Waypoint
Paul-1
Paul-2
Paul-3
Paul-4
Paul-5
Paul-6
Paul-7
Paul-8
Paul-9
Paul-10
Submit Samples
Admin area

**Fig. 3.** Sample tracking screen of database.



## Discussion

The two principal objectives of this project were to develop a high-throughput diagnostic assay for ASBVd and to then use this assay in surveys to determine the incidence of ASBVd in Australia. Initially, the surveys were to be nationwide but the discovery of an infected plant at Mt. Tamborine led to a request by the Avocado Industry Advisory Committee to broaden the surveys at Mt Tamborine.

A high throughput diagnostic assay for ASBVd using a laboratory automation workstation and a magnetic-bead based RNA extraction kit was successfully developed. Using this diagnostic assay, more than 3,000 trees were tested. The rate-limiting step in the surveys was sample collection and not testing. Although it would be feasible to expand the surveys to all of Australia, extra assistance would be needed to collect the samples and probably this could be done most efficiently and cost-effectively by eliciting the help of the growers for this task.

During surveys of Mt Tamborine, an asymptomatic ASBVd-infected tree was identified. This tree was strongly positive and the pathogen could easily be detected, no matter where on the tree the leaf samples were taken and in what month of the year the tree was sampled. This result is consistent with previous studies that have shown that asymptomatic strains are uniformly distributed around the tree (Semancik 2003). Two of the sequence variants isolated from this tree were identical to those isolated from an asymptomatic cv. Lima plant in the USDA germplasm collection in Florida, and the third sequence variant was near-identical. All three sequence variants did not have the extra U or UU between bases 115 and 118 that typifies bleaching strains of the viroid (Schnell *et al.* 2001a).

No other ASBVd-infected trees were found on either the original or other commercial properties on Mt Tamborine. The avocado orchards on Mt Tamborine are isolated from other production areas by distances of 50 km or more and the most plausible explanation for how this tree became infected is through infection pathways in the nursery (an infected scion or rootstock used for grafting) from where the tree originated. The tree in question was at least 20 years of age and the fact that neighbouring trees in the same block and also in all of Mt Tamborine were negative for ASBVd suggests that secondary spread of this pathogen in the field is negligible. This result has implications for how pest-free production areas are defined for ASBVd.

International Standards for Phytosanitary Measures (ISPM) No. 10: *Requirements for the establishment of Pest free places of production and Pest free production sites* (1999) states that a pest-free production area can be any premises or collection of fields operated as a single production unit. Thus, an individual farm could be considered a pest-free production area. ISPM no. 10 further states that "Where the biology of the pest is such that it is likely to enter the place of production or production site from adjacent areas, it is necessary to define a buffer zone around the place of production or production site within which appropriate phytosanitary measures are applied. The extent of the buffer zone and the nature of the phytosanitary measures will depend on the biology of the pest and the intrinsic characteristics of the place of production or production site." ASBVd is so poorly

transmissible in the field that a fence line dividing two properties would be sufficient buffer zone to divide a pest-free production area from an unregistered area.

Methods developed during the course of this project could be adapted for a range of avocado pathogens and for incursion responses where pathogen delimitation surveys are needed. Once nucleic acid extracts have been prepared, a range of PCR assays could be done, which would be relatively generic in nature, the major differences being in the sequences of the primers and probes. The web-based database we have created could also be expanded to capture information on all types of pathogens. Claims of area-freedom do need to be supported by data, and one of the greatest challenges in providing this data is collation, especially when work has been done by independent research groups or laboratories.

## Technology Transfer

To encourage the self-reporting of ASBVd, a free ASBVd-testing service for a maximum of five plants was provided to any farmer in Australia until 30 June 2009. To promote this offer, information on disease symptoms and how to submit samples was published in *Talking Avocados* (see Appendix 1). A follow-up circulation of this extension article was done by email or by hard copy to 'Regional Study Groups' as part of the related HAL project led by Simon Newett.

Upon completion of surveys at each property, the results were directly reported back to the growers by phone and by letter. When the ASBVd-infected tree was discovered at Mt Tamborine, a request was received from the Avocado Industry Advisory Committee to negotiate removal of this tree and this was done with the assistance of Avocados Australia Limited (AAL). The cost of removal of the tree was borne by AAL and limited compensation provided (in the form of replacement trees) to the grower.

Results from this project were presented in seminars at:

(i) the 4th Australian and New Zealand Avocado Growers Conference in Cairns 21-24 July 2009.
(ii) the Avocado R&D Workshop in Canberra on 24 June 2010.

Survey data collected in this project, including the raw data files, have been loaded onto the web-based database created during this project. This database will be given to AAL, enabling this organisation to interrogate the database and generate reports on a needs basis. The database will also be able to be used for future operation of ANVAS.

A research paper entitled "New RT-PCR assays for *Avocado sunblotch viroid* and utilisation in broad scale surveys" is in an advanced stage of preparation for publication in the international journal *Plant Disease*.

## Recommendations

The recommendations from this project are:

(1) It is not yet possible to conclude that ASBVd has been eradicated from Australia, although the results from this project have demonstrated that this pathogen is very rare and under active control. Within a matter of 20-30 years, ASBVd will disappear as old trees die and are replaced by pathogen-free planting material from ANVAS-accredited nurseries. Under the International Plant Protection Convention, ASBVd would be considered a regulated, non-quarantine pest and thus Australia could impose phytosanitary measures on avocado fruit imports to reduce the pest risk to an acceptable level (ISPM No. 2 – 2007). If the pest risk was considered unacceptable and there were no measures available to mitigate the risk, then the imports could be prohibited. At the other end of the spectrum, if the pest risk was considered negligible, the import may be permitted with few if any phytosanitary measures.

(2) It would not be cost-effective to survey all commercial orchards in Australia for ASBVd and impractical to survey backyard plants. Blocks planted with avocado plants produced by ANVAS-accredited nurseries should be regarded as ASBVd-free production areas and freedom maintenance surveys would not be needed as long as rules were adhered to such as prohibiting the movement of pruning equipment between non-accredited and accredited blocks. Even if a block had not been planted with ANVAS-accredited nursery plants, it would be possible to test the block post-planting on a fee for service basis if a plant health certificate was needed by the farmer for the purpose of exports. The major limiting factor to doing such surveys would be sampling of the trees and this task would need to be done by the avocado grower.

(3) Testing procedures for ASBVd are now very sensitive and reliable. There are, however, weaknesses in the ANVAS scheme, as there is no ‘paper trail’ to link diagnostic results with plants in the nursery. Samples that are received for testing as part of the ANVAS scheme are often inadequately or unclearly labelled and after a year or so, it would be difficult to cross-reference test results to a particular nursery plant. Improvements to the scheme could be gained by adopting some of the same processes used in the QBAN banana nursery accreditation scheme. Furthermore, use of the web-based database developed in this project for ANVAS will force stakeholders to standardise procedures. For example, use of the online sample submission form will ensure a consistency in the information collected. Information held in the database will also be more easily retrievable if there was a need to query it. The database would also provide a central storage location if more than laboratory was to become accredited for ASBVd testing (e.g. a Western Australia laboratory). Finally, if there was a need to demonstrate active surveillance for ASBVd for biosecurity purposes, then a report showing recent activities could easily be generated.

(4) The web-based database could be enlarged to become a central portal for all diagnostics work done for the avocado industry. Other features could also be added to the database including:

(i) credit card charging facilities

(ii) email notification capabilities e.g. notification when samples were submitted or received, when tests were completed and when routine testing was required to fulfil the requirements of ANVAS.

(iii) better reporting capabilities e.g. generation of reports of testing done for ANVAS within a specified time period.

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# Appendix 1: Sunblotch disease – past, present or never a problem?

By Andrew Geering and Vish Steele
Queensland Department of Primary Industries and Fisheries

The avocado industry has the dubious honour of having the smallest pathogen of any plant, animal, insect or other living organism in the world. This pathogen is *Avocado sunblotch viroid* (ASBVd), which at 247 nucleotides long, has about one ten millionth of the genetic content of humans. ASBVd is so small that it cannot even be seen using the most powerful microscope and therefore molecular detection assays must be used to reveal its presence in a tree. However, the disease caused by ASBVd, sunblotch disease, is much more obvious and reason for concern.

ASBVd causes a range of disease symptoms but is most typically associated with discoloured (white, yellow or pink) and depressed stem streaks, grooves on the older branches and lesions on the fruit. Symptoms on leaves are generally uncommon but in the early stages of infection, bleached or yellow areas may form around the leaf veins, which can progress to generalised variegation in the later stages of infection. The yield of infected trees has been estimated to be depreciated by 30% and further economic losses are incurred through the downgrading of fruit because of disfigurement and quarantine barriers imposed on fruit bound for some export markets.

Avocado trees may be symptomless carriers of ASBVd. Over time, trees with acute symptoms may recover and conversely, symptomless trees may develop typical symptoms following severe pruning or stumping. Seedlings from trees with typical symptoms may also be symptomless carriers of the pathogen. This reversion in symptoms appears to be related to minor changes in the genetic make-up of ASBVd – different strains of ASBVd may be present in different branches. Even in the absence of foliar symptoms, yield loss from ASBVd may still be significant.

The principal way that ASBVd moves around is in contaminated nursery plants. Once introduced onto a property, ASBVd can then be transmitted from tree to tree on the blades of pruning tools and probably also by natural grafting of the roots of neighbouring trees but if the block is clean at planting, then it is likely to remain this way for the duration of its life. Importantly, ASBVd is not transmitted by insects. For those unlucky enough to have trees affected by ASBVd, there is no cure and the only option is to destroy the diseased trees to prevent further spread.

To combat ASBVd and also *Phytophthora cinnamomi*, the Avocado Nursery Voluntary Accreditation Scheme (ANVAS) was introduced in 1978. ANVAS obliges participating nurseries to implement strict hygiene practices and to test nuclear stock for ASBVd on a regular basis. Judging by the infrequency at which ASBVd is now reported, it is likely that ANVAS has been successful in progressively eradicating ASBVd from Australia but claims of area-freedom need to be evidence-based. The last published report of ASBVd was in 1989. However, this was also the last time that anything approaching an industry-wide survey was done.

To try and provide data on the incidence and distribution of ASBVd in Australia, we are undertaking surveys as part of a small project commissioned by

Horticulture Australia Limited on behalf of the avocado industry. As one can imagine, looking for ASBVd is like looking for a needle in a haystack. Consequently, help is needed to find ASBVd as no one knows their avocado trees better than the farmer! If you suspect a tree to have sunblotch symptoms, we would be grateful to receive leaf samples for testing (see accompanying instructions on how to send a sample). Testing of a small number of trees is free and results will be kept confidential unless consent is given to publish the results.

Symptoms of *Avocado sunblotch viroid*. Photograph courtesy of the Department of Primary Industries and Fisheries, Queensland.


## Information on the whereabouts of *Avocado sunblotch viroid*

**Free testing** of a maximum of 5 trees per property
(until 30 June 2009)

To take up this offer:

1. Collect a single leaf from eight different branches.
2. Wrap shoots in moistened newspaper or paper towelling and seal in a plastic bag. Separately package leaves from different trees. Keep leaves out of sun.
3. On a piece of paper, provide
    a. Your name and contact details.
    b. Location of the tree (property and block or GPS coordinates).
    c. Cultivar and approximate age of tree.
    d. Symptoms.
4. Send samples by express post to :

Ms Vish Steele, Department of Primary Industries and Fisheries, 80 Meiers Road, Indooroopilly QLD 4068.

5. Test results will be kept confidential unless consent is provided to release the results.