An overview of avocado sunblotch viroid disease in South Africa from 2008 to 2013

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ABSTRACT

The presence of avocado sunblotch viroid disease (ASBVd) leads to massive losses in yield, as well as discoloured reddish avocado with black skin. This severely reduces the marketability of avocado. Thus, detection of ASBVd is of the utmost importance in controlling this disease, as symptomless trees represent a great danger in terms of spreading of the viroid. A rapid and more sensitive method for the routine detection of infected avocado tissues by ASBVd is by reverse transcription-polymerase chain reaction (RT-PCR) assay. At the Agricultural Research Council – Institute for Tropical and Subtropical Crops (ARC-ITSC), detection of ASBVd is performed using a real-time PCR machine which is more sensitive to detect low viroid titer. Over a period of five years, 24 685 mother trees have been tested from 14 different commercial nurseries operating in the Limpopo and Mpumalanga provinces. Shockingly, 15.6% of trees tested positive. This percentage is considered too high if compared to 2% reported by Korsten and co-workers in 1987. The objective of this paper will be to give an overview of the ASBVd prevalence in South Africa from 2008 to 2013. In addition, management strategies in the control of ASBVd will be discussed with the long term objective of an avocado industry free from ASBVd.

INTRODUCTION

Avocado, *Persea americana* Mill, Sunblotch was first described in 1928 by Coit. This researcher attributed the disease to physiological stress caused by sun burn (Coit, 1928). However, in 1932 Horne and Parker described it as graft-transmissible. Palukaitis and co-workers established the causal agent Avocado Sunblotch Viroid (ASBVd) as an infective single-stranded circular RNA molecule of 247 base pairs nucleotides (Horne & Parker, 1932; Palukaitis *et al.*, 1979).

Transmission of the viroid can occur mechanically or naturally through seed, pollen and root grafting. To date, no known insect vector has been identified. The symptoms of the disease vary considerably and are influenced by host cultivar, the environment and the strain of ASBVd that infect a given plant. These include discoloured and depressed stem streaks, lesions and discolouration of the fruit, and diverse foliar symptoms. Small changes in the nucleotide sequence of the viroid can have a dramatic effect on symptom expression. Sequence variants have been categorised according to their association with the different symptom types: those associated with bleached (ASBVd-B), variegated (ASVBd-V) and asymptomatic tissues (ASBVd-SC) (Semancik & Syzchowski, 1994). The economic impact of ASBVd can be devastating, if not controlled. The long term effects can result in: smaller and fewer fruits; fruit disfigurement (fruit downgraded on quality standards); quarantine restrictions that may affect export of fruit; and finally lead to massive losses in yield and profit to the industry (Mohammed & Thomas 1980; Schnell *et al.*, 1997).

The question can be asked how significant a problem ASBVd is currently in South Africa. The last published report of ASBVd was by Korsten and co-workers in 1987, when 3 125 nursery trees were tested using the dot-blot hybridisation technique and each tree was tested twice. From that study, only 2% of the trees tested positive. No sign of Sunblotch or symptoms of the disease was observed on the infected trees, which can basically then be classified as symptomless carriers of the viroid (Korsten *et al.*, 1987). Therefore, the objectives of this study was to highlight the disease prevalence of ASBV in South Africa from 2008 to 2013, as well as to propose management strategies for the control of ASBVd, with the long term objective of a ASBVd-free avocado industry.

MATERIALS AND METHODS

Sample collection

Young and old leaves from symptomless trees were received for ASBVd analysis from fourteen nurseries in the Mpumalanga and Limpopo provinces over a period of five years (2008 to 2013). Five leaves were sampled from each of the compass points per tree and these were combined with three more samples from different trees to make one sample consisting of twenty leaves. A total of 24 685 trees were tested. Fourteen nurseries were grouped into three groups i.e. A, B and Others. This grouping was purely based

on the volume of samples that were received and processed from all fourteen nurseries.

RNA extraction

Twenty leaves were punched four times with sawn off blue pipette tip to obtain about 400 mg of leaf tissue collected into Agdia bags. Five ml of avocado extraction buffer and 2.5 ml chloroform/iso-amyl alcohol (24:1) were added into Agdia bags containing 400 mg of leaf tissue. This was grinded with a drill press to mush and 1600 µL added to a 2 mL Eppendorf tube, filled with 200 µL of chloroform/iso-amyl alcohol. After centrifugation for 3 minutes, 700 µL of clean upper phase was transferred to a new Eppendorf, followed by 300 µL of Ethanol. The sample was then loaded onto small CF-11 (Whatman) columns (Ben-Shaul *et al.*, 1995) (500 µl CF-11 packed in 1 ml syringe barrels), washed twice, each with 1 ml of a solution containing 1 x STE (50 mM Tris-HCI, 0.1 M NaCI, 1 mM EDTA, pH 6.8) and 35% ethanol, eluted with 500 µl of 1 x STE and precipitated with 1/I0 volume of 3 M sodium acetate (pH 5.2) and 3 vol-

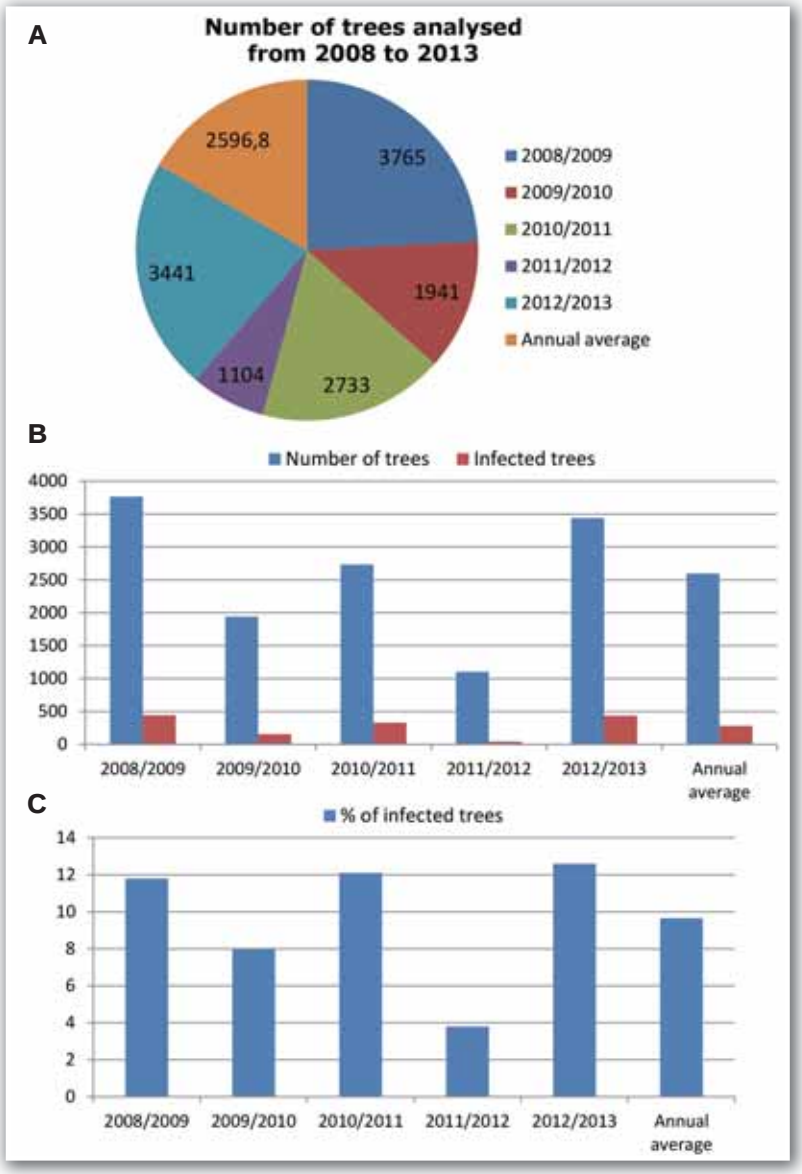

Figure 1. Occurrence of Avocado Sunblotch Viroid disease (ASBVd) in South Africa, Nursery A, over a five year period. (A) Number of trees analysed; (B) Number of trees tested compared to number of infected trees; (C) % of the infected trees with an average of 9.7%.

Table 1. Synthetic oligonucleotide primers complementary or homologous to specific ASBV sites used in this study.

Primer sequence (5'- 3')	Amplicon	Reference
Forward: AGAGAAGGAGGAGTCGTGGTGAAC Reserve: TTCCCATCTTTCCCTGAAGAGACGA	99 bp	(Manicom BQ, pers. comm)
Forward: ATCACTTCGTCTCTTCAGGGAAAGA Reserve: CAAGAGATTGAAGACGAGTGAACTA	247 bp	(Luttig & Manicom, 1999)

umes of ethanol. After centrifugation for 10 minutes, the RNA pellet was washed with 70% ethanol, dried and re-suspended in 100 µl sterile distilled water.

Viroid detection by RT-PCR analysis

Two µl of the sample was added to 18 µl of a PCR reaction mixture containing: master mixture containing 1 x RT-PCR buffer, 200 µM of each dNTP and 5 mM DTT-solution; primer (2 µM) (Table 1) and enzyme mix. Amplifications were performed in a qPCR machine and PCR conditions as follows: 30 min/50°C, 3 min/94°C, 35 cycles of 1 min/94°C, 1 min/60°C, 1 min/68°C. Twenty µl of amplified PCR products were analysed by electrophoresis in 2% agarose gel in 1 x TBE and visualised with ethidium bromide.

RESULTS AND DISCUSSION

Nursery A had the biggest bulk of the trees submitted for testing, on average 2 597 trees were tested annually. The lowest number of trees analysed was in the 2011/12 season, while the highest was in the 2008/09 season (Fig. 1A). The lowest number of ASBVd infected trees was recorded in the 2011/12 season with the highest in the 2012/13 season (Fig. 1B). On average 9.7% of the trees tested were infected with ASBV (Fig. 1C).

Nursery B submitted on average 1 008 trees annually to be tested. The lowest number of trees analysed was in the 2008/09 season, while the highest was in the 2011/12 season (Fig. 2A). The lowest number of ASBVd infected trees was recorded in the 2010/11 season, with the highest in the 2011/12 season (Fig. 2B). On average, 23.9% of the trees tested were infected with ASBV (Fig. 2C).

The other 12 nurseries submitted on average 506 trees annually to be tested. The lowest number of trees analysed was in the 2008/09 season, while the highest was in the 2012/13 season (Fig 3A). Here, an increase in the number of trees tested was observed compared to other years. This can be attributed to

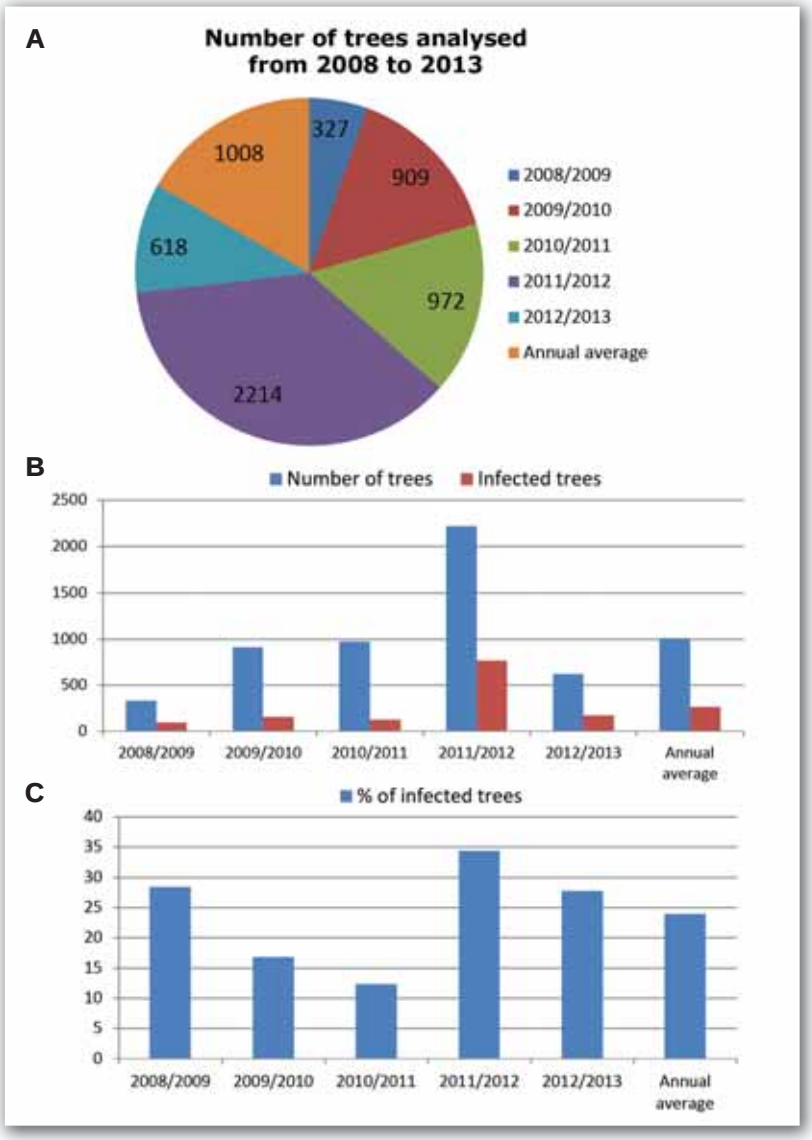

Figure 2. Occurrence of Avocado Sunblotch Viroid disease (ASBVd) in South Africa, Nursery B, over a five year period. (A) Number of trees analysed; (B) Number of trees tested compared to number of infected trees; (C) % of the infected trees with an average of 23.9%.

the emergence of new nurseries or old nurseries re-testing. The lowest number of ASBVd infected trees was recorded in the 2008/09 season, with the highest in the 2012/13 season (Fig. 3B). On average 18.9% of the trees tested were infected with ASBV (Fig. 3C).

On average, all 14 nurseries submitted 4 114 trees annually for testing. The lowest number of trees analysed was in the 2009/10 season, while the highest was in the 2012/13 season (Fig. 4A). The lowest number of ASBVd infected trees was recorded in the 2009/10 season, with the highest in the 2011/12 season (Fig. 4B). On average, 15.6% of the trees tested from all fourteen nurseries were infected with ASBV (Fig. 4C). Interestingly, the percentage of infected trees in all nurseries is fluctuating. Here, an abnormal trend of "increase-decrease-increase-decrease" phenomena is observed (Figs. 1C, 2C, 3C). Because of continuous testing throughout the years, expectations are that there must be a continuous and gradual decline annually in the percentage of infected trees. An in-depth study is needed to understand whether current management strategies for control of ASBVd are a cause for concern. The possibility exists that there is perhaps a new strain of ASBVd or that there are other factors that lead to the "increase-decrease-increase-decrease" phenomena. In 1995, Querci and co-workers reported that Potato spindle tuber viroid (PSTVd) was detected in avocado trees and some trees were co-infected with ASBV (Querci *et al.*, 1995). This finding needs to be investigated in South Africa and whether PSTVd in any way influence the outcomes of local testing.

CONCLUSIONS

In conclusion, 15.6% of the trees submitted for ASBV analysis during the past five years tested positive, i.e. 3 851 of 2 4685 trees tested. This percentage of infected trees is particularly high when compared

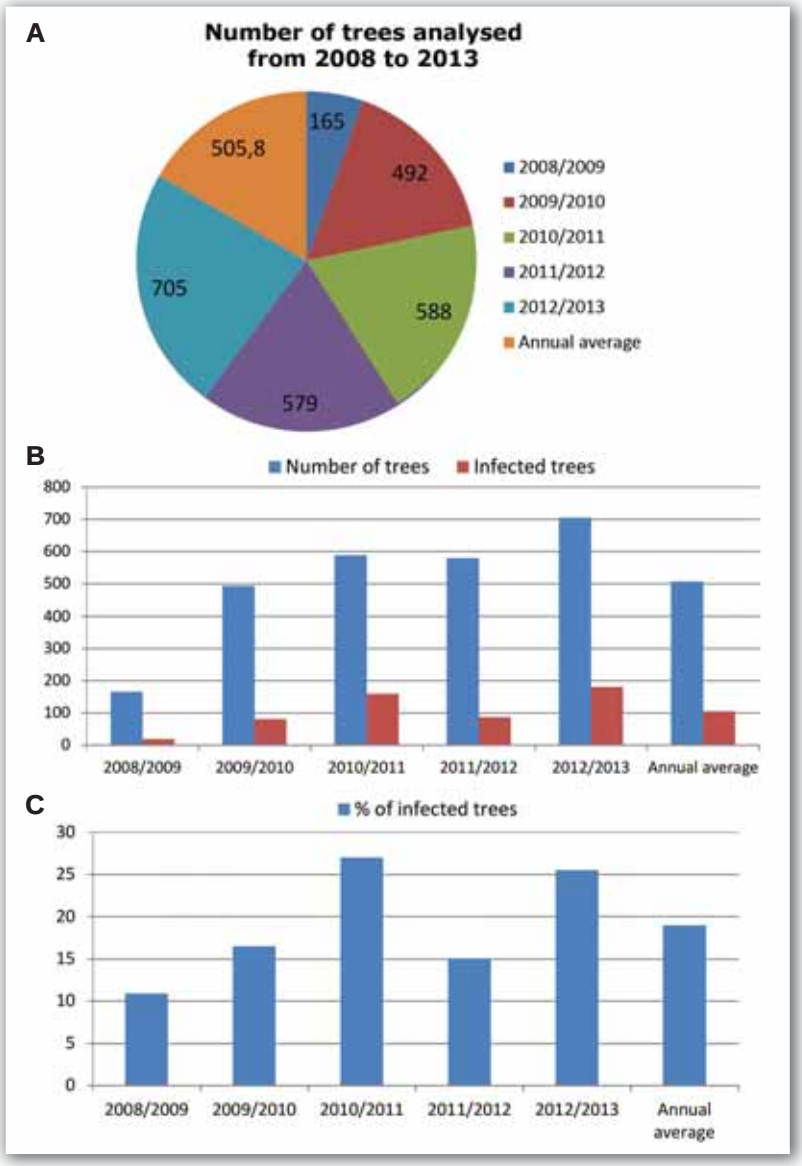

Figure 3. Occurrence of Avocado Sunblotch Viroid disease (ASBVd) in South Africa, Other nurseries, over a five year period. (A) Number of trees analysed; (B) Number of trees tested compared to number of infected trees; (C) % of the infected trees with an average of 18.9%.

with the study done by Korsten *et al.*, 1987, where they recorded 2% of the infected trees of about 3 124 trees. In the present study, an increase in viroid detection can be ascribed to the addition of Polyvinylpyrrolidone (PVP) in the extraction buffer. PVP removes polyphenols (Maliyakal, 1992) and as a result viroid extracts contain no PCR inhibitors, making ASBV detection more sensitive and reliable. In addition, the new RT-PCR machine increased detection of infected trees by multiplying the viroid up to detectable levels and targets the conserved region of ASBVd variants (Luttig & Manicom, 1999).

RECOMMENDATIONS: MANAGEMENT STRATEGIES

Viroid infections are incurable, therefore all ASBVd infected plants must be destroyed to prevent further spread of the disease. New blocks should always be established with plants certified to be free of ASBVd. In addition, testing of trees twice a year could reduce the possibility of missing ASBV due to seasonal variations in viroid concentrations (Allen & Dale, 1981; Korsten *et al.*, 1987). To ensure freedom from ASBVd, the ARC-ITSC lab is testing nursery and field trees at a cost. For more information, contact Dr. Desmond Ncango (+27 13 753 7125 or Ncangom@arc.agric.za).

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REFERENCES

ALLEN, R.N. & DALE, J.L. 1981. Application of rapid biochemical methods for detecting avocado Sun-

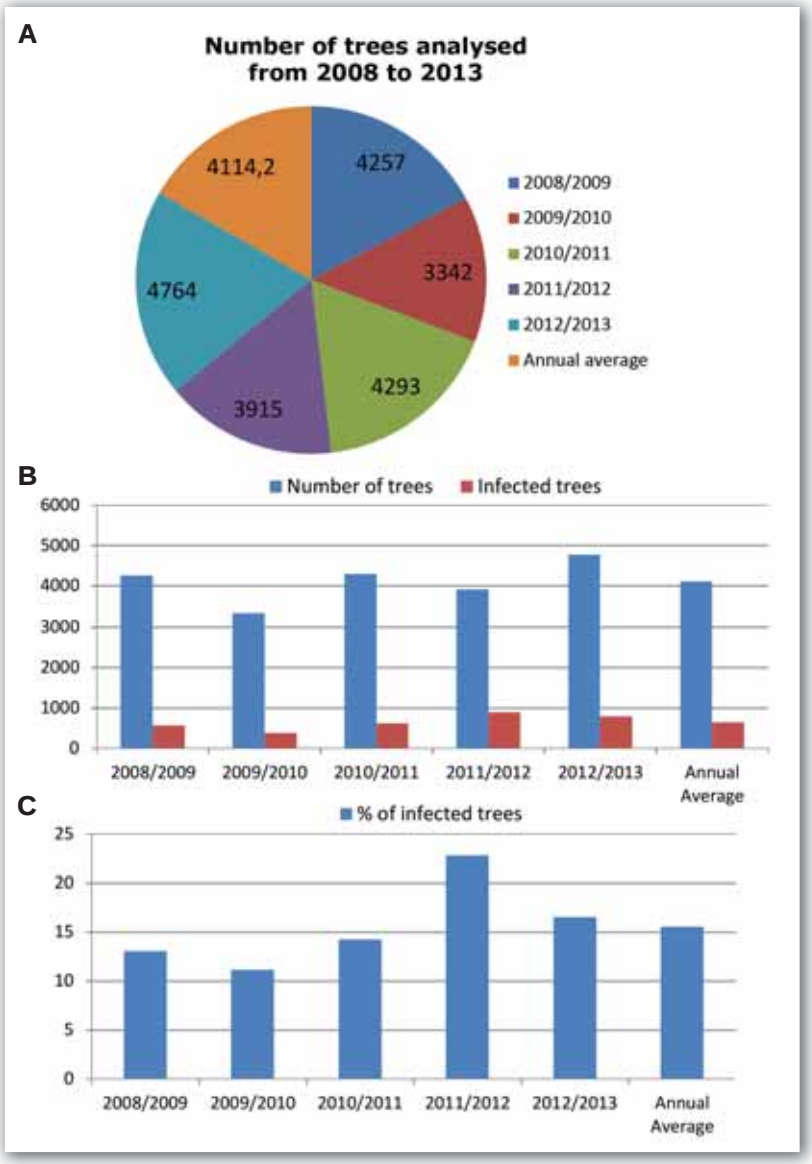

Figure 4. Occurrence of Avocado Sunblotch Viroid disease (ASBVd) in South Africa, fourteen nurseries combined, over a five year period. (A) Number of trees analysed; (B) Number of trees tested compared to number of infected trees; (C) % of the infected trees with an average of 15.6%.

blotch disease. *Annuals of Applied Biology* 98: 451-461.

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.

COIT, J.E. 1928. Sun blotch of the avocado, a serious physiological disease. *California Avocado Society Yearbook* 27-32.

HORNE, W.T. & PARKER, E.R. 1932. The avocado disease called Sunblotch. *Phytopathology* 21: 235-238.

KORSTEN, L., BAR-JOSEPH, M., BOTHA, A.D., HAYCOCK, L.S. & KOTZÉ J.M. 1987. Commercial monitoring of avocado Sunblotch viroid. *South African Avocado Growers' Association Yearbook* 9:63.

LUTTIG, M. & MANICOM, B.Q. 1999. Application of a highly sensitive Avocado Sunblotch Viroid Indexing method. *South African Avocado Growers' Association Yearbook* 22: 55-60.

MALIYAKAL, E.J. 1992. An efficient method for isolation of RNA and DNA from plants containing polyphenolics. *Nucleic Acid Research* 20: 23-8.

MOHAMED, N.A. & THOMAS, W. 1980. Viroid-like properties of an RNA species associated with the Sunblotch disease of avocados. *Journal of General. Virology.* 46: 157-167.

PALUKAITIS, P., HATTA, I., ALEXANDER, D.M.C.E. & SYMONS, R.H. 1979. Characterization of a viroid associated with avocado Sunblotch disease. *Virology* 99: 145-151.

QUERCI, M., OWENS, R.A., VARGAS, C. & SALAZAR, C. 1995. Detection of Potato Spindle Tuber Viroid in Avocado Growing in Peru. *Plant disease* 79: 196-202.

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. *Journal of General Virology* 75: 1543-1549.