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Virology

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

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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. As with 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 behavior of honey bees can provide a complementary strategy to 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.

Keywords: biosecurity, eDNA, high-throughput sequencing, plant viruses, pollen

Avocado sunblotch viroid (ASBVd; genus *Avsunviroid*) has a circular, single-stranded RNA genome of 238 to 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 have 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 infected trees do not show obvious leaf or developmental alterations, 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 to 100% have been observed in seed from asymptomatic carrier trees, but the rates are about 20-fold lower in seed from symptomatic trees (Wallace and Drake 1962). Pollen transmission does occur, but this results in infection of only the seed (1 to 4% infection) but not the pollen-recipient tree (Desjardins et al. 1979, 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 (Geering 2018). A similar certification scheme, the Avocado Plant Improvement Scheme, managed jointly by the South African Avocado Growers' Association and the Avocado Nurserymen's Association, 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 favor 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

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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 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 and are even more difficult to find when trees are not bearing fruit. An avocado tree can reach a height of 5 to 18 m at maturity, making it difficult to thoroughly inspect a tree for symptoms and collect leaves for laboratory testing. Finally, it is common 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 honey bee (*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 underutilized (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 are 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%.

Fig. 1. Hive locations in relation to four avocado sunblotch viroid-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.

Materials and Methods

Field collection of hive pollen and bees—Queensland, Australia

Samples of stored pollen and adult worker bees were collected from managed 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, 200, 300, and 400 m from a group of four ASBVd-infected trees (Fig. 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 (Fig. 2). 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.

Field collection of hive pollen and bees—South Africa

Samples of stored pollen and adult worker bees were collected in October 2020 from managed beehives 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 26 and 179 m (Fig. 3). In October 2021, single hives were placed under 10 infected trees at one orchard (the same orchard as SA1-SA3 in 2020) (Fig. 4) and sampled for bees and pollen as previously. 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.

RNA extraction

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

Fig. 2. Collection of pollen from beehives for avocado sunblotch viroid detection.

For HTS, 50 QLD bees per hive were macerated using a Stomacher 80 (Seward, U.K.) and extraction bag in 10 ml of 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, U.S.A.).

Individual foragers were added to a 2-ml Beadbug tube (Benchmark Scientific, U.S.A.) with 1.5-mm zirconium beads and 500 µl of homogenization buffer (Promega). Foragers with pollen loads were first separated with tweezers, and the pollen loads/hind legs were placed in separate tubes. Foragers and pollen loads were macerated in a FastPrep instrument for 45 s at 6 m/s, then centrifuged (22,000 × *g*, 2 min) before using 350 µl of supernatant for RNA extractions with the Maxwell RSC simplyRNA Tissue Kit (Promega).

Pollen from each hive was first mixed in 5 ml of 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 of homogenization buffer (Promega) was added and transferred to a 2-ml Beadbug tube (Benchmark Scientific) with 1.5-mm zirconium beads and

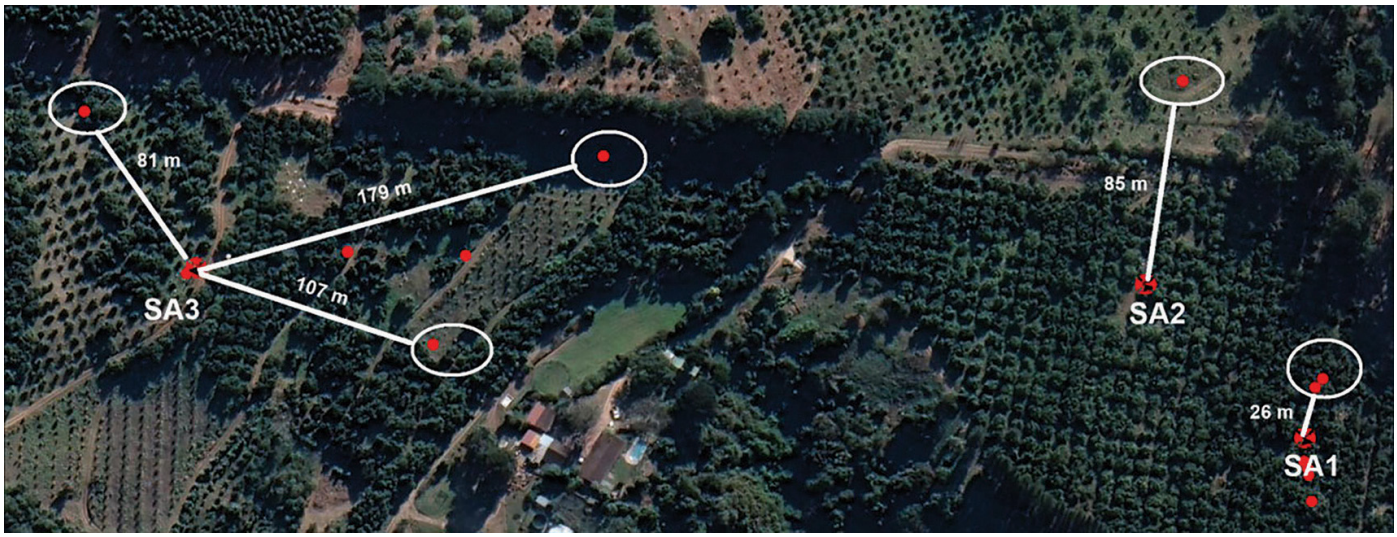

Fig. 3. Three hive positions in relation to infected trees (red dots) in 2020 from a South African avocado orchard.

Fig. 4. The spatial position of positive trees (red dots) in relation to beehives that were placed underneath infected trees (yellow dots) in 2021. Avocado sunblotch viroid-infected trees in the neighboring farm indicated with blue dots.

macerated in a FastPrep instrument for 45 s at 6 m/s twice. Samples were centrifuged at 22,000 × *g* for 2 min, then 350 µl of supernatant was used for RNA extraction with the Maxwell RSC simplyRNA Tissue Kit (Promega).

South African (SA) 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 RT-PCR and HTS detection for ASBVd

Bee and pollen samples were 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 kit (PCR Biosystems, U.K.) and primers (5′-AGAGAAGGAGGAGTCGTG GTGAAC-3′; 5′-TTCCCATCTTTCCCTGAAGAGAC-3′) to amplify a 99-bp fragment using a Rotor-Gene Q instrument (QIAGEN, Germany). C_T values were analyzed 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 (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.

HTS analysis

Sequence analyses were carried out with CLC Genomics Workbench v20 (CLC Bio, Denmark) with raw data first quality trimmed and adapter sequences removed. Small RNA sequencing data were also size selected for 21 to 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., New Zealand). Raw HTS data and annotated virus sequences were deposited in GenBank (PRJNA868881).

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 nt 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.

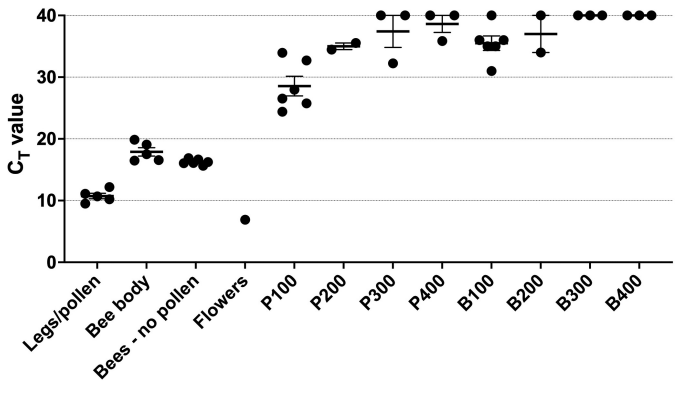

Fig. 5. Detection of avocado sunblotch viroid by real-time RT-PCR from foraging bees and pollination hives in an Australian avocado orchard. Samples with no amplification were given a value of $C_T = 40$. Bees with pollen loads were dissected to test the legs/pollen loads separately to the bee body. Pollen (P) and bees (B) were collected from hives distanced at 100, 200, 300, and 400 m from four infected trees.

Results

ASBVd bee surveillance—QLD orchard

The QLD orchard in which the study was undertaken only contained four ASBVd-infected trees, all near neighbors 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 (Fig. 5). ASBVd levels in the pollen loads of foraging bees were significantly higher than levels in bee bodies ($P = 0.003$) and were similarly as high as levels in the flowers ($P = 0.952$). Detection of ASBVd was much lower in pollination hives (Fig. 4). Only pollen from hives within 100 m of infected trees yielded a positive detection of ASBVd below $C_T = 30$ and was significant compared with hives at farther distances ($P < 0.05$).

ASBVd bee surveillance—SA orchards

ASBVd was consistently detected in pollen and bees from pollination hives at four SA 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 (Fig. 6). The prevalence of ASBVd-infected trees at SA4 was unknown but was presumed to be 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 at other sites ($P = 0.0112$ to 0.0001), reflecting the higher prevalence of ASBVd-infected trees within 100 to 200 m of these hives (Fig. 3).

Pollination hives positioned underneath infected trees in 2021 were also tested and showed a significant difference between pollen and bee samples ($P = 0.017$, Fig. 7). The positions 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 that tested negative, and all trees had adjacent ASBVd-positive trees (Supplementary Table S1). Pollen and bee samples were also tested from these hives 3 months later, and three hives still returned positive ASBVd detections.

HTS of hive samples

Pollen and bee samples were also analyzed by HTS for detection of ASBVd and other viruses. Using both total RNA sequencing and small RNA sequencing approaches, ASBVd was detectable

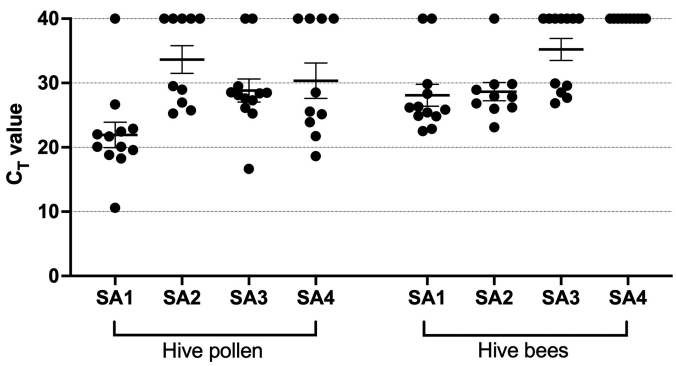

Fig. 6. Detection of avocado sunblotch viroid by real-time RT-PCR from pollen and bees collected from South African pollination hives in 2020. Samples with no amplification were given a value of $C_T = 40$.

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 SA 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.

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). Importantly, HTS detection alone is not confirmation of plant infection or transmission by bees or pollen and would require additional experimental evidence. 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. 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 was 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 would also be new to Australia.

The SA pollen samples, which underwent dsRNA extraction before small RNA sequencing, had higher recovery of virus reads (3,577 to 20,182 reads) than the QLD pollen samples (<2,400 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 virus sequences detected at lower abundance were ageratum latent virus, alfalfa mosaic virus, and tobacco streak virus.

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 and QLD4 hives, with lower presence in QLD1, where ASBVd was detected. This is likely a reflection of the relatively low attractiveness of avocado flowers to bees compared with other flowering species present (McGregor 1976).

Discussion

This study demonstrates that bee-assisted surveillance is a useful tool to test for the presence 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 3 months later, 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 titer, even when the leaves have browned and become desiccated (Pretorius and Geering 2023).

Apart from ASBVd, PBCVd (genus *Apscaviroid*) was detected in a pollen sample from a hive in Queensland, emphasizing the utility of bees for viroid surveillance. The source of this viroid isolate was most likely *Pyrus* × *bretschneideri* (Asian white pear or nashi; syn. *Pyrus serotina*), which was represented in pollen samples from three of the four hives, although PBCVd is not known to be pollen transmitted. Nashi is a recorded host of PBCVd in Australia (Joyce et al. 2006), but this detection would 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. Although these detections would 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 cultivar 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 with 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.

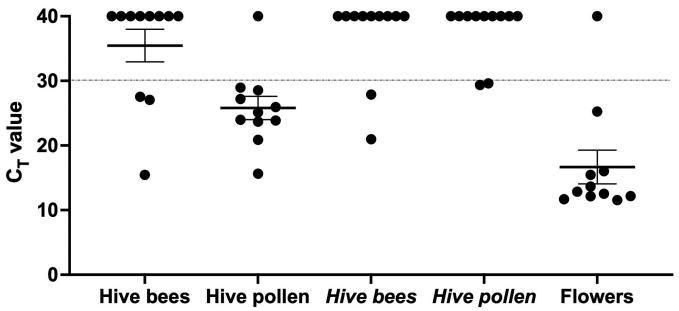

Fig. 7. Detection of avocado sunblotch viroid by real-time RT-PCR from pollen and bees collected from South African pollination hives in 2021 positioned underneath infected trees and resampled 3 months later (italics). Samples with no amplification were given a value of $C_T = 40$.

TABLE 1. Detection of avocado sunblotch viroid (ASBVd) in reads per kilobase million (RPKM) from high-throughput sequencing (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

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. The ToRSV sequence 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 into how best to implement honey bee surveillance at the orchard level. Our data from Australia and South Africa showed that hives positioned 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 100 m of hives during avocado pollination (McGregor 1976). Hive stocking rates of 2 to 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 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. Certainly, the low and variable detection of *Persea americana* compared with other pollen sources could have been influenced by our sampling method. 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 homogenize and accumulate in food stores through internal hive activities and is well suited for persistent pathogens such as ASBVd. Further examination of different pollen sampling strategies will help to determine and optimize 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 hive pollen and bees and forager 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

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 × *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

TABLE 2. Plant and bee virus genomes detected by high-throughput sequencing (HTS) of hive samples and foragers during avocado pollination in Queensland[z]

				Total RNA sequencing			Small RNA sequencing		
Plant viruses/viroids	Family	% Maximum identity	% Genome coverage	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	+	+	+		+	+

[z] Viruses marked with an asterisk have a known association with pollen according to Card et al. (2007).

hives. However, there are clear benefits to taking an HTS approach to obtain a holistic view of the pathogen landscape. Several 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 that found a greater recovery of viroids (Pecman et al. 2017). We saw some evidence for this in our data, although the SA samples were only tested with small RNA sequencing and could not be compared. 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 plant's immune response to actively replicating viruses/viroids (Ding and Voinnet 2007). Although 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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