

# Application of a Highly Sensitive Avocado Sunblotch Viroid Indexing Method

**M. Luttig and B.Q. Manicom**

*Institute for Tropical and Subtropical Crops, Private Bag X11208, Nelspruit, 1200*

## ABSTRACT

In South Africa, ASBV indexing techniques such as biological indexing, polyacrylamide gel electrophoresis, radiolabeled oligonucleotide probes and DNA and RNA hybridisation with digoxigenin (DIG) labeled probes have been used over the years. Testing became increasingly more sensitive and less time consuming, but some known positives were still missed due to the low viroid titre. Our objective was to develop a RT-PCR test, to selectively amplify the pathogen from avocado total nucleic acid extracts. Each of the extraction, isolation and detection stages was improved. PCR inhibitors were removed using mini CF-I7 columns. First strand cDNA was synthesised and amplified in one tube to save time. Various primers were tested and the PCR process optimised. This test can detect ASBV from 25pg of total double strand RNA.

## INTRODUCTION

Sunblotch is an economically important disease of avocado, first described by Parker and Home (1931) as graft-transmissible. The causal agent, avocado sunblotch viroid (ASBVd), was established by Palukaitis *et al.* (1979) as an infective single-stranded circular RNA molecule of 247 nucleotides. Symons (1981) determined the sequence and proposed a rod-like RNA structure.

Symptoms of infection are yellow or white, usually sunken, streaks on twigs. Fruit symptoms are similar though the discoloration is reddish with black-skin avocados. Roughened, cracked bark is common on major limbs. Leaves may be distorted or variegated (Dale *et al.*, 1982; Desjardins, 1987; Horne and Parker, 1931). Differences in symptoms have been associated with changes in RNA sequence (Semancik and Szychowski, 1994). The most important aspect of infection is the massive losses in yield evinced by infected trees. This is so even where trees do not show any symptoms (the symptomless carriers). ASBVd is transmitted by grafting of infected budwood, pollen and seed; but has no known insect vector. It is therefore of vital importance for nurserymen to know that they are not using symptomless infected trees as budwood or seed source.

Several methods have been described for the detection of ASBVd. In the 1950's a bioassay was developed (Wallace, 1958). This method; however, was laborious and

time consuming, as 6 months to 2 years were required for characteristic symptoms to appear. By 1980 polyacrylamide gel electrophoresis (PAGE) was introduced when the viroid nature of ASBV was accepted (Palukaitis *et al.*, 1979; Mohamed & Thomas, 1980). This technique failed to live up to expectations as known positives were often missed (Moll *et al.*, 1984).

Molecular techniques became available and sensitive techniques such as a cDNA probe (Palukaitis *et al.*, 1981; Allen & Dale, 1981) or a synthesized oligonucleotide (Bar-Joseph *et al.* 1986) were used in dot blot hybridizations. The latter test was used commercially (Korsten *et al.*, 1986), but only 47-55% of known positives were detected and radioactive labeling was required (Bar-Joseph *et al.*, 1986).

In the 1990s we developed and commercially used a digoxigenin labeled RNA based dot blot hybridization test (Manicom & Luttig, 1996). This test could detect one infected leaf in a leaf sample of 1000. Even this sensitive test occasionally missed known positives.

Detection methods based on the reverse transcription-polymerase reaction (RT-PCR) have been reported (Semancik and Szychowski, 1994; Schnell *et al.*, 1997). These protocols rely on two-step RT-PCR procedures and crude total nucleic acid isolation, which is time consuming and not suitable for processing large numbers of samples. Recently, one-step RT-PCR systems have been commercialized by companies such as Roche Products, allowing both reactions (reverse transcription and DNA amplification) to be conducted in the same tube without any addition of primers or enzymes between the RT and the amplification steps, thus reducing the handling steps and the risk of contamination. A critical step for routine use of PCR technology is template isolation. An improved small-scale extraction procedure for viroid RNA has been reported (Ben-Shaul *et al.*, 1995).

Our objectives were to find suitable primers specific for ASBVd and to develop a one-step RT-PCR assay to detect the pathogen in clarified dsRNA extracts. The one-step RT-PCR procedure was then compared with our digoxigenin labeled RNA based dot blot hybridization test.

## **MATERIALS AND METHODS**

### **Sample collection**

As positive control, two infected leaves were mixed with eight healthy leaves. Freeze dried leaf tissue from Australia (RT-PCR tested) and several tree samples from ITSC orchards (dot blot tested) were used as negative controls. Leaves from trees with typical fruit and stem symptoms as well as infected symptomless trees that occasionally tested negative with dot blot indexing were randomly collected. To optimize the test, young and old infected leaves were indexed.

### **Template preparation**

Ten leaves from each tree were stacked and by punching a hole with a sterile metal cap (20 mm diameter), ca. 1 g of leaf tissue was obtained. Leaf disks were crushed into smaller pieces in liquid nitrogen. Five milliliters of extraction buffer (Manicom and Luttig,

1996) and 100 mg polyvinylpyrrolidone (PVP) per gram fresh weight of tissue were added and ground with a Polytron in 30 ml tubes. The slurry was stirred for 10 min on ice. Approximately 1.5 ml of slurry was added to 250µl Tris-HCl buffered phenol (pH 8.0) in 2 ml eppendorf tubes, shaken and centrifuged at 12000g for 10 min. One ml of the aqueous phase was transferred to a new tube and precipitated with an equal volume of isopropanol at -20°C for 1 h. Nucleic acids were recovered by centrifugation, dissolved in 2 ml 1 x STE/35% ethanol solution and loaded on small CF-11 (Whatman) columns (Ben-Shaul *et al.*, 1995) (300µl CF-11 packed in 1 ml syringe barrels), washed twice, each with 1 ml of a solution containing 1 x STE (50 mM Tris-HCl, 0.1 M NaCl, 1 mM EDTA, pH 6.8) and 35% ethanol, eluted with 50Qµl of 1 x STE and precipitated with 1/10 volume of 3 M sodium acetate (pH 5.2) and 3 volumes of ethanol. The RNA pellet was washed with 70% ethanol, dried, and resuspended in 50µl water.

Primer	Nucleotide sequence (5' to 3')	Reference
ASBV1	AAG TCG AAA CTC AGA GTC GG	(Schnell <i>et al.</i> , 1997)
ASBV2	GTG AGA GAA GGA GGA GT	
SEM1	TCT TTC CCT GAA GAG ACG A	(Semancik & Szychowski, 1994)
SEM2	TGG GAA GAA CAC TGA TGA G	
AVFL1	CAA GAG ATT GAA GAC GAG TGA ACT A	(Hafner, pers. comm.)
AVFL2	ATC ACT TCG TCT CTT CAG GGA AAG A	

## Primers

Synthetic oligonucleotide primers complementary or homologous to specific ASBV sites were tested (Table 1). The primer pairs cover ASBVd sites, which had shown no sequence heterogeneity in 5l cDNA clones analyzed by Rakowski and Symons (1989).

## RT-PCR analysis

The Titan™ One Tube RT-PCR System (Roche Products) was used according to manufacturer's protocol with the following modifications: In each reaction, 4 µl of each primer (2.5 µM) and 2 µl of template (dsRNA) were used. The primers were annealed to the viroid template by incubation at 100°C for 5 min, chilled on ice for 5 min, then allowed to stand at room temperature for 30 min. Thirty-one µl of a master mixture containing 1x RT-PCR buffer, 200 µM of each, dNTP and 5mM DTT-solution, was added to each annealing reaction. Finally one µl of enzyme mix (AMV and Expand™ High Fidelity PCR-System) was added to each tube and mixed. The following reverse transcription and PCR cycles were performed: 30 min/50°C, 3 min/94°C, 35 cycles of 1 min/94°C, 1 min/60°C, 1 min/68°C and one final cycle of 5 min/68°C. Ten microlitres of PCR product was analyzed by 1% agarose gel electrophoresis.

## Dot-blot hybridization

Ten microlitres of each ASBV RNA sample was additionally analyzed by dot blot hybridization with an ASBV specific digoxigenin labeled (DIG) RNA probe (Manicom and Luttig, 1996).

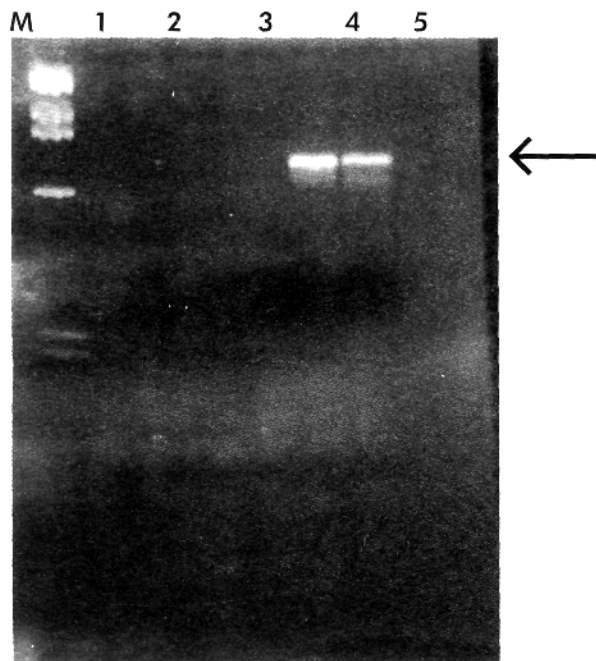


Figure 1. Agarose gel electrophoretic analysis of reverse transcription-polymerase chain reaction (RT-PCR)-amplified avocado sunblotch viroid (ASBVd) cDNA synthesized from crude extracts of young and old avocado leaves. A positive result is scored by the presence of a 247 bp DNA fragment. Lane 1, healthy tree; lanes 2 and 3, infected old leaves; lanes 4 and 5, infected young leaves. Lane M, molecular weight markers with size 1419bp, 517bp, 396bp, 214bp, 75bp, 65bp. The arrow indicates position of ASBVd (247 bp).

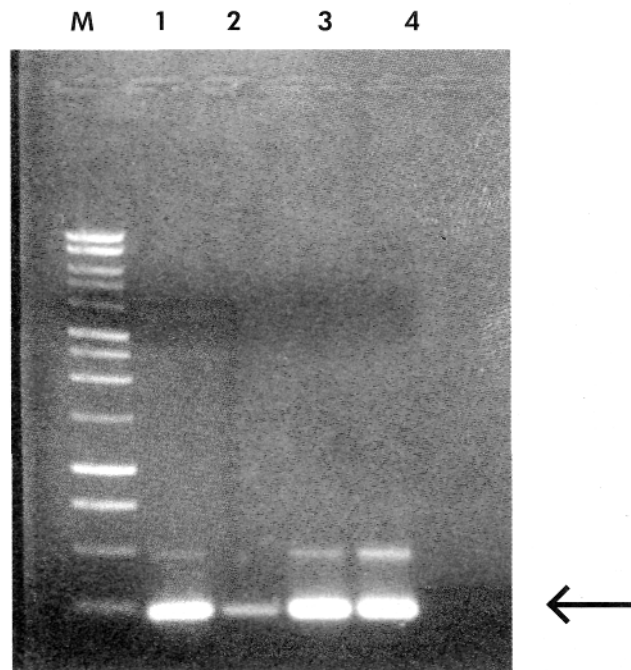
## RESULTS AND DISCUSSION

Initially RT-PCR could detect ASBVd only from crude extracts of young leaves (Fig. 1). Mature avocado leaf tissue has a high level of polysaccharides, polyphenols and phenol oxidases, which interfere with ASBV detection. This is not the case with infected young leaves. Detection of the viroid increased dramatically when PVP, which removes polyphenols (Maliyakal, 1992), was added to the avocado extraction buffer. Lycine as additive did not improve viroid detection as well as PVP. Small scale CF-1 1 chromatography (Ben-Shaul *et al.*, 1995) was adopted to clean the viroid RNA further from PCR inhibitors (Fig. 2).

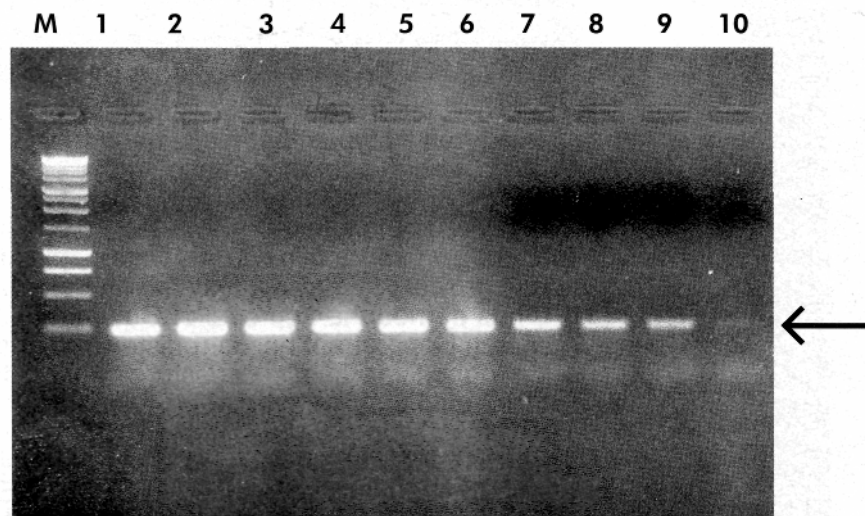
The Titan One-Tube RT-PCR System was evaluated with three sets of ASBVd specific primers (Table 1). To prevent amplification of false positives due to the presence of high concentrations of host 5s RNA, the 25-mer AVFL1 and AVFL2 primers together with a high annealing temperature were used.

The sensitivity of the protocol allowed avocado viroid detection from as little as 25 pg of total dsRNA (Fig. 3), compared to 1 .0 ng of total nucleic acids in the crude extract, two-step RT-PCR method by Schnell *et al.* (1997). The dot blot hybridization test with an ASBV specific digoxigenin labeled (DIG) RNA probe (Manicom and Luttwig, 1996), could detect ASBV from 1.0 ng of total dsRNA (data not shown). We could repeatedly detect

leaves from one infected tree mixed with leaves from three healthy trees (data not shown). In future research we will determine at what level leaves can be pooled into even larger lots.



**Figure 2.** Successful detection of ASBVd from young and old leaf extracts after removal of polyphenols with PVP and small scale CF-11 chromatography. Lane 1 and 2, lypine as additive to remove PCR inhibitors, young leaves, old leaves. Lane 3 and 4, removal of polyphenols with PVP, young leaves, old leaves. Lane M, 250-bp ladder. Larger products from viroid-infected leaves correspond to multimeric forms known to occur in ASBVd infection. The arrow indicates position of ASBVd (247 bp).



**Figure 3.** Sensitivity study for the new one-step RT-PCR indexing test. The ASBV total dsRNA concentrations of lanes 1 to 10 are 1000 ng, 500 ng, 250 ng, 25 ng, 2,5 ng, 1,0 ng, 500-pg, 250-pg, 25-pg, 2,5-pg. Lane M, 250-bp ladder. The arrow indicates position of ASBVd (247 bp).

## CONCLUSIONS

Schnell *et al.* (1997) could repeatedly detect ASBVd positive trees from crude nucleic acid extracts with accuracy of only 85%, probably because of PCR inhibitors carried over in non-column-purified nucleic acids. Previously column chromatography purification was considered to be a difficult and time-consuming extraction procedure. By using small-scale CF-11 chromatography (Ben-Shaul *et al.*, 1995), the viroid extracts not only are concentrated, but also cleaned from remaining RT-PCR inhibitors. The mini-columns are reusable and more samples can be handled simultaneously. The addition of PVP to the avocado extraction buffer effectively removed polyphenols from old leaves. As a result viroid extracts contain no PCR inhibitors, making ASBV detection sensitive and reliable.

The level of viroid concentrations in avocado trees with symptoms can vary by 1000 times from branch to branch of one tree (Allen & Dale, 1981) and by 10000 times between trees (Palukaitis *et al.*, 1981). Variants of ASBVd associated with bleached and variegated leaf symptoms or symptomless carrier tissue were found (Semancik & Szychowski, 1994). The RT-PCR technique multiplies the viroid up to detectable levels and targets the conserved region of ASBVd variants (Semancik & Szychowski, 1994; Schnell *et al.*, 1997). The newly introduced one-step Titan RT-PCR System is more sensitive, quick and less expensive than the classical two-step RT-PCR system.

The results obtained indicate that the new one tube RT-PCR indexing test coupled with small-scale CF-11 chromatography is a large improvement on all previous known tests. The PCR test is a practical and valid method and is available now for the detection of ASBVd. Our laboratory is willing to test for ASBVd in nursery or field trees. For details and cost, contact Michael Luttig or Dr. Barry Manicom at (013) 753 2071.

## ACKNOWLEDGEMENTS

Thanks to Gerhard Erasmus for his interest and for sending coded samples. The sharing of plant material and techniques by Greg Hafner from Australia is sincerely appreciated. We wish to acknowledge the financial support received from The Avocado Nurserymen's Association to conduct the experiments in this project.

## REFERENCES

- ALLEN, R.N. & DALE, J.L. 1981. Application of rapid biochemical methods for detecting avocado sunblotch disease. *Ann. Appl. Biol.* 98:451-461.
- BAR-JOSEPH, M., YESODI, V, FRANCK, A., ROSNER, A. & SEGEV, D. 1986. Recent experiences with the use of synthetic DNA probes for the detection of avocado sunblotch viroid. *South African Avocado Growers' Association Yearbook* 9:75-77.
- BEN-SHAUL, A., GUANG, Y, MOGILNER, N., HADAS, R., MAWASSI, M., GAFNY, R. & BAR-JOSEPH, M. 1995. Genomic diversity among populations of two citrus viroids from different graft-transmissible dwarfing complexes in Israel. *Phytopathology* 85:359-364.

- DALE, J.L., SYMONS, R.H. & ALLEN, R.N. 1982. Avocado sunblotch viroid. CMI/AAB. Descriptions of plant viruses, No. 254.
- DESJARDINS, P.R. 1987. Avocado sunblotch. In: The viroids, pp229-313. Edited by T.O. Diener. New York: Plenum Press.
- HORNE, W.T. & PARKER, E.R. 1931. The avocado disease called sunblotch. *Phytopathology* 21:235-238.
- KORSTEN, L, BAR-JOSEPH, M., BOTHA, A.D., HAYCOCK, L.S. & KOTZÉ J.M. 1986. Commercial monitoring of avocado sunblotch viroid. *South African Avocado Growers' Association Yearbook* 9:63.
- MALIYAKAL, E.J. 1992. An efficient method for isolation of RNA and DNA from plants containing polyphenolics. *Nucleic Acid Research* 20:23-81
- MANICOM, B.Q. & LUTTIG, M. 1996. Simplification and improved sensitivity of avocado sunblotch viroid detection. *South African Avocado Growers' Association Yearbook* 19:68-69.
- MOHAMED, N.A. & THOMAS, W. 1980. Viroid-like properties of an RNA species associated with the sunblotch disease of avocados. *J. Gen. Virol.* 46:157-167.
- MOLL, J.N., HUSSEY, K.M. & VAN VUUREN, S.P 1984. Sunblotch indexing for the plant improvement scheme. *South African Avocado Growers' Association Yearbook* 7:24.
- PALUKAITIS, R, HATTA, I, ALEXANDER, D.MCE. & SYMONS, R.H. 1979. Characterization of a viroid associated with avocado sunblotch disease. *Virology* 99:145-151.
- PALUKAITIS, P, RAKOWSKI, A.G., ALEXANDER, D.MCE. & SYMONS, R.H. 1981. Rapid indexing of the sunblotch disease of avocados using complementary DNA probe to avocado sunblotch viroid. *Ann. Appl. Biol.* 98:439-449.
- RAKOWSKI, A.G., & SYMONS, R.H. 1989. Comparative sequence studies of variants of avocado sunblotch viroid. *Virology* 173:352-356.
- SCHNELL, R.J., KUHN, D.N., RONNING, C.M. & HARKINS, D. 1997. Application of RT-PCR for indexing avocado sunblotch viroid. *Plant Disease* 81:1023-1026.
- SEMANCIK, J.S. & SZYCHOWSKI, J.A. 1994. Avocado sunblotch disease: a persistent viroid infection in which variants are associated with differential symptoms. *J. Gen. Virol.* 75:1543-1549.
- SYMONS, R.H. 1981. Avocado sunblotch viroid: primary sequence and proposed secondary structure. *Nucleic Acids Res.* 9:6527-6537.
- WALLACE, J.M. 1958. The sunblotch disease of avocados. *J. Rio Grande Vall. Hort. Soc.* 12:69-74.