Distribution, Epidemiology and Management strategies for Avocado sunblotch disease in South Africa

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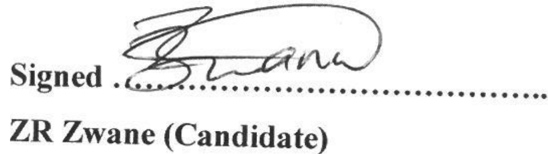

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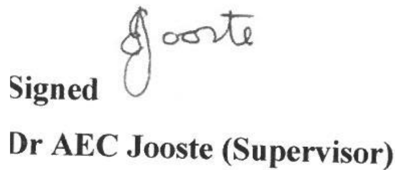

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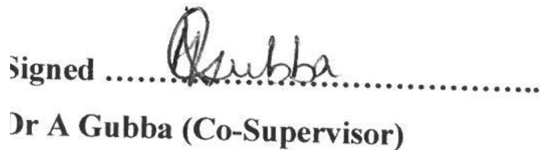

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Dissertation summary

Avocado (*Persia americana* Mill.) is an economical important subtropical fruit worldwide. In the republic of South Africa (RSA) avocado contributes approximately 29% to the total gross value of subtropical fruits. Avocado sunblotch disease caused by *Avocado sunblotch viroid* (ASBVd) is an important disease that affects yield and quality in avocado production worldwide. Typical symptoms are found on leaves, fruit and bark of the tree. However, some trees do not display any visible symptoms and these are termed symptomless carrier trees. The most important control measure for Sunblotch disease is careful selection of pathogen-free bud wood and seed that are used for propagation which is achieved through indexing. The objectives of the current study were to (1) validate the sensitivity of ASBVd detection techniques used for indexing, (2) study the distribution of ASBVd in a single infected tree and (3) conduct an online- and field survey on commercial farms for sunblotch disease incidence, management and strain variations in the Limpopo and Mpumalanga provinces, RSA.

To validate the sensitivity of ASBVd detection techniques, an ASBVd infected tree with typical ASBVd symptoms on the leaves and stem was selected from the glasshouse at the Agricultural Research Council- Institute for Tropical and Subtropical Crops (ARC-TSC). A single ASBV infected leaf was selected as a positive control and mixed with 9, 19, 29, 39, and 49 healthy avocado leaves, respectively. RNA was extracted from the leaves using two methods, a small-scale cetyltrimethylammonium bromide (CTAB) - based RNA extraction method that was compared to a large-scale cellulose column chromatography extraction method. From each method, cDNA was amplified using a fluorescent-based one-step real-time RT-PCR reaction, in a Rotor Gene Q instrument. Two primer sets were compared in separate reactions, firstly the Bar- Joseph *et al*. (1985) primer pair that resulted in a 247 bp product and secondly the Jooste (unpublished) primer pair that generates a 99 bp product. Of all the methods tested, RNA extraction with the cellulose column chromatography and amplification using the Jooste (unpublished) primer pair was the most sensitive and reliable for large scale ASBVd indexing. Further, cDNA was amplified using a two-step conventional RT- PCR. Two primer pairs were compared in a conventional RT-PCR: first the Bar- Joseph *et al*. (1985) primer pair resulting in a 247 bp product and secondly the published primer pair from Luttig and Manicom (1999) with a 250 bp product. The products were visualised on a 1.5% agarose gel, stained with ethidium bromide. The most sensitive results were obtained using the Bar- Joseph *et al*. (1985) primer set from RNA extracted using both the CTAB and

1 Cellulose column chromatography extraction methods. In this study the sensitivity and
2 reliability of a large scale indexing method for ASBVd was validated.

3 The distribution of ASBVd in a single infected tree was studied from avocado trees collected
4 at three nurseries in the Limpopo province that included symptom bearing trees and known
5 ASBVd positive symptomless carrier trees. Branches of the same tree were sampled
6 separately collected (young and old) with fruits being included when present. Further,
7 ASBVd distribution within a single infected fruit between the green skin (healthy part) and
8 yellow skin (symptom bearing) was investigated. RNA was extracted using the large-scale
9 cellulose column chromatography method and amplified in a fluorescent based one-step real-
10 time RT-PCR reaction in the Rotor Gene Q instrument using the Jooste (unpublished) primer
11 pair. In this study, an even distribution of ASBVd between the branches of the symptomless
12 trees and symptomless fruits was demonstrated. An uneven distribution of ASBVd in
13 symptom bearing trees was observed. These findings will improve the sampling method thus
14 increase the reliability of ASBVd indexing. This will also lead to improved management of
15 Sunblotch disease in RSA.

16 Two surveys were conducted during this study, firstly an online survey which was created
17 using Google sheets and submitted to the South African Subtropical Growers' Association
18 (Subtrop) website. The survey was conducted to determine the knowledge farmers have about
19 Sunblotch disease. From the responses it was discovered that not all avocado growers are
20 familiar with Sunblotch disease symptoms; some farmers do not take precautions with their
21 cutting tools and removal of infected trees from the field, which could pose a serious threat in
22 disease spread. A field survey was conducted to determine the spread of Sunblotch disease
23 and to determine the commonly occurring ASBVd variants in RSA. A total of 310 trees were
24 randomly sampled from 20 commercial farms in the Limpopo and Mpumalanga provinces.
25 RNA was extracted using a large-scale cellulose column chromatography method, samples
26 were indexed using a fluorescent based one-step real-time RT-PCR reaction in a Rotor Gene
27 Q instrument using the Jooste (unpublished) primer pair that generates a 99 bp product. Only
28 32 (10.3%) of trees tested positive for ASBVd, four of which manifested symptoms and the
29 rest were symptomless carrier trees. All positive samples were further amplified using a two-
30 step conventional RT- PCR using the Bar- Joseph *et al*. (1985) primer pair and PCR products
31 were sent for sequencing. The evolutionary history was inferred using the Neighbor-Joining
32 method. Sequences detected in the current study aligned with other ASBVd sequences

already deposited in the GenBank® database with a 98% identity. Different ASBVd variants were detected which were the result of minor changes in the sequence nucleotides.

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First and foremost I would like to thank my creator, the one above it all, the king, my everything. If it wasn't for you I wouldn't be here, this is a blessing and I never saw myself here but here I am. All things are possible with you, ungubaba uMvelingqangi, uyinkosi yamakhosi akekho ofana nawe, abangakaboni uyinkosi bayoze babone bavuma, ngyabonga, Nkosi yamaKhosi.

This thesis is dedicated to me; it is a reminder of my journey throughout my years of studying. I would also like to dedicate it to one person who would probably not even see this, Thank you for the motivation throughout we both know if it wasn't for you I wouldn't be here. Thank you again beautiful lady I hope all your dreams come true and you live to see many more and keep on being a great influence to others as well.

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It is easy to make a choice but the hardest part is living with our choices (stolen)

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Introduction to Dissertation

Avocado (*Persia americana* Mill.) is rated amongst the most important fruit crops in the South African and global subtropical industry (Wolstenholme and Whiley, 1999; Tondo *et al.*, 2010). The avocado industry in Republic South Africa (RSA) is one of the most developing industries in that a steady increase in the production has been observed over the years (Nortje, 2012). The production area in RSA had already increased from 2000 hectares in 1970s to 15 388 hectares by 2012 accounting for 0.01% of the total agricultural area (Nortje, 2012). During the 2012/13 season, avocados contributed 29% (R0.78 billion) to the total gross value of subtropical fruits (R2.6 billion) in RSA with an increase of 12.58% from 2013 to 2014 on total production (DAFF, 2014). The steady increase in the production area is a due to its high marketability which is a result of the nutritional value of avocado, with their oil content reaching up to 30% (Dreher and Davenport, 2013).

Improved overall diet quality, improved nutrient intake, and reduced risk of metabolic syndrome are some of the benefits associated with avocado consumption (Fulgoni *et al.*, 2013). Compared to other fruits, avocado contains more nutrients, dietary fiber and vitamins (K, and E), potassium, and magnesium making them one of the most attractive and nutritional fruits to consumers worldwide (Ochoa, 2009; Dreher and Davenport, 2013). However, there are limiting factors associated with avocado production. Disease infestation is a major concern in the production of any crop which leads to both direct and indirect economic losses, if uncontrolled (Manicom, 2001). The effect of different diseases on avocado vary, based on type of the disease involved, some diseases are more important than others in terms of the economic losses they can cause (Manicom, 2001). There are also a number of insects and pests that infest avocados in the process affecting the yield and fruit quality of avocados (Manicom, 2001).

Pathogenic agents causing economic losses in avocado production include fungal, bacterial, viral/viroid pathogens (Manicom, 2001). The majority of important avocado diseases are associated with fungal pathogens. These diseases include Cercospora spot caused by the fungus *Pseudocercospora purpurea* a fruit-spotting disease which can cause up to 70% losses when uncontrolled (Manicom, 2001). Root rot, caused by *Phytophthora cinnamomi* with effects that could lead to killing the entire tree if uncontrolled (Manicom, 2001). *Anthracnose* which leads to pre and post-harvest problem that can cause losses up to 37% (Manicom, 2001), and this fungus can interact with other avocado infecting fungi such as

Dothiorella which causes stem end rot and pepper spot (*Colletotrichum*). Stem canker which is caused by a combination of *Phytophthora* spp *P. citricola, P. cactorum and P. cinnamomi*, which causes fruits downgrading and rejections of up to 30% (Manicom, 2001). These diseases are regarded as important avocado diseases; however, the research has been successful in management of these diseases. For example Root rot, caused by *Phytophthora cinnamomi* of avocado was one of the most devastating avocado diseases which are now well controlled (Manicom, 2001).

Viroids are the smallest self-replicating non- protein coding molecules that possess unusual structural features making them hard to study and control (Flores *et al.*, 2009). The only effective control against the viroid diseases is to exclude or eradicate the infected material from the field (Hammond and Owens, 2006). Avocado sunblotch disease is the only important viroid disease associated with economic losses in avocado production (Semancik, 2003; Wallace, 1958). It is a chronic infection of avocados induced by *Avocado sunblotch viroid* (ASBVd) and is regarded as one of the most devastating diseases of avocado which can lead up to 82% fruit reduction (da Graca, 1985; Manicom, 2001). The infection manifests in two forms, one is where the characteristic symptoms are physically expressed on the young green stem, leaves and the fruits of the infected tree. Secondly, the trees do not show any visible symptoms on any plant tissues, and this is termed symptomless carrier trees (Thomas and Mohamed, 1979). The latter infection is the most complicated because infected trees remain undetected in the field since no symptoms are displayed. The only way to protect against Sunblotch disease is to produce viroid-free propagative material that is achieved through indexing of all propagative mother material.

Significance of the research

Agricultural Research Council–Tropical and Subtropical Crops (ARC-TSC) has a long history in the indexing for ASBVd. Different nurseries and farms send suspect leaf material for indexing. Recently several complaints about the inconsistencies of indexing results have been filed by some nurseries. For instance, trees with clear Sunblotch disease symptoms tested negative for the presence of ASBVd. Some of the trees tested negative one year would test positive the next and negative again the following year. This false negative/positive result could lead to the further propagation of Sunblotch disease in South Africa. From the previous studies, an increase in disease occurrence from 2% to 15.6% for the past 28 years in RSA has been recorded (Korsten *et al.,* 1986; Ncango *et al.*, 2014). Due to these facts, the ARC-TSC

concluded that an investigative study was necessary to validate the current ASBVd detection techniques, and to study the disease in more depth in order to keep up with the spread of Sunblotch disease. Most avocado profits rely on the exportation of good quality fruits. Since the South African avocado industry is export oriented, it is of utmost importance that disease-free avocados are produced all the time.

Research aim and objectives

The aim of this research was to investigate the epidemiology and management of Sunblotch disease in South Africa.

Objectives of the current study were as follows:

1. Investigate the sensitivity of the current indexing method used at the ARC-TSC based on the limit of detection (LOD) to optimize a cost effective, rapid, quality assured diagnostic tool.
2. Survey commercial farms and nurseries in two major avocado growing provinces in South Africa (Limpopo and Mpumalanga) to determine the incidence and spread of ASBVd.
3. Investigate the distribution of ASBVd between branches of the same tree on different avocado cultivars using fluorescence based real time reverse transcription polymerase chain reaction (RT-PCR).
4. Investigate ASBVd strain variation in different avocado cultivars in two provinces by sequencing and phylogenetic analysis using ASBVd specific primers.

Dissertation structure

This dissertation is divided into four chapters. The first chapter is a review of literature highlighting the importance of avocado and Avocado sunblotch disease as an economically important disease of avocados in South Africa. Chapter 2 deals with the validation and optimization of ASBVd detection techniques based on the limit of detection. This chapter also looks at the distribution of ASBVd within the branches of a single infected tree using fluorescent based real time qPCR. Chapter 3 describes the results of the survey and genetic variations of the causal agent, ASBVd, in the Limpopo and Mpumalanga provinces. Chapter 4 is the general overview of the study including the major findings and the way forward.

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14

15

1 # CHAPTER 1

2 # LITERATURE REVIEW

3 ## 1.1 The host - Avocado (*Persia americana* mill.)

4 ### 1.1.1 Origin and distribution

5 Avocado has been known since the year 1499 and is native to Central America (Sippel,
6 2001). To date avocados are commercially grown in North- and South America, Australia,
7 Israel, and Africa (Bertelsen *et al,* 1995). America (North and South) is the leading producer
8 of avocado, accounting for 76% of worldwide production. Africa accounts for 11% of the
9 total production followed by Asia with 9% and Europe with 2% (DAFF, 2012).

10 In Republic of South Africa the first orchard consisted of West Indian seedlings of about 10
11 000 trees (Sippel, 2001). These trees were introduced by an Indian farmer in Durban in 1933
12 (Sippel, 2001). To date, avocados are produced in four provinces namely Limpopo,
13 Mpumalanga, KwaZulu-Natal and Eastern Cape (Nortje, 2012). Most avocado production is
14 in the Northern provinces of RSA, Limpopo and Mpumalanga. Limpopo leads with 61% of
15 total production including the emerging growers. Mpumalanga province is the second largest
16 producer with 30% of the total production. The least production occurs in KwaZulu-Natal
17 with 8% and the Eastern Cape with only 1% of the total avocado production (Nortje, 2012).
18 The area in hectares covered by avocado production in South African growing areas is
19 indicated in Table 1.1.

20 **Table 1.1** South African avocado growing provinces and the total of land used (ha) in each
21 province (Nortje, 2012)

Province	Area (ha)	Percentage
Limpopo (commercial)	7 568	49%
Limpopo (Emerging growers)	1 833	12%
Mpumalanga and Swaziland	4 554	30%
KwaZulu-Natal	1 319	8%
Eastern Cape	114	1%
TOTAL	**15 388**	

22 ### 1.1.2 Production and economic importance

23 In South Africa, avocado production is ranked low; however, it is amongst the top five
24 avocado exporters (Ntombela *et al.*, 2013). According to the profile of the South African

avocado market value chain by DAFF (2013), approximately 110 000 tons of avocados are produced yearly. About 45 % of total production is exported, 15 % is processed (guacamole, oil, etc.), 25 % is destined for local markets and 15 % is sold on the informal markets. A total of 97.8% of South Africa's avocados were exported to Europe in the fourth quarter of 2014, and the rest went to other African countries (DAFF, 2014). This is evident that the avocado industry in South Africa is export oriented. The industry alone creates approximately 6 000 permanent jobs and an additional 2000 casual labourers during peak periods. The industry has played a major role in rural upliftment as the majority of the jobs created are within rural areas. Avocado production provides a livelihood to 36 000 individuals who rely on selling avocado to generate income and support households (DAFF, 2012).

1.1.3 Physiology and cultivation

Avocados are classified under the family *Lauraceae* together with camphor (*Cinnamomum camphora* Nees) and cinnamon (*C. zeylanicum* Nees) (Wolstenholme and Whiley, 1999). Avocado has three botanical varieties based on the country of origin (Bergh and Ellstrand, 1986). The Mexican type (var. *drymifolia*) originates from Central Mexico, Guatemalan type (var. *guatemalensis*) from Guatemala and West Indian type (var. *americana*) from America (Bergh and Ellstrand, 1986). Avocados are grown in tropical, subtropical and semi-tropical conditions, and the three types are all adapted to different climatic conditions (Table 1.2). In RSA areas are divided into cool and warm subtropical, the cultivars grown in these temperatures belong to Mexican and Guatemalan types (DAFF, 2012; Nortje, 2012). All commercial cultivars and their distribution are depicted in (Figure 1.1).

Avocado production is most favoured in temperatures between 20-25°C and are sensitive to water stress (Kotzé, 1979; DAFF, 2012). The average annual rainfall required is >1000 mm p.a., however, semi-arid regions with >400 mm p.a. can be sufficient for avocado production (Vorster, 2001). They can only tolerate light frost after flowering and before set (DAFF, 2012). Avocados are produced from the end of February until the beginning of November; however, most production occurs until the beginning of September (DAFF, 2014). The different traits each cultivar possesses is based on the country of origin as demonstrated in Table 1.2

1 **Table 1.2** Different tree traits expressed by cultivars from the three different avocado
2 botanical types (Nortje, 2012)

Trait	Mexican	Guatemalan	West Indian
Climatic adaptation	semi-tropical	subtropical	tropical
Cold tolerance	most	intermediate	least
Salt tolerance	least	intermediate	most
Hairiness	most	less	less
Leaf anise	present	absent	present
Leaf color	medium	often redder	paler

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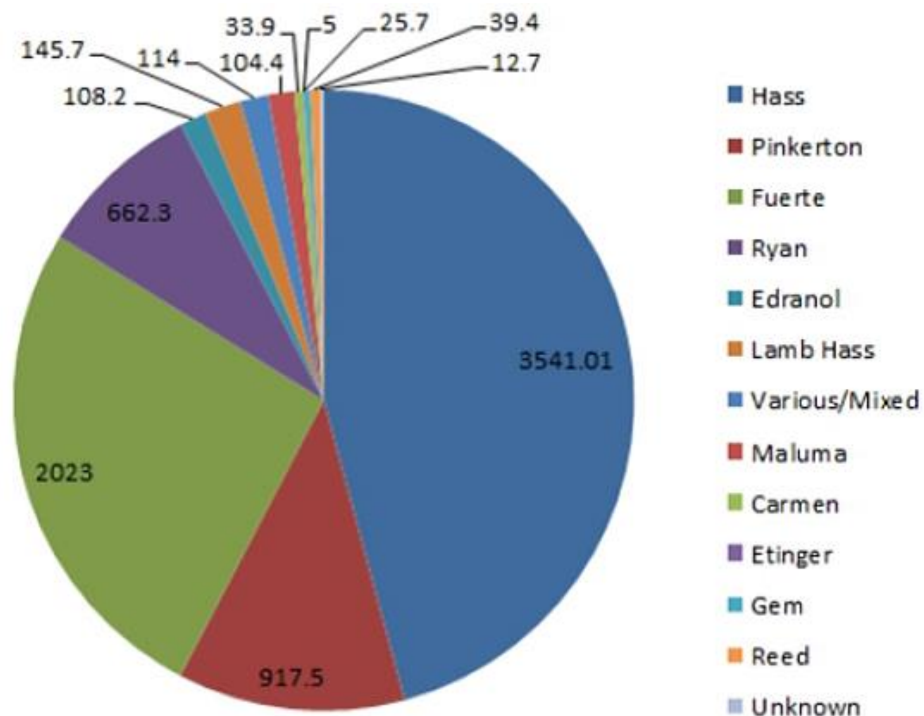

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5 **Figure 1.1** Avocado commercial cultivars and their distribution in South African growing
6 regions in hectares (Nortje, 2012)

7 ## 1.2 THE DISEASE - Avocado sunblotch disease

8 ### 1.2.1 History

9 Avocado sunblotch disease was first discovered in Southern California in 1914 (Whitsell,
10 1952; Horne and Parker, 1932). The green skin of the fruits displayed yellow sunken areas

resembling sunburn which later turned brown (Whitsell, 1952). This was therefore confused for a physiological disorder caused by sunburn (Coit, 1928). Later the disease was formerly described as graft transmissible which also led to it being mistaken again for a viral disease (Horne and Parker, 1932). Due to failed attempts to detect ASBVd using virus detection methods, Thomas and Mohamed (1979) investigated the possibility of the causal agent being a viroid. These researchers reported the presence of a low molecular weight (60-70 000 g/mol) molecule which they compared to other viroid diseases such as *Potato Spindle tuber viroid* (PStVd) (50 000 g/mol), *Citrus exocortis viroid* (CEVd) (50-60 000 g/mol) and *Coconut cadang-cadang viroid* (CCCVd) (84 000 g/mol).

However, Palukaitis *et al.* (1979) was the first to report the causal agent of Sunblotch disease as a viroid, *Avocado sunblotch viroid* (ASBVd). The first primary and a secondary structure of ASBVd were proposed to be 247 base pairs long (Figure 1.2; Symons, 1981). Symons (1981) compared ASBVd to the structures of viroids *Potato spindle tuber viroid* (PSTVd) and *Chrysanthemum stunt viroid* (CSVd) which were already known. The length of ASBVd appeared to be much smaller than the other two viroids (Figure 1.2). Furthermore, ASBVd shared a homology of only 18% with the two viroids whereas the two viroids shared a homology of 69% with each other (Symons, 1981). In RSA the disease was only discovered in 1954, however it was believed to have been present way before (Loest and Stofberg, 1954; da Graca and van Vuuren, 2003). In 1983, Sunblotch disease was reported to be present in all commercial cultivars in RSA (da Graca and Mason, 1983).

1.2.2 Causal agent – *Avocado sunblotch viroid* (ASBVd)

Avocado sunblotch viroid (ASBVd) is an infective single stranded covalently closed circular RNA molecule (Palukaitis *et al*., 1979; Symons, 1981; Saucedo-Carabez *et al*., 2015). ASBVd consists of a small rod like secondary structure between 239 and 251 base pairs (Hammond and Owens, 2006). ASBVd is the type member of family Avsunviroidae, the family does not consist of a central conserved region and the members of this family have self-cleavage ability (Delan-Forino *et al*., 2014). It is classified in the same family as *Peach latent mosaic viroid* (PLMVd), *Chrysanthemum chlorotic mottle viroid* (CChMVd) and *Eggplant latent viroid* (ELVd). ASBVd is structurally and functionally distinct from other family members, and differences between these viroids are demonstrated in Table 1.3. It is also the single member of genus *Avsunviroid* and it is the only viroid that processes RNA transcripts from cDNA clones at specific sites in the absence of enzymes (Pallas *et al*., 1988; Hutchins *et al*., 1986; Steger and Riesner, 2003).

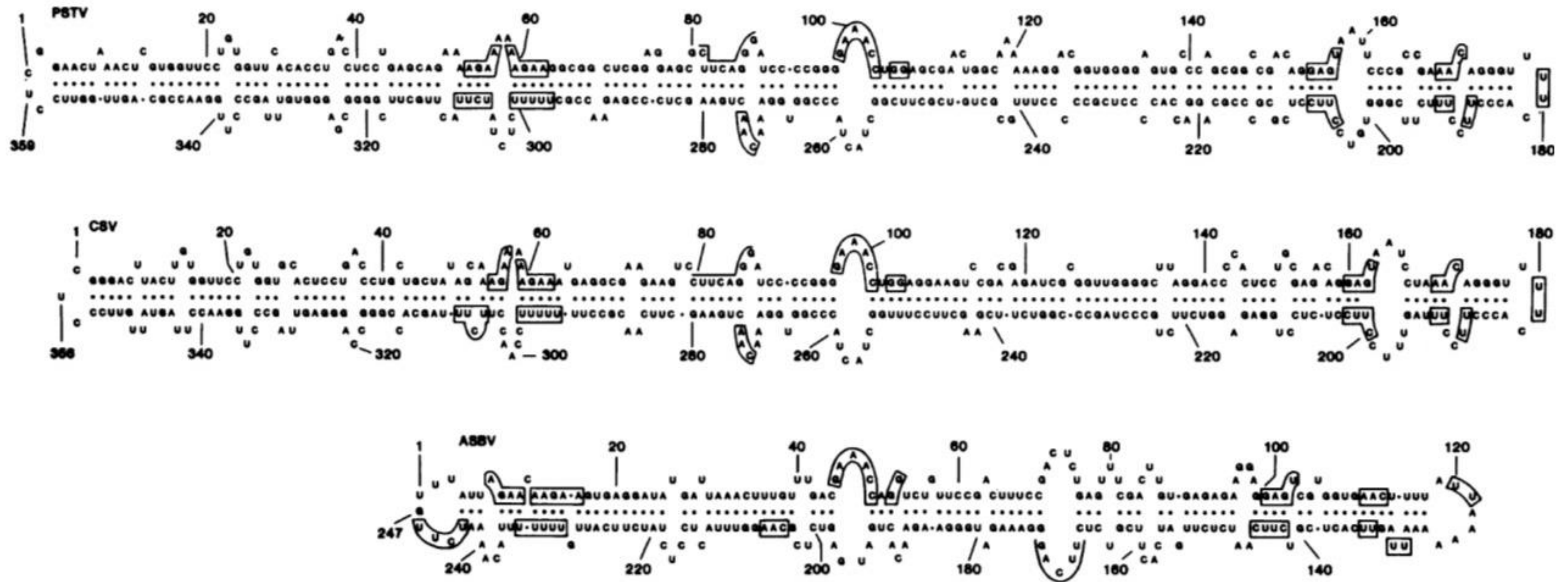


Figure 1.2 Proposed *Avocado sunblotch viroid* (ASBVd) secondary structure compared to that of *Potato spindle tuber viroid* (PStVd) and *Chrysanthemum stunt viroid* (CSVd) (Symons, 1981).

1 ASBVd consist of 108 different variants, which are grouped into four groups. The first group
2 is Avocado sunblotch variants; this is the larger group with 87 variants. The other three
3 group's namely symptomless carrier (ASBVd-Sc), Bleached (ASBVd –B) and Variegated
4 (ASBVd-V) are smaller with 10, 6 and 1 variant, respectively (Semancik and Szychowski,
5 1994). Different variants of ASBVd arise from slight sequence variations on the ASBVd
6 sequence (Running *et al*., 1996). Most of the changes occur between U-A bases leading to the
7 sequence variations (Shnell *et al*., 1997). All the variants share similar biological properties
8 and are thus regarded as the same strain (Semancik and Szychowski, 1994). The variation
9 found between ASBVd variants is a crucial factor to consider when developing molecular
10 techniques to detect this viroid.

11 1.2.3 Epidemiology

12 ASBVd is a systemic pathogen but its concentration can vary widely between the branches,
13 leaves and flowers within a single avocado tree (Running *et al*., 1996; Bruening *et al*., 1982).
14 The variation can be influenced by temperature and growing seasons (Running *et al*., 1996).
15 Damage of viroid diseases is known to be more severe in hotter climates compared to cooler
16 climatic regions (Singh, 1983). Increasing day/night temperatures of 28-30°C can accelerate
17 symptom development in Sunblotch disease indicator avocado trees (da Graca and Van
18 Vuuren, 1981). However, a combination of higher temperatures with consistent light at day
19 and night is a more effective in symptom development (Desjardins, 1987). Cutting back of
20 trees can also effectively accelerate symptom development in the infected avocado seedlings
21 (da Graca and Van Vuuren, 1980).

22 1.2.4 Economic impact

23 Viroid diseases lead both to direct and indirect losses in crop production (Randles, 2003).
24 However, there is a difficulty in stating the exact percentages on yield losses due to the lack
25 of essential quantitative data on diseases caused by viroids (Singh *et al.*, 2003). Generally,
26 ASBV infected trees are lower yielding and the infected fruits are discarded (Randles, 2003).
27 Similar with symptomless carrier trees, they produce lesser yield with most of the fruits being
28 downgraded on quality standards. No cultivar has been found to be tolerant to ASBVd
29 although symptom development is delayed on a Zutano cultivar (Wallace, 1958). Indexing
30 costs during the selection of parent material for propagation and eradication of infected trees
31 in the field have a direct economic impact on to avocado production (Wallace, 1958; Randles,
32 2003).

33

1 **Table 1.3** Species classified under the family *Avsunviroidae* together with ASBVd
2 (Hammond and Owens, 2006)

Genus	**Species**	**Variants**	**Length** (nt)	**Natural host**(s)
Avsunviroid	*Avocado sunblotch viroid* (ASBVd)	108	239- 251	avocado
Pelamoviroid	*Chrysanthemum chlorotic mottle viroid* (CChMVd)	21	397- 401	chrysanthemum
	Peach latent mosaic viroid (PLMVd)	168	335- 351	peach, nectarine
Elaviroid	*Eggplant latent viroid* (ELVd)	9	332- 335	eggplant

3

4 Several studies have been conducted worldwide to evaluate yield losses associated with
5 ASBVd infections (da Graca, 1985; Running *et al*., 1996; Randles, 2003; Tondo *et al* 2010;
6 Saucedo-Carabez *et al*., 2014; Ncango *et al*., 2014). ASBV affects the postharvest quality of
7 symptomatic fruits but asymptomatic fruits satisfy the international quality standards
8 (Saucedo-Carabez *et al*., 2015). However, the latter could have implications towards
9 quarantine restrictions since South African avocados are exported. In terms of yield
10 reduction, da Graca (1985) conducted a three year trial testing the effect of ASBVd in Fuerte
11 cultivars both in symptomless carrier trees and symptomatic trees. Symptomatic fruits
12 showed a reduction in yield of 14% while yield on the symptomless carrier trees was reduced
13 by 82%.

14 Running *et al*., (1996) conducted a study in Miami based on detection of ASBVd and
15 estimated infection rates among accessions in the national germplasm. They indexed 429
16 trees using a reverse transcription-polymerase chain reaction (RT-PCR) to determine the
17 incidence of infection, and found that 18.9% of the trees were infected with ASBVd. They
18 also discovered that infection rate between different races is the same; however the West
19 Indian race had higher ASBV infection rates (Running *et al*., 1996). A similar study was
20 conducted in 2009 on the same accessions using ASBVd specific RT-PCR (Tondo *et al.,*
21 2010). In this study, they discovered an increase in the infected number of infected trees from
22 19% in 1996 and 2006 to 24% in 2009. The newly infected plants were either adjacent to
23 previously infected plants, adjacent to plots from which infected plants had been removed, or

1 adjacent to other newly infected plants that are adjacent to previously infected plants or
2 contaminated plots. They related these infections to root grafting. Other new infections were
3 random and these were related to pollen transmission and contamination during pruning
4 (Tondo *et al*., 2010).

5 Korsten *et al.* (1986) conducted a study on occurrence of ASBVd in South African nursery
6 trees using a dot-blot hybridization technique. A total of 3 125 trees were tested and only 2%
7 of those trees tested positive for ASBVd. A similar study was repeated recently by Ncango *et*
8 *al.* (2014) using a reverse transcription polymerase chain reaction (RT-PCR) assay. A study
9 was conducted over a five year period from 24 685 avocado mother trees from 14 different
10 nurseries in Limpopo and Mpumalanga, RSA. From the tested trees 15.6% of the trees tested
11 positive for ASBVd. After 28 years the disease occurrence had already increased from 2% to
12 15.6%. ASBVd infection is not only increasing in RSA but also in other growing regions
13 worldwide. It is possible that ASBVd is more widespread because sunblotch symptoms vary
14 and symptomless carriers of ASBVd are common (Luttig and Manicom, 1999).

15 1.2.5 Geographical distribution

16 Avocado sunblotch disease has been reported in all avocado growing areas worldwide and
17 infects all avocado cultivars (Saucedo-Carabez *et al*., 2015). The disease has been reported in
18 Mexico, Guatemala and Central America (Sippel, 2001), North and South America,
19 Australia, Israel, South Africa (da Graca, 1980) and Ghana (Acheampong *et al*., 2008). This
20 is also presented in a map below (Figure 1.3)

21 1.2.6 Transmission

22 The use of infected propagative material is the most important mode of spreading Sunblotch
23 disease but there is not much evidence on the natural infection of Sunblotch disease in the
24 field from an infected tree to the healthy tree (Wallace, 1958). ASBVd can be transmitted via
25 seed from the infected tree used for propagative rootstock, scion used for grafting and via
26 root grafts and pollen. To date, no vector has been reported for the transmission of ASBVd.
27 Avocado is the only natural host for ASBVd (Shnell *et al*., 1997). The following modes of
28 ASBVd transmission were described:

29 *1.2.6.1 Graft transmission*

30 Sunblotch disease can be transmitted through the grafting of unhealthy scion onto a healthy
31 rootstock or vice versa. This type of transmission is regarded as the most important for the
32 spreading ASBVd (Bar-Joseph *et al.*, 1986). ASBVd was first discovered as graft

transmissible by Horne and Parker (1931). They described Sunblotch disease as graft transmissible and discovered that after grafting the disease will manifest after three to two years.

1.2.6.2 Root graft transmission

Spread by root grafting from an infected tree to an adjacent healthy tree has been reported for Sunblotch disease (Wallace, 1958). As the trees grow older the roots intersect, and this has been suspected to be one of the methods Sunblotch disease can be transmitted from an infected tree to a healthy one (Wallace, 1958). However, the frequency of root grafting in the field is unknown and could be of minor importance (Semancik, 2003).

1.2.6.3 Seed transmission

Seed transmission was described by Zentmyer (1946) and confirmed by Wallace (1950). There are two types of seed transmission, seed transmission from symptom-bearing trees and from symptomless carriers (Wallace, 1950). In 1953, Wallace and Drake indicated that seed obtained from symptomless carriers could transmit up to 100% of disease to the seedlings. The progeny of the symptomless carriers are all symptomless carriers (Wallace, 1958). Mathews (2011) mentioned that symptomless carrier trees have been found to maintain high levels of viroid in leaves, fruit and seed which could explain the high transmission rate. Seed obtained from plants showing symptoms can only transmit 0-5% of the disease to the new trees which develop visible typical Sunblotch disease symptoms (Wallace, 1958).

1.2.6.4 Pollen transmission

Pollen transmission occurs when a healthy avocado tree is pollinated by infected pollen, in this case only the fruits exhibit symptoms and the rest of the tree remain disease-free (Dodds, 2001). Desjardins *et al.* (1979) experimentally demonstrated pollen transmission on avocado plants and found a low transmission rate between 1.8% and 3.125%. It has been suggested that symptomless carrier trees may be the main sources of pollen transmission in the field since they maintain higher concentrations of ASBVd (Mathews, 2011).

1.2.6.5 Transmission by vectors

To date, no vector has been reported to transmit the viroid from one tree to next (Shnell *et al.*, 1997; Luttig and Manicom, 1999). However pollen transmission has been demonstrated using honeybees in caged trees, this could imply that honeybees could be possible vectors for ASBVd (Desjardins *et al.*, 1979; Dodds, 2001).

1.2.6.6 Mechanical transmission

Sunblotch disease can be transmitted on sap-contaminated pruning blades, injection material and harvesting clippers (Semancik, 2003). An 8% to 30% mechanical transmission by cutting blades has been reported (Dodds, 2001). Da Graca and Van Vuuren (1980) successfully transmitted ASBV by grafting infected tree bark strips to the cinnamon seedling stems. Slash inoculations and leaf rub with extracts from Sunblotch disease infected tissues has also proven to successfully transmit ASBVd to other members of *P. americana* (Semancik, 2003).

1.2.7 Symptoms and signs

The symptoms of Sunblotch disease manifests in two forms, one form is where the characteristic symptoms are displayed on the young green stem, leaves and the fruits of the infected tree. Sunblotch disease -infected trees may appear stunted, with branches spreading unevenly to the sides and the sprawling of the lateral branches (Dodds, 2001). The sprawling habit of the plants exposes the tree to the sunburn (Acheampong *et al*., 2008). The trees have abnormal growth where they grow in a flattened shape with limbs bending towards the ground (Wallace, 1958). The thinning of the tree canopy has also been described as one of Sunblotch disease signs (Dodds, 2001). The second form is where no symptoms are displayed and these trees are termed symptomless carriers (Thomas and Mohamed, 1979).

1.2.7.1 Stem symptoms

The symptoms appear as yellow or colourless sometimes reddish sunken longitudinal streaks on the green stems of young growth (Parker and Horne, 1931). On the older trees, the trunk usually develops rectangular cracking also referred to as alligator bark. This is one of the common Sunblotch disease symptoms on the field, and is of value in the diagnoses of Sunblotch disease (Wallace, 1958).

1.2.7.2 Fruit symptoms

Sunblotch disease fruit symptoms are caused by anatomical and chemical changes in the structure of the exocarp and mesocarp cells which results from cellular disorganisation, accumulation of phenolic compounds in the cytoplasm and cell walls and reduction in cytoplasmic content leading to cell collapse and death (Vallejo-Perez *et al*., 2014). Fruits develop streaks similar to those on the stem, depressed streaks with yellow or pink colour, which reduces fruit marketability (Vallejo-Perez *et al*., 2014). Streaks extend from the stem end to the entire fruit, sometime fruits appear small and misshapen (Wallace, 1958). A recent study by Vallejo-Perez *et al*., (2014) showed an increase of up to 62% in the phenolic compounds of the symptomatic fruits compared to the asymptomatic fruit, also a reduction of

up to 28% of both chlorophyll A and B. Chlorophyll reduction and increase in phenolic compounds leads to the development of yellow and pink symptoms on the rind (Vallejo-Perez *et al*., 2014). Fruit symptoms appear as indicated in Figure 1.4 (A-E).

1.2.7.3 Leaf symptoms

Leaf symptoms are expressed as white/yellow variegation and bleaching of the leaves, however these are very rare in the field (Semancik and Szychowski, 1994). The symptoms are associated with three ASBVd variants namely ASBVd-B associated with bleached symptoms, ASBVd-V associated with variegation and ASBVd-SC associated with symptomless carriers (Palukaitis *et al*., 1979; Dann *et al*., 2013) (Fig. 1.5). The variants differ in a small region in their nucleotide sequences but have different molecular weight (Semancik and Szychowski, 1994). The leaves may appear chlorotic under severe infections (Dodds, 2001).

1.2.7.4 Symptomless carriers

A symptomless carrier generally refers to a tree that is infected with ASBVd but doesn't show any visible symptoms and signs of the disease. These trees have recovered from the infection but they still carry the viroid in their tissues (Da Graca, 1980). The tree appears normal except that it produces lesser yield and has smaller leaves than an uninfected tree. Wallace and Drake (1962) demonstrated that symptomless carries arises from an infected symptomatic tree. The tree sends up new shoots that appear healthy; the leaves dominate the tree and replace all the symptomatic leaves. However, symptomless carriers can also arise from parent symptomless carrier trees that underwent a recovery during an early greenhouse stage and the appearance also becomes dominant. However, these trees can exhibit symptoms when they are subjected to stress for instance fire, or when the trees are cut back and when a healthy scion is grafted on a symptomless carrier tree (Dodds, 2001).

1.2.8 Host range

ASBVd has a narrow host range restricted to family Lauraceae, *Persia amaricana* species is the only natural host (da Graca and Van Vuuren, 1980). Cinnamon was the first alternative host to be described for ASBVd (da Graca and Van Vuuren, 1980). Da Graca and Van Vuuren (1981) reported three more hosts of ASBVd from the same family Lauraceae. These were Coyo (*Persea schiedeana*), stinkwood (*Ocotea bullata*) and camphor (*Cinnamomum camphora*). All three species developed typical ASBV symptoms however, no natural infection has been reported for all the alternative hosts and a low disease transmission rate was reported (da Graca and Van Vuuren, 1981).

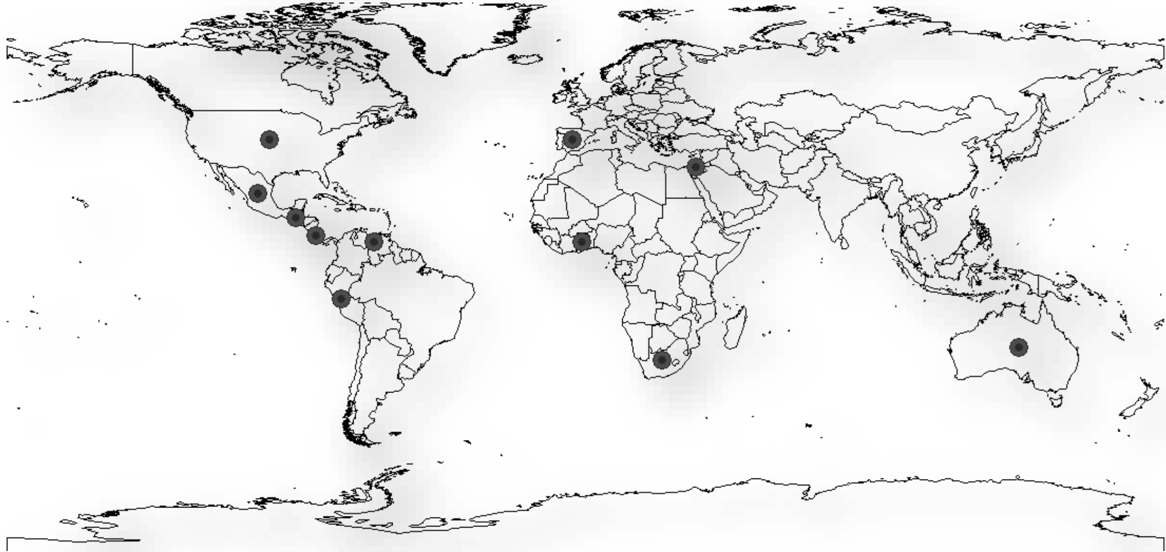


Figure 1.3 Distribution map of Avocado sunblotch disease (ASBV) in different avocado growing regions worldwide (Plant wise)

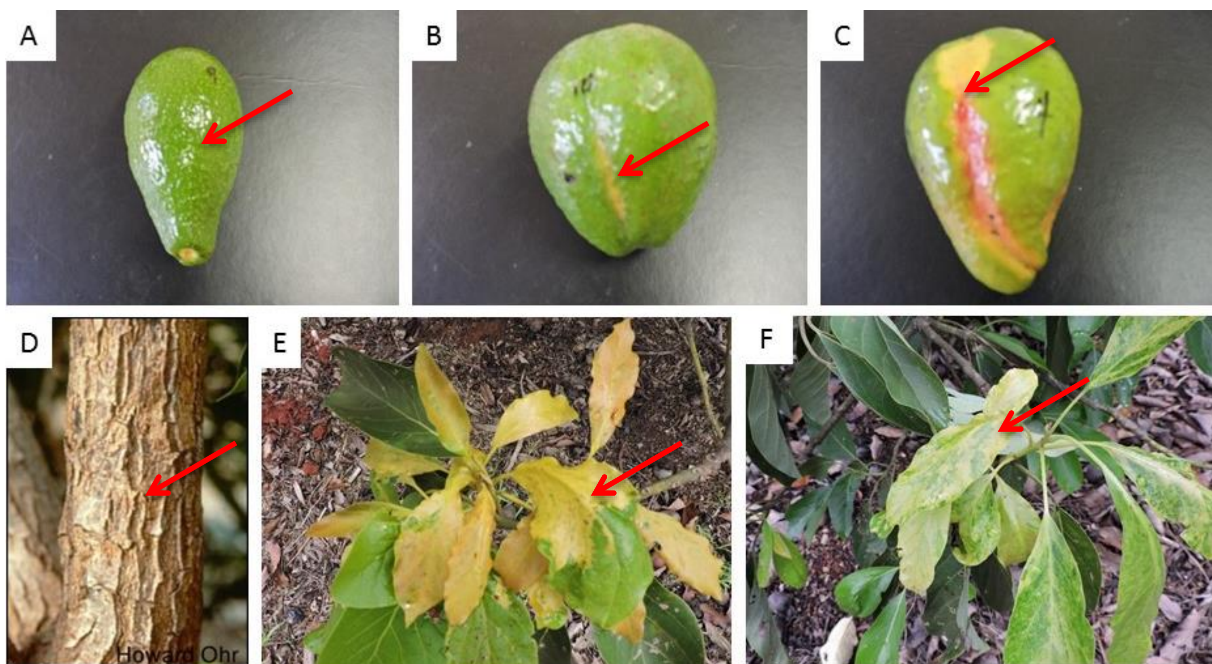
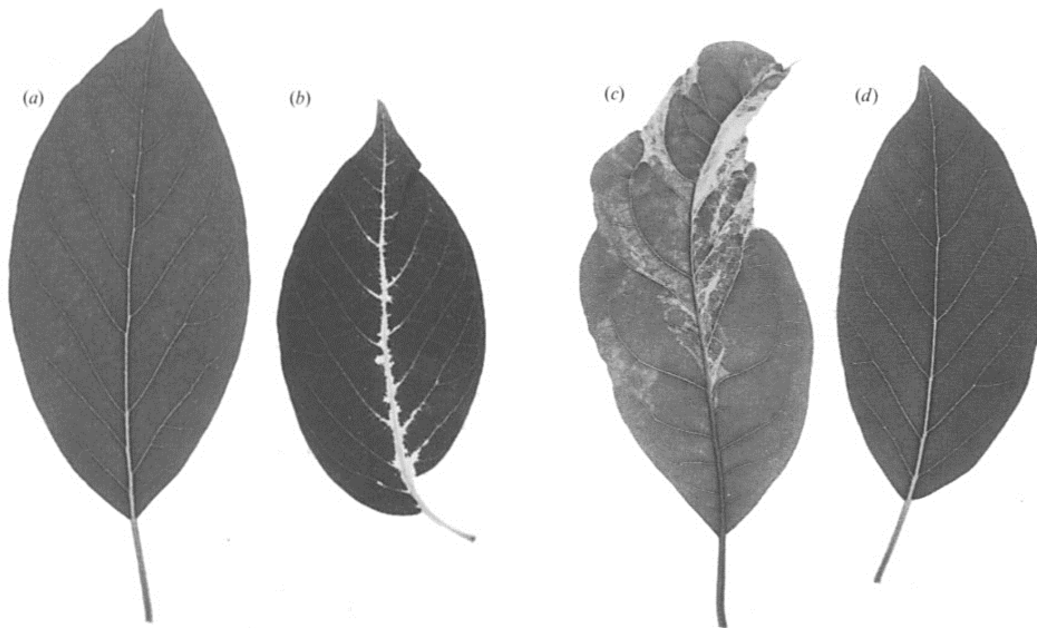


Figure 1.4 Avocado sunblotch symptoms observed in the field. Compared to (A) infected symptomless fruit, a (B) slightly infected fruit showing yellow sunken streak and a (B) severely infected fruit showing yellow sunken lesions with reddish streaks. (D) Rectangular bulk cracking and (E) and (F) severely infected leaves, with (E) severe variegation symptom and (F) severe bleaching symptom on leaves.

1



2 **Figure 1.5** Avocado sunblotch leaf symptoms associated with different variants, (a) a healthy
3 avocado leaf, (b) Variegated symptom associated with ASBVd-V variant, (c) a bleached
4 symptom associated with the ASBVd-B variant and (d) a symptomless carrier symptom
5 associated with ASBVd-SC variant. (Semancik and Szychowski, 1994).

6 1.2.9 Replication

7 ASBVd self cleaves in both polarities on a hammerhead ribozyme and replicates
8 autonomously when inoculated on avocado (Delan-Forino *et al.,* 2014). The replication cycle
9 of ASBVd is extremely dependent on enzymatic host activities (Navarro *et al*., 1999).
10 ASBVd replicates through an RNA-RNA symmetrical rolling circle mechanism in the
11 chloroplast (Fig. 1.6) (Delan-Forino *et al*., 2011; Saucedo-Carabez *et al*., 2014). Both the
12 negative and positive dimeric ASBVd RNA molecules fold on different directions and reach
13 their active self-cleaving structures (Delan-Forino *et al*., 2011). The negative strand is easily
14 cleaved using the double- hammered structure during *in vitro* transcription and by a single –
15 hammerhead structure after purification of dimeric RNA. A positive strand requires more
16 stable double-hammerhead structures for self-cleavage during and after *in vitro* transcription
17 (Delan-Forino *et al*., 2014). Positive strands are more dominant in the infected tissues
18 compared to the negative strands (Delan-Forino *et al*., 2014). Delan-Forino *et al* (2014)
19 presented new ASBV structures for both strands (Fig. 1.7).

20 1.2.10 Detection and management

21 The most important control measure for ASBV is careful selection of pathogen-free bud
22 wood and seed that are used for propagation, thus the importance of indexing (Wallace,

1958). Diagnosis of ASBVd are divided into two indexing techniques, these are the biological indexing and molecular indexing techniques.

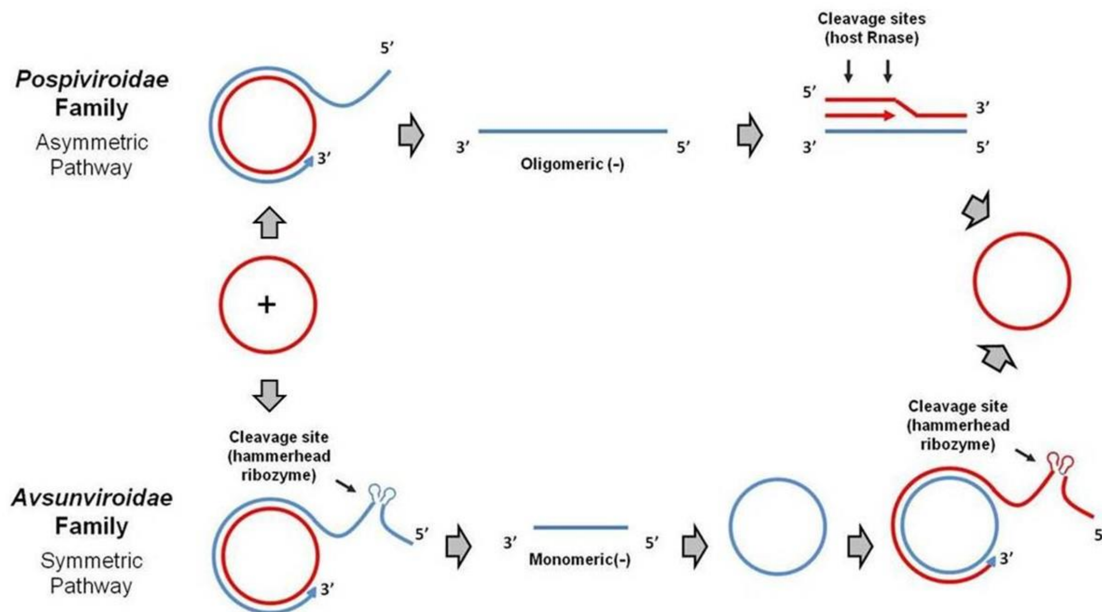


Figure 1.6 Replication cycle of Pospivioidae and Avsunviroidae family via a rolling circle mechanism (Delan-Forino *et al.*, 2011)

1.2.10.1 Diagnosis

1.2.10.1.1 Biological indexing

Biological indexing aims at planting of ASBVd disease-free trees, both the scion and the root stock must be disease free (Wallace, 1958). When undertaking biological indexing at least ten seedlings are desirable which about 3-5 months from germination. Indexing involves grafting a diseased bud or bark patches into a healthy indicator tree either Hass or Collison cultivar (da Graca and van Vuuren, 1981). Regrowth is forced by cutting 2-3 buds from the seedlings above germination. After 6 months of observation the top of the tree must be cut again to force new growth. The experiment can be undertaken both in the field or the greenhouse and if the experiment is undertaken in the field, the trees must be shaded to prevent sunburn which could mask early Sunblotch disease symptoms (Wallace, 1958). This method is effective for indexing however, due to delay in the infection and symptom development which can take up to 18 months makes this method unsuitable for commercial indexing (Burns *et al.*, 1969). Even though exposing the trees to higher temperatures (28-30°C) can accelerate symptom development to 8 months (Shnell *et al.*, 1997). This is still too long for commercial indexing.

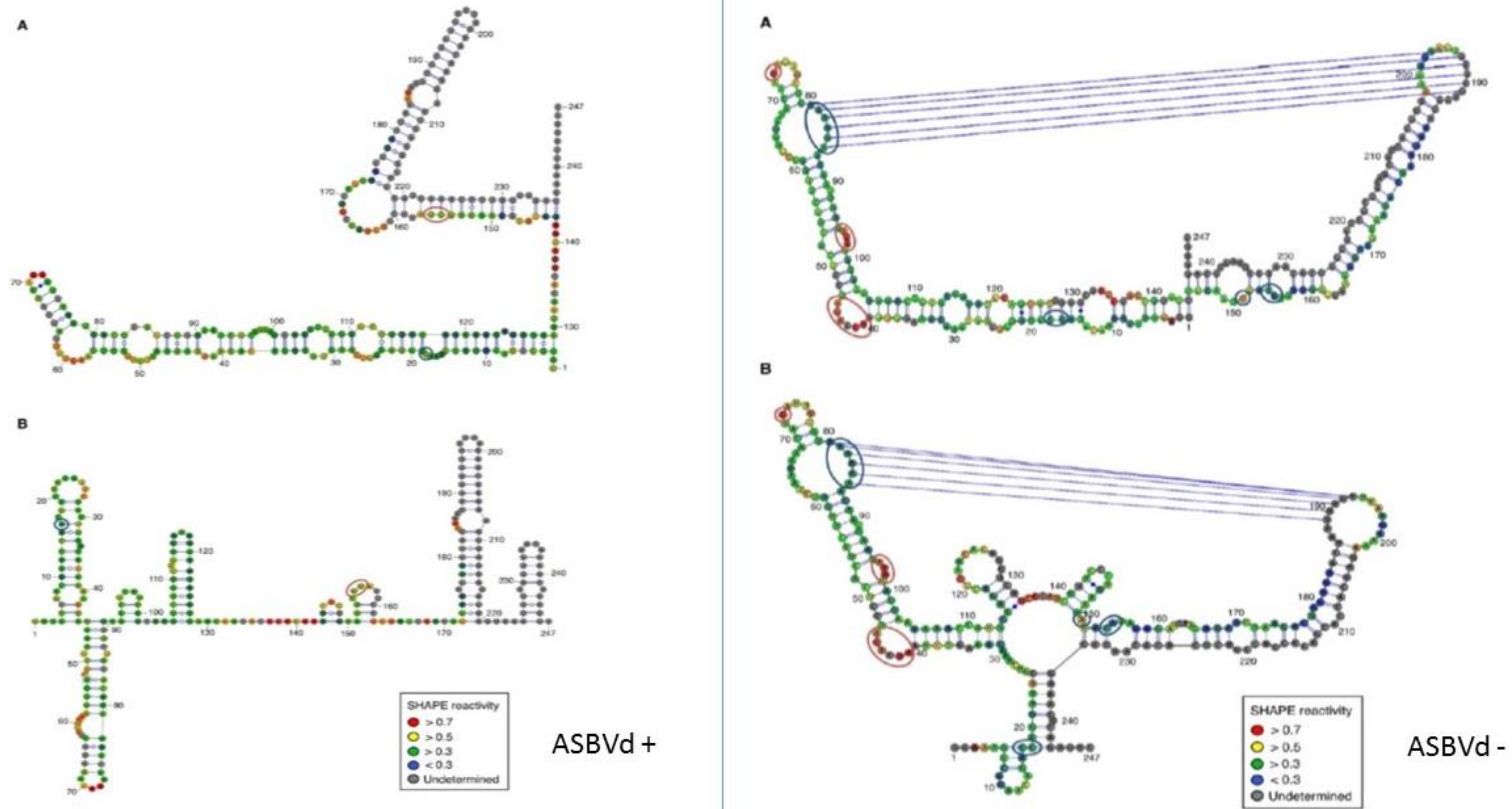


Figure 1.7 Proposed secondary structure models of both negative and positive strands of ASBVd (Delan-Forino *et al*., 2014).A and B are both proposed ASBVd different structures of both a positive and negative strands

1.2.10.2 Molecular techniques

1.2.10.2.1 Standard polyacrylamide gel electrophoresis (sPAGE)

Standard polyacrylamide gel electrophoresis (sPAGE) is based on separation of the RNAs in 5% polyacrymide gels (da Graca and van Vuuren, 1981). The method requires the use of stains, one of the stains is 0.01% toluidine blue stain. This stain requires to be destained with several changes of 5% acetic acid and viewed the gels under UV light. The other stain is the ethidium bromide. Ethidium bromide proved to be the most suitable compared to the toluidine due to its quick reaction. However, using this method for indexing had limitations; the lower concentrations of ASBVd couldn't be detected thus making the method unreliable for detection (da Graca and Trench, 1985; Running *et al*., 1996). Moreover, the method failed to detect ASBVd from the symptomless carrier plants (Shnell *et al*., 1997).

1.2.10.2.2 Hybridization analysis with a 32P labelled cDNA probe

A research by Running *et al*. (1996) proved that the hybridization with a 32P labelled cDNA probe method was more sensitive and reliable than the sPAGE method in ASBVd indexing. The method was rapid and sensitive but it was costly and used radioactivity which can be hazardous to human health when screening large amounts of samples.

1.2.10.2.3 Dot-blot hybridization with 32P labelled ASBVd

This method works by spotting the healthy and infected leaf extracts on nitrocellulose paper and hybridized with 32P labelled synthetic probes (Bar-Joseph *et al*., 1985). Dot blot hybridization is a simple and quick method for indexing but it is less sensitive and some results could be interpreted as false positive (Acheampong *et al*., 2008).

1.2.10.2.4 DNA hybridization with digoxigenin (DIG) labelled probes

Methods used were described as short-lived, expensive and ecologically unfriendly (Manicom and Luttig, 1996). An alternative method was discovered by Boehringer by the substitution of radioactive labelling. Manicom and Luttig (1996) conducted and experiment using dimeric clones of ASBVd, labelled with DIG which was used as probes in DNA-RNA dot-blot hybridization. Although safer to use, this method also missed some ASBVd positive samples (Luttig and Manicom, 1999).

1.2.10.2.5 RT-PCR

A test that was sensitive, safe and can be used in a large commercial scale was required for the indexing of ASBVd. RT-PCR was adapted from the detection of *Apple scar viroid* (ASSVd), *Dapple apple viroid* (DAVd), *Citrus exocortis viroid* (CEVd), and *Cachexia viroid*

(CCaVd) and was also developed for the detection of ASBVd (Schnell *et al*., 1997). Hadidi and Yang (1990) used 0.5-1 ng/µl of total nucleic acid of ASBVd with ASBVd specific primers. Indexing time was extremely decreased and no safety hazards were reported (Shnell *et al*., 1997). The method was then adapted for ASBVd indexing, both purified and unpurified RNA samples were suitable for the detection of ASBVd (Shnell *et al*., 1997). To date RT-PCR is the most commonly used method for ASBVd indexing. There are two types of RT-PCR used for Sunblotch disease indexing; these are conventional RT-PCR and quantitative real time PCR (qRT-PCR).

In conventional RT-PCR clone DNA is synthesized using two ASBVd specific primers. The cDNA is run on an agarose gel to check for the size of the band using a molecular weight marker. The conventional RT-PCR is sensitive enough but it takes a longer time. In addition, the chances of contamination of DNA with the pathogenic DNA is possible when loading samples. Moreover, the variations between the samples are very hard to detect based on the band size only.

Real-time PCR is more sensitive and faster than conventional PCR, and results are detected on the early stages of the reaction and the variations between samples can be detected (Bar-Joseph *et al*, 1986). There are two types of real time qRT-PCR, one method uses probes which is expensive and thus impossible to use for commercial indexing. The most used method is the fluorescent based qRT-PCR; the commonly used dye is SYBR Green 1 (Ncango *et al*, 2014). The dye binds to the double stranded DNA minor groove and the intensity of the fluorescence increases with an increase in DNA amplicons in the reaction. However, the dye binds to any double stranded DNA in the reaction, therefore specific primers are used, a known positive and the standards which help with the accurate detection.

1.2.10.3 Viroid inactivation by sanitisation

The use of 5% commercial bleach (sodium hypochloride) has been proven to be effective in inactivation of some viroids, and the use of the commercial bleach for the inactivation of ASBVd was demonstrated by Desjardins *et al*. (1980). The use of a 1:1 mixture of 2% sodium hydroxide and 2% formaldehyde or 6% solution of hydrogen peroxide were effective in the inactivation of the viroid on pruning tools, harvesting clippers and injection equipment (Desjardins *et al*. (1987).

1 *1.2.10.4 Eradication*

2 All the suspected trees should be indexed and all the infected trees and those within a 15m
3 radius to the infected trees must be removed. Symptomless carriers are not easy to spot in the
4 field thus making detection of the disease very difficult (Schnell *et al*., 1997).

5 1.3 CONCLUSIONS

6 Most avocado profits rely on the exportation of good quality fruits. Since the South African
7 avocado industry is export oriented, it is of utmost importance that disease-free avocados are
8 produced all the time. Avocado sunblotch disease affects the quality and the yield of the
9 fruits. Ensuring sensitive detection of ASBVd is crucial to avoid losses and restrictions that
10 could arise when infected material is exported. According to the findings of most studies
11 conducted for ASBV worldwide, an increase in the occurrence of Sunblotch disease was
12 reported over the years. Shipping of avocados is affected by factors such as storage
13 temperatures with a minimum of 28 days cold storage (Blakey, 2011). Therefore,
14 unnecessary shipping delays and quarantine procedures could affect the fruits thus leading to
15 economic losses for avocado industry. Therefore, keeping up with disease advancement is
16 important in order to generate knowledge about Sunblotch disease. The lack of information
17 about the management of the disease can be a hindrance in future production. Propagation of
18 disease-free trees is considered the first step in establishing healthy plants (Saucedo-Carabez,
19 2014). This is achieved through regular and precise indexing of propagation material to
20 prevent unnecessary Sunblotch disease outbreaks.

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24

CHAPTER 2

Validating the sensitivity of detection techniques against *Avocado sunblotch viroid* (ASBVd) and studying the distribution of ASBVd in a single infected tree using fluorescent based real- time RT- PCR

Abstract

Avocado sunblotch disease is a chronic, infectious disease of avocado induced by *Avocado sunblotch viroid* (ASBVd). Sunblotch disease is the only viroid disease of economic value infecting avocados worldwide leading to losses and fruit being degraded on quality standards. Elimination of Sunblotch disease is considered the first step to control the disease. This is achieved through indexing of all propagation material whether using biological control or molecular diagnostics. The objective of the current chapter was to validate the sensitivity of the ASBVd indexing method currently used at the ARC-TSC laboratory and to study ASBVd distribution between branches of a single infected tree. To validate the sensitivity of ASBVd detection techniques, an ASBVd infected tree was identified in the glasshouse at the ARC-TSC, Nelspruit, showing typical ASBVd symptoms on the leaves and stem. A single Sunblotch disease infected leaf was selected and combined with 9, 19, 29, 39, and 49 ASBVd negative leaves, respectively. RNA was extracted from the leaves using two methods; a small-scale cetyltrimethylammonium bromide (CTAB) - based RNA extraction method which was compared to a large-scale cellulose column chromatography extraction method. From each method RNA was amplified using a fluorescent based one-step real-time RT-PCR reaction, in a Rotor Gene Q instrument. Here, two primer sets were compared in separate reactions: a published primer set by Bar- Joseph *et al*. (1985) amplifying a 247bp product and a primer set modified by Jooste (unpublished) resulting in a 99bp product. The most sensitive results were obtained using the Jooste (unpublished) primer set from RNA extracted with the cellulose column chromatography method making it the more sensitive and reliable ASBVd detection method. Tested secondly, RNA was amplified using a two-step conventional RT-PCR in a thermocycler. Two primer pairs were compared that included two published primer sequences by Bar- Joseph *et al*. (1985), amplifying a 247bp product, and primer sequences by Luttig and Manicom (1999) resulting in a 250bp amplicon. The more sensitive results were obtained using the Bar- Joseph *et al*. (1985) primer set from both the CTAB and Cellulose column chromatography extraction methods. In this study we demonstrated the reliability of a large scale indexing method for ASBVd indexing.

To study the distribution of ASBVd in a single infected tree, leaf and fruit samples were collected from three nurseries in Limpopo, including symptom-bearing trees and known ASBVd positive symptomless carrier trees. Branches of the same tree were sampled separately by collecting young and old leaves and fruits were sampled when present. ASBVd distribution within a single infected fruit was investigated. The fruits were divided into three categories: symptomless fruits; slightly infected fruits and severely affected fruits, dividing the skin into yellow infected part and green part in symptom bearing fruits. RNA was extracted using a large-scale cellulose column chromatography method; purified RNA was amplified with a fluorescent based one-step real-time RT-PCR reaction on a Rotor Gene Q instrument using the Jooste (unpublished) primer set. In this study an even distribution of ASBVd between the branches of the same tree and symptomless fruits was demonstrated. A variation in ASBVd distribution in symptom - bearing trees was observed, these showed an uneven distribution between the branches and between the leaves and fruits. The variations in the distribution were also observed within single symptom-bearing fruits. The uneven distribution of ASBVd between the branches of an avocado tree has a direct implication on the accuracy of the sampling method for indexing avocado trees. ASBVd was found to be evenly distributed between the branches of symptomless carrier trees; these trees were also found to contain high ASBVd concentrations compared to the symptom-bearing trees. The latter could pose a threat in disease spread and could be the source of new infections in the orchards. These findings will improve the sampling method thus increasing the reliability of indexing. This will also lead to improved management of Sunblotch disease in South Africa.

2.1 Introduction

Avocado sunblotch disease is a chronic, infectious disease of avocado induced by *Avocado sunblotch viroid* (ASBVd) (Manicom, 2001). Avocado sunblotch is the only viroid disease of economic value infecting avocados worldwide leading to losses and fruit being degraded on quality standards (Acheampong *et al.*, 2008). Avocado sunblotch-infected trees may appear stunted, with branches spreading unevenly to the sides and sprawling of the lateral branches (Dodds *et al.*, 2001). The sprawling habit of the plants exposes the tree to sunburn (Acheampong *et al.*, 2008). The infected trees sometimes display an abnormal growth where they grow in a flattened shape with limbs bending towards the ground (Wallace, 1958). The symptoms of Sunblotch disease manifests in two forms, one form is where the characteristic symptoms are displayed on the young green stem, leaves and the fruits of the infected tree. The second is where no symptoms are displayed and these trees are termed symptomless

carriers (Thomas and Mohamed, 1979). The thinning of the tree canopy has also been described as Sunblotch disease symptom (Dodds *et al.*, 2001).

The use of infected propagative material is the most important mode of spread of Sunblotch disease, and there is not much evidence on the natural infection of Sunblotch disease in the field from an infected tree to the healthy tree (Wallace, 1958). Sunblotch disease can be transmitted via seed from the infected tree used for propagative rootstock, scion used for grafting and via root grafts and pollen. To date, no vector has been reported for the transmission of Sunblotch disease (Shnell *et al.*, 1997; Luttig and Manicom, 1999). Avocado is the only natural host for ASBVd and can only be transmitted to other trees by mechanical inoculation (Shnell *et al.*, 1997). Elimination of Sunblotch disease is considered the first step to control the disease. This is achieved through indexing of all propagation material whether using biological control or molecular diagnostics.

Many methods have been tested over the years and RT-PCR proved to be the most reliable and sensitive diagnostic tool for ASBVd. Since the discovery of using RT-PCR to detect ASBVd, the sensitivity of this method has never been doubted. However, complaints of inconsistencies in results have been reported by farmers in the past few years. The objectives of the current study were to investigate the sensitivity of the method using both the real-time and conventional RT-PCR to optimize a cost effective, rapid, quality assured diagnostic tool. The distribution of ASBVd between the branches of a single tree was also investigated to establish the correct sampling procedure when samples are being sent for indexing.

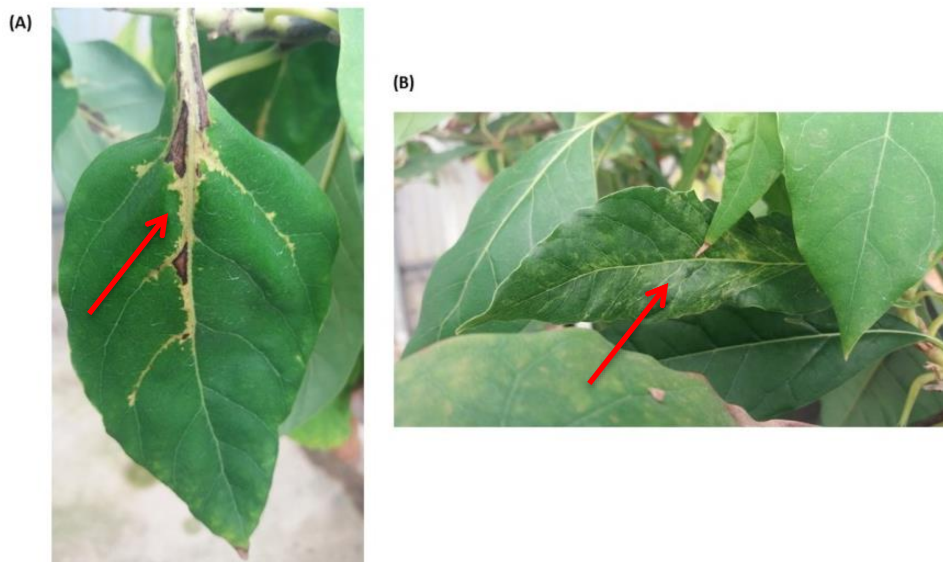
2.2 Materials and methods

2.2.1 Sample collection

For the limit of detection study, an ASBVd infected tree was identified in the glasshouse at the ARC-TSC (Nelspruit) showing typical ASBVd symptoms on the leaves and stem (Fig. 2.1). A single Sunblotch disease infected leaf was selected and combined with 9, 19, 29, 39, and 49 healthy avocado leaves, respectively, obtained from ARC-TSC field sources (Table 2.1).

For the distribution studies, infected trees were selected from three nurseries in the Limpopo province. Plants were either symptom-bearing or had been previously diagnosed as positive for ASBVd. In a single tree, each branch was indexed separately. Furthermore, the distribution within a single fruit was investigated by separating the infected, yellow part of the skin from the green part within the same fruit. The fruits were divided into three

1 categories: infected symptomless fruits, slightly infected- and severely infected fruits (Fig.
2 2.2).



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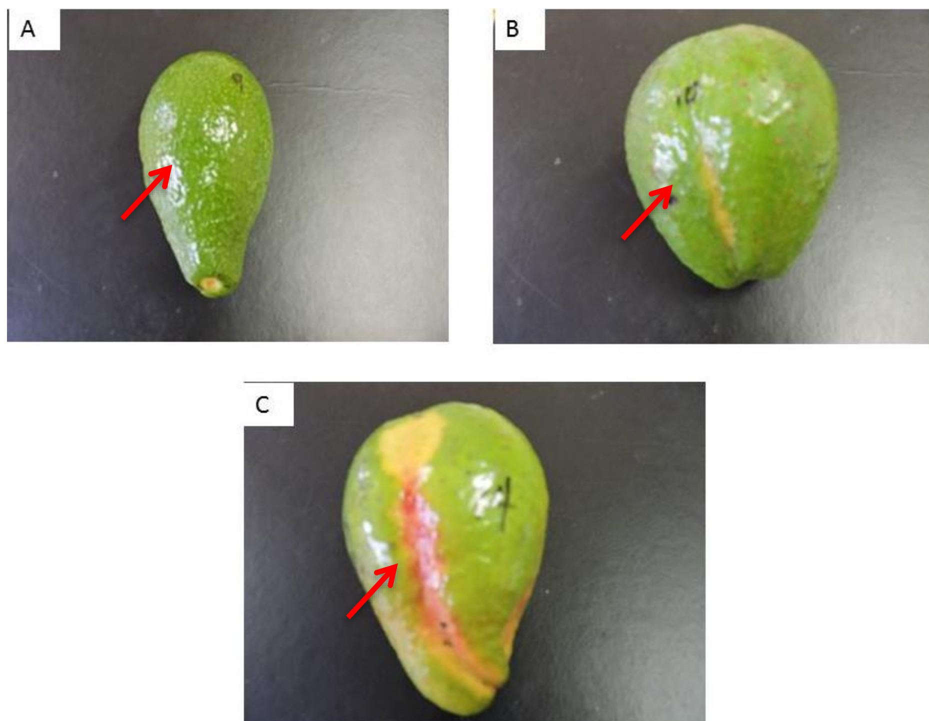
4 **Figure 2.1** ASBV positive tree that was used to select a single infected leaf to be used in the
5 limit of detection experiment. **(A)** Leaf from one branch of the tree showing variegated
6 ASBV symptom and **(B)** leaf from a different branch of the same tree showing a bleached
7 ASBV symptom.

8



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10 **Figure 2.2** Avocado sunblotch disease symptoms on avocado fruits: A- symptomless infected
11 fruit; B-slightly infected fruit showing yellow sunken patches on green skin; and C- severely
12 infected fruit showing yellow and pink patches on infected fruits.

1 **Table 2.1** Description of how the ASBVd infected leaf was combined with negative leaves to
2 study the limit of detection of a single infected leaf

Sample	Infected number of leaves	Negative number of leaves
Positive control	1	0
Negative control	0	1
10	1	9
20	1	19
30	1	29
40	1	39
50	1	49

3

4 2.2.2 ASBVd RNA extraction
5 Two different extraction methods were compared; one was a small-scale RNA extraction
6 method using Cetyltrimethylammonium bromide (CTAB) (White *et al*., 2008). This was
7 compared to a Cellulose column chromatography extraction method (Luttig and Manicom,
8 1999) currently being used at the ARC-TSC laboratory for large scale indexing. The two
9 extraction methods were as follows:

10 *2.2.2.1 Cellulose column chromatography method*
11 Fresh leaf and fruit tissue (400mg) was mixed with 5ml avocado extraction buffer and 2.5ml
12 chloroform/iso-amyl alcohol (CL/iso) (24:1) into Agdia grinding bags. The samples were
13 ground using a drill press; approximately 1.8ml of the supernatant was transferred into 2ml
14 Eppendorf tubes containing 200µl CL/iso (24:1). The tubes were spun for 3 min at 13500
15 rpm, 700µl of the supernatant was mixed with 300µl of EtOH into a new 2ml Eppendorf tube
16 and loaded onto a column of Cellulosepulver MN 2100 (450µl Cellulosepulver MN 2100
17 slurry packed in 1ml Avacare syringes. Tubes were allowed to drain, washed with 1xSTE_{35}
18 (50 mM Tris-HCI, 0.1 M NaCl, 1mM EDTA, pH 6.8 and 35% alcohol), allowed to drain and
19 eluted with 500µl of 1x STE into a new 2ml tube. RNA was precipitated by adding three
20 volumes of EtOH and 50µl of 3M NaAc (pH 5.2); this was stored overnight at -20°C.
21 Samples were centrifuged for 10 minutes, the supernatant was discarded, and the pellet was
22 dried for 30 minutes and re-suspended in 50µl dH_2O.

23 *2.2.2.2 Cetyltrimethylammonium bromide (CTAB) based RNA isolation method – small scale*
24 *(White et al., 2008)*
25 Four millilitres of preheated CTAB containing 3% β-Mercaptoethanol was added to 200mg
26 fresh avocado leaf tissue in Agdia grinding bags. Using a drill press the mixture was
27 thoroughly ground and transferred into a 2ml Eppendorf tube, vortexed and incubated in a
28 water bath for 30min. The samples were spun at 13500 rpm for 10min at 4°C and 1000 µl
29 supernatant was transferred to a new tube containing 1000 µl chloroform/Iso-Amyl alcohol

(CL/iso) (24:1). The mixture was vortexed for 30s and spun at 13 000 rpm for 15 min at 4°C. The step was repeated using 800 µl of supernatant to 800 µl of chloroform/Iso-Amyl alcohol (CL/iso) (24:1). A total of 600 µl of the supernatant was transferred into a new 2 ml tube; RNA was precipitated by adding 200 µl 8M LiCl, and stored overnight at -20ºC. The precipitate was spun down at 13000 rpm for 60 min at 4°C and the supernatant was discarded. The pellet was washed with 500µl 70% EtOH, spun for 15 min and the EtOH discarded. The tube was spun for 5 min and the remaining EtOH was pipetted out. The pellet was dried on ice for 15 min and re-suspended in 50µl dH_2O.

2.2.3 Amplification of ASBVd using a one-step real time reaction

A Fluorescent based one-step real-time RT-PCR was optimised to amplify samples using the qPCRBIO SyGreen 1-Step Go Lo-ROX kit (PCRBIOSYSTEMS), according the manufacturer's instruction, in a QIAGEN Rotor Gene Q instrument. ASBVd specific primer sets, Jooste (unpublished) and Bar- Joseph *et al*. (1985), amplifying a 99bp and 250bp product respectively, were used at final concentration of 400nM in the reaction. Two microliter of a variable final concentration of template RNA was added in a 20µl reaction. The cycling conditions were as follows: reverse transcription at 50°C for 10 min, followed by a polymerase activation of 95°C for 2 min. The PCR step had 35 cycles with denaturation step at 95°C for 5 sec and annealing step at 60°C for 30 sec.

Results were analysed using four standards that were the serial dilutions of a known positive control diluted to 1:10, 1:100, 1:1000 and 1:2000, respectively. Positive-, negative- and non-template controls were included. These controls were previously confirmed templates. Cyclic threshold (Ct) values of the standards were used to determine whether the samples were positive or negative, samples with Ct value lower than the 1:2000 standard was considered negative.

2.2.4 Amplification of ASBVd for conventional RT-PCR detection

Samples were amplified using a two-step RT-PCR in a Go Script™ Reverse Transcription system (Promega) followed by a PCR using the GoTaq® Flexi DNA Polymerase system (Promega). Two different ASBVd primer sets were used in separate reactions Bar- Joseph *et al*. (1985) and Luttig and Manicom (1999) (Table 2.2). The cDNA strand was synthesised from annealing 2 µl RNA template to 0.3 µl of the respective reverse primer at 70°C for 5 min, in a total volume of 5 µl, followed by cooling on ice for 5 min. Five microliter of the annealed products was added to the reverse transcription (RT) mix containing 2 µl GoScript™ 5x RT buffer, 0.6 µl $MgCl_2$ (25mM), 1 µl dNTP (10mM), 0.05 µl RNase

inhibitor (40U), 0.5 µl GoScript™ RT in a total volume of 7.5 µl. The RT products were synthesized in a ProFlex PCR system thermocycler (Applied Biosystems by Life technologies) with the following cycling conditions: annealing at 25°C for 5min; extension at 42°C for 60 min and inactivation at 70°C for 15 min.

Four microliters of the RT reaction was used as template in a Go Taq® PCR reaction adding 5 µl 5 x GoTaq® Flexi DNA Polymerase buffer, 2 µl $MgCL_2$ (25mM), 0.5 µl dNTP (10mM), 0.5 µl forward and reverse primers (10µM each) and 0.25 µl GoTaq® Flexi DNA Polymerase (5U/ µl) in a 25 µl total volume reaction mix. Amplification with the Luttig and Manicom (1999) primer set was carried out using the following conditions: polymerase activation at 95°C for 5 min; denaturation at 95°C for 30 sec; annealing and extension at 60°C for 1 min, repeated for 30 cycles, and the final extension at 72°C for 5 min. Amplification with the Bar-Joseph *et al*. (1985) primer set was carried out using the following conditions: polymerase activation at 95°C for 5 min; denaturation at 95°C for 30 sec; annealing at 60°C for 30sec; extension at 60°C for 30sec min, repeated for 30 cycles, and the final extension at 72°C for 5 min. PCR products were visualised on a 2% agarose gel using 1x TAE buffer and stained with ethidium bromide.

Table 2.2 Three different ASBVd-specific primer pairs used in the study

Primer source	Primer sequence	Amplicon (bp)
Bar- Joseph *et al.* (1985)	F: 5'-AAGTCGAAACTCAGAGTCGG-3' R: 3'-GTGAGAGAAGGAGGAGT-5'	247
Luttig and Manicom (1999)	F: 5'-ATCACTTCGTCTCTTCAGGGAAAGA-3' R: 3'-CAAGAGATTGAAGACGAGTGAACTA-5'	250
Jooste, unpublished	Unpublished	99

2.3 Results

2.3.1 Limit of detection study

2.3.1.1 Amplification of ASBVd using a one-step real time reaction

Figure 2.3 represents cycling data obtained from using a real-time fluorescent-based quantitative PCR for two ASBVd specific primers. Firstly, ASBVd was amplified using RNA extracted with the cellulose column chromatography method using the Jooste (unpublished) primer set, amplifying a 99bp product (Fig. 2.3 A). The sample containing one infected leaf in 49 healthy leaves was detected before the 1:2000 standard cut off threshold with the positive control being amplified within 5 cycles of the start of the run (Fig. 2.3A). The

quantitative data indicated that a positive control had a Ct value of 4.61 and a calculated concentration of 26 433 copies/µl (Table 2.3). As the number of healthy leaves per single infected leaf increased from one to 1 in 49, Ct values increased from 4.61 to 10.51, there was also a drastic decrease in the concentration from 26433 copies/µl of a positive control to 1064 copies/µl of one infected in 49 healthy leaves. There was a low variation between the calculated concentration and the given concentration that ranged from 0.2% to 8.6% validating the accuracy of the run. A decrease in sensitivity of the primers was observed when using the same primer set to amplify RNA extracted from a CTAB method. The variation percentage between the calculated concentration and the given concentration was increased to a higher range of 7.9% to 21.9% (Table 2.4). There was a decrease in concentration of the positive control, detected at 5 977 copies/µl, and an increase in the Ct value to 8.06. A decrease in calculated concentration and increase in the Ct values was observed when the number of negative leaves increased (Table 2.4). Here the samples were still detected as positive but the raw cycling data showed that the samples started to amplify after 20 cycles (Fig. 2.3 B).

Comparison of the two extraction methods continued and the primer set (Bar- Joseph *et al*., 1985) amplifying the 247 bp product was used to amplify template RNA extracted with the cellulose chromatography method. A calculated concentration of 18,526 (copies/µl) with a Ct value of 18.67 was detected for the positive control. The Ct values for all the samples were very high and the percentage variation between the given and calculated standards was too high (Table 2.5). Quantitation data (Table 2.4) did not correspond to cycling data (Fig. 2.3C). One infected leaf in 49 healthy leaves presented as positive in the quantification data had a Ct value lower than the 1:2000 standard. In the cycling data, 1:49 was amplified after 1:2000 and this is regarded as a negative. These results are highly unreliable and this could easily represent a false negative/ positive result. The same primer set was used to amplify cDNA from RNA extracted from a CTAB method (Fig 2.3D). A positive control amplified after 15 cycles. Ct values obtained were high and the positive control had a Ct value of 14.98. The concentrations obtained were also very high reaching 311,714 (copies/µl) for a positive control (Table 2.6).

2.3.1.2 Amplification of ASBVd for conventional RT-PCR detection

In addition, two primer sets were compared in a conventional RT-PCR for ASBVd (Bar-Joseph *et al*. (1985) and Luttig and Manicom (1999) (Table 2.2) using RNA template extracted with both methods. Results can be seen in Figure 2.4 and Figure 2.5. In both RT-

PCR systems the correct size amplicon was amplified, namely 247 bp and 250 bp, respectively. The primer pair resulting in the 247 bp amplicon produced bright and clearer bands for RNA extracted using both the CTAB and cellulose chromatography methods (Fig. 2.4). The primer pair amplifying the 250bp product amplified a clear product and detected all the samples (Fig. 2.5). Both extraction methods yielded the same quality product and no difference was observed in conventional PCR.

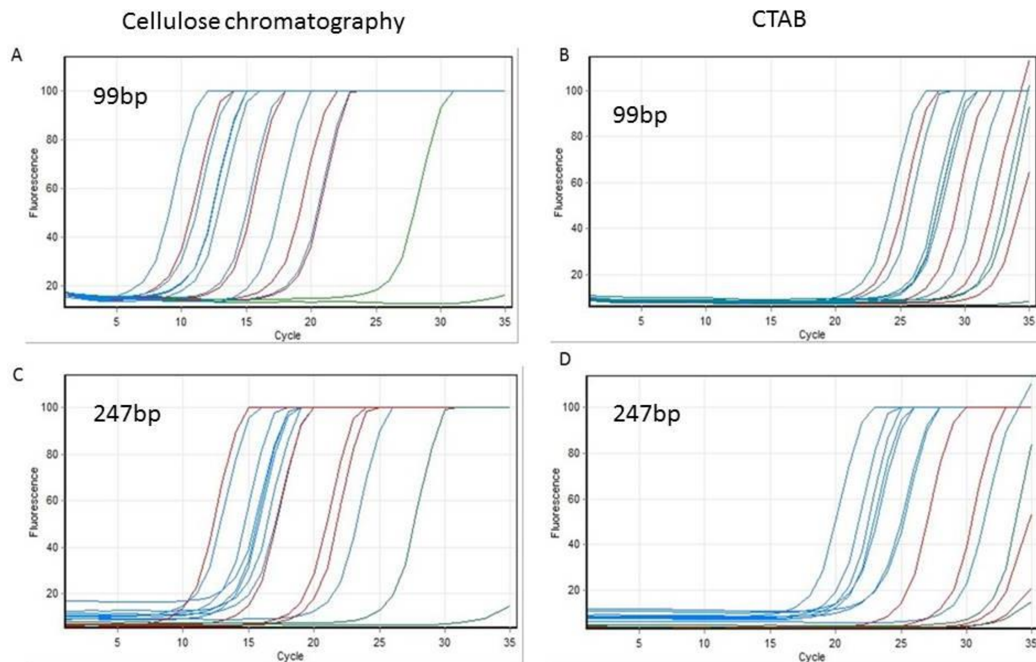
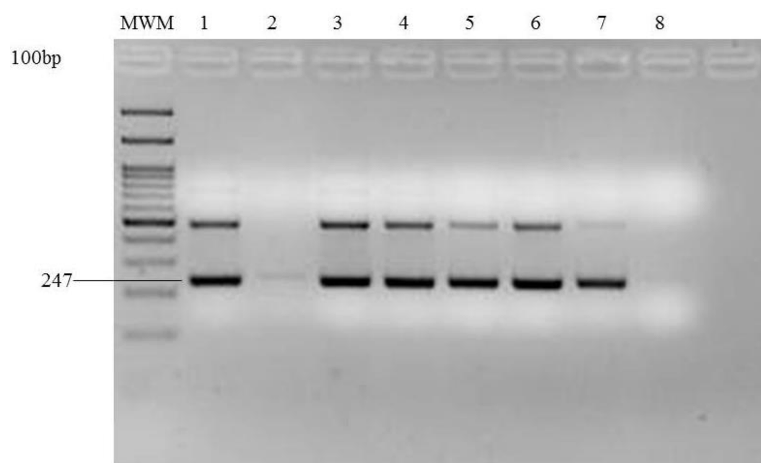


Figure 2.3: Cycling data for ASBVd obtained from a real-time fluorescent based quantitative RT-PCR for two ASBVd specific primers. A- ASBVd RNA extracted using column chromatography and cDNA amplified using the Jooste (unpublished) primer set; B- ASBVd RNA extracted using the CTAB method and cDNA amplified using Jooste (unpublished) primer set; C- ASBVd RNA extracted using the column chromatography method and cDNA amplified using the Bar- Joseph *et al*. (1985) primer set; D- ASBVd RNA extracted using CTAB method and cDNA amplified the Bar- Joseph *et al*. (1985) primer set. Blue lines indicate the samples and the red lines indicate the standards.

2.3.2 Distribution of ASBVd within avocado trees

The distribution results are presented in Table 2.7. A total of 21 trees were sampled from three nurseries in Limpopo. All trees either had visible Sunblotch disease symptoms or were symptomless carrier trees that were previously indexed and tested positive for ASBVd. Variations in the distribution of ASBVd were observed in all the symptom-bearing trees during ASBVd amplification using fluorescent based real-time RT-PCR. Trees that were only displaying fruit symptoms tested positive for the fruits and the leaves tested negative (Trees 2, 5 and 7). Uneven distribution of ASBVd between the branches of the same tree was

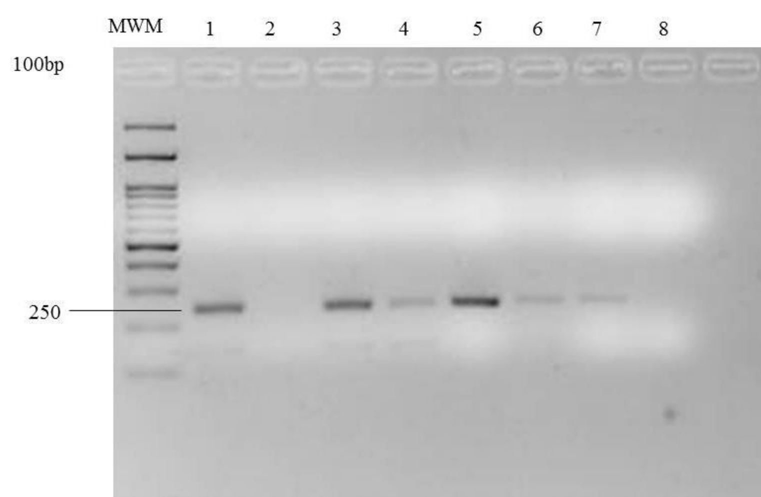
1 observed in trees showing symptoms all over (fruits, leaves and stem). Tree 1 had two
2 branches, the symptoms were displayed on one of the branches and the other branch had no
3 symptoms. The symptom-bearing branch tested positive for ASBVd and the other branch
4 tested negative. Tree 3 displayed bark cracking on the older stem, and five of the six branches
5 tested negative for ASBVd and only one tested positive.



6

7 **Figure 2.4** Gel electrophoresis of ASBVd on a 2% agarose gel using the Bar- Joseph *et al*
8 (1985) primer pair from RNA extracted with column chromatography. Positive results were
9 scored by the presence of a 247bp DNA fragment using a 100bp molecular weight marker
10 (MWM); lane 1, Positive control, lane 2, healthy tree; lanes 3 to 7 represents mixing1
11 ASBVd positive leaf in 9 healthy leaves, 1-19,1-29,1-39,1-49, respectively; and lane 8, non-
12 template control.

13



14

15 **Figure 2.5** Gel electrophoresis of ASBVd on a 2% agarose gel using the Luttig and Manicom
16 (1999) primer pair from RNA extracted with column chromatography. Positive results were
17 scored by the presence of a 250 bp DNA fragment using a 100bp Molecular weight marker
18 (MWM); lane 1, Positive control; lane 2, healthy tree; lanes 3 to 7 represents mixing1
19 ASBVd positive leaf in 9 healthy leaves, 1-19,1-29,1-39,1-49, respectively; and lane 8, non-
20 template control.

Tree 4 had leaf symptoms in all branches, however, only one branch tested positive for ASBVd and the other branch tested negative. Tree 15 displayed leaf symptoms in all the tree branches, out of three branches sampled only one tested positive while the other two remained negative. Trees 16 and 18 were dwarfed (dwarfing is regarded as one of ASBVd symptoms in the field) and both trees tested positive for ASBVd and in both trees ASBVd was found unevenly distributed between the branches. Tree 16 tested positive in one branch out of the three branches tested and Tree 18 tested positive in two branches and two other branches tested negative. Trees 17 and 19 had creeping branches, this is also considered as one of the ASBV symptoms in the field, and both these trees tested negative for ASBVd.

Trees 9 and 20 were the symptomless carriers that were previously indexed and tested positive for ASBVd, but later tested negative. These trees tested negative in this study. Tree 11 was tested as a possible symptomless carrier tree as it was growing next to tree 10 for more than 30 years; it was sampled to investigate the possibility of root grafting or other transmission from the neighbouring infected tree. This tree however, tested negative for ASBVd. Trees 6, 8, 10, and 12 were all symptomless carrier trees that tested positive, in these trees all the branches and fruits (when available) tested positive displaying an even distribution of ASBVd.

Fruits were further tested individually for ASBVd distribution (Table 2.8) and symptoms were separated into two categories: symptoms on the yellow infected part and the seemingly green part of the fruit (Fig 2.2). For the slightly infected fruits, the yellow part tested positive while the green part tested negative for ASBVd. The severely infected fruits however showed different results where both the green and the yellow infected parts tested positive. Similar results were obtained with the symptomless fruits where the fruits positive all around the skin showing an even distribution of ASBVd.

2.4 Discussion

It was demonstrated that the primers used currently are sensitive enough to detect ASBVd from a single infected leaf in 49 healthy leaves using both a fluorescent based one-step real-time RT-PCR in a QIAGEN Rotor Gene Q instrument and a conventional RT-PCR in a thermocycler. However, the accuracy and reliability of ASBVd indexing depend on the type of extraction method used and the selected primer set. Results showed that RNA extracted using a CTAB method was highly incompatible with a fluorescent based one-step real-time RT-PCR in a QIAGEN Rotor Gene Q instrument. Using the RNA template extracted with the

column chromatography method and Jooste (unpublished) primer pair produced the best results for ASBVd detection in a fluorescent based real-time RT-PCR (Fig. 2.3 A). Previous studies proved that RNA extracted from cellulose column chromatography is concentrated and are free of inhibitors. It was also reported as a valid and practical method for ASBVd indexing (Luttig and Manicom, 1999).

Detection using the fluorescent based real-time RT-PCR and the ASBVd specific primers for indexing is currently the best option to use in routine diagnosis. Real-time applications are more sensitive and faster than the conventional PCR and results are detected in the early stages of the reaction and the variations between the samples can be detected (Bar-Joseph *et al.*, 1986). The most used method is the fluorescent based RT-PCR; and the commonly used dye is SYBR Green 1 (Ncango *et al.*, 2014). The dye binds to the double stranded DNA minor groove and the intensity of the fluorescence increases with an increase in DNA amplicons in the reaction. The dye binds to any double stranded DNA in the reaction, therefore specific primers are used, a known positive and the standards which help with the accurate detection. In conventional RT-PCR cDNA is synthesised using ASBVd specific primers, and thereafter agarose gel electrophoresis is run to confirm the size of the amplicon using molecular weight marker. The conventional RT-PCR is sensitive enough, however the process takes longer to obtain results, chances of contamination are increased and it is labour intensive with many steps and preparation of agarose gels are not desirable for large scale indexing.

Distribution studies indicated that ASBVd is unevenly distributed between the branches of the same symptom-bearing trees and evenly distributed between the branches of the symptomless carrier trees. The latter also proved to have higher concentration copy numbers compared to the symptom-bearing trees. The results further indicated that symptomless carrier trees can test positive one year and test negative again the following year. However, according to farmers this is also true for the symptom–bearing trees which were not demonstrated in this study. Similar findings were reported by Tondo *et al*. (2010) that related this to the accuracy of the assay used. In the current study, a very sensitive indexing method was presented and two of these trees still tested negative, therefore, it is recommended to immediately remove positively indexed trees from the field as they may pose threats for disease spread.

Table 2.3 Amplification of ASBVd using the real-time fluorescent based RT-PCR showing the threshold values (Ct) and calculated concentration (copies/µl) results with the Jooste (unpublished) primers and RNA extracted with the column chromatography method.

Name	Type	Ct	Given Conc (copies/ul)	Calc Conc (copies/ul)	% Var
+	Positive Control	4.61		26,433	
1:10	Standard	6.31	10,000	10,465	4.7%
1:100	Standard	10.79	1,000	914	8.6%
1:1000	Standard	14.78	100	104	4.4%
1:2000	Standard	16.12	50	50	0.2%
neg	Negative Control	23.41		1	
water	NTC				
10	Unknown	6.72		8,395	
20	Unknown	7.93		4,347	
30	Unknown	7.94		4,312	
40	Unknown	8.28		3,586	
50	Unknown	10.51		1,064	

NTC- Sample cancelled, as efficiency were less than the reaction efficiency threshold.

Table 2.4 Amplification of ASBVd using the real-time fluorescent based RT-PCR showing the threshold values (Ct) and calculated concentration (copies/µl) results with the Jooste (unpublished) primers and RNA extracted with the CTAB method.

Name	Type	Ct	Given Conc (Copies)	Calc Conc (Copies)	% Var
+	Positive Control	8.06		5,977	
10	Standard	6.87	10,000	11,626	16.3%
100	Standard	11.69	1,000	781	21.9%
1000	Standard	15.51	100	92	7.9%
2000	Standard	16.28	50	60	19.7%
neg	Negative Control	22.56		2	
water	NTC	31.72			
10	Unknown	9.97		2,049	
20	Unknown	10.92		1,201	
30	Unknown	11.61		817	
40	Unknown	12.04		642	
50	Unknown			503	

Table 2.5: Amplification of ASBVd using the real-time fluorescent based RT-PCR showing the threshold values (Ct) and calculated concentration (copies/µl) results with the Bar- Joseph *et al*. (1985) primers and RNA extracted with the column chromatography method.

Name	Type	Ct	Given Conc (copies/ul)	Calc Conc (copies/ul)	% Var
+	Positive Control	18.67		18,526	
10	Standard	19.61	10,000	10,394	3.9%
100	Standard	23.69	1,000	857	14.3%
1000	Standard	26.60	100	145	45.2%
2000	Standard	28.76	50	39	22.7%
neg	Negative Control	28.25		53	
water	NTC				
10	Unknown	20.25		7,058	
20	Unknown	22.29		2,024	
30	Unknown	22.47		1,807	
40	Unknown	22.50		1,774	
50	Unknown	25.04		376	

NTC- Sample cancelled, as efficiency was less than the reaction efficiency threshold.

Table 2.6: Amplification of ASBVd using the real-time fluorescent based RT-PCR results showing the threshold values (Ct) and calculated concentration (copies/µl) results with the Luttig and Manicom (1999) primers and RNA extracted with the column chromatography method.

Name	Type	Ct	Given Conc (Copies)	Calc Conc (Copies)	% Var
+	Positive Control	14.98		311,714	
10	Standard	20.92	10,000	8,927	10.7%
100	Standard	24.25	1,000	1,214	21.4%
1000	Standard	28.37	100	103	3.2%
2000	Standard	29.76	50	45	10.5%
neg	Negative Control	28.00		128	
water	NTC	30.47		29	
10	Unknown	16.63		115,772	
20	Unknown	17.66		62,663	
30	Unknown	18.42		39,666	
40	Unknown	19.77		17,729	
50	Unknown	20.52		11,295	

1 **Table 2.7** Avocado sunblotch disease (ASBV) - infected trees with known indexing status from the three nurseries from Limpopo and
2 Mpumalanga provinces in South Africa.
3

Tree number	Symptom description	qPCR results	Number of branches	Number of infected branches	Number non-infected branches	Fruits	Number of infected fruit
1	Leaf symptoms on infected branch	Positive	2	1	1	0	
2	Fruit symptoms	Positive	3	0	3	3	3
3	Cracking of bark	Positive	6	1	5	0	
4	Fruit and leaf symptoms	Positive	2	1	1	0	
5	Fruit symptoms only	Positive	7	0	7	4	4
6	Symptomless	Positive	2	2	0	0	
7	Fruit symptoms	Positive	2	0	2	2	2
8	Symptomless	Positive	5	5	0	2	2
9	Symptomless	Negative	4	0	4	2	0
10	Symptomless	Positive	7	7	0	0	
11	Symptomless next to tree 10	Negative	6	0	6	0	
12	Symptomless	Negative	5	0	5	0	
13	Symptomless	Positive	2	0	2	2	2
13a	Recovery growth on tree 14	Positive	1	1	0	0	
14	Leaf symptoms	Positive	3	1	2	0	
15	Offspring 15 (symptomless)	Negative	4	0	4	0	
16	Dwarf	Positive	3	1	2	0	
17	Creeping growth	Negative	4	0	4	0	
18	Dwarf	Positive	4	2	2	0	
19	Creeping	Negative	4	0	4	0	
20	Symptomless	Negative	2	0	2	0	
21	Bark cracking	Positive	3	1	2	3	3

4

1 **Table 2.8** ASBVd distribution within a single infected avocado in trees with symptomless-
2 and symptom - bearing fruit

Tree number	Fruit number	Type of infection	Skin description	RT-PCR results
Tree 1	1	Symptomless	Green	Positive
	2	Symptomless	Green	Positive
	3	Symptomless	Green	Positive
Tree 2	1	Slight infection	Yellow	Positive
			Green	Negative
	2	Slight infection	Yellow	Positive
			Green	Negative
	3	Severe infection	Yellow	Positive
			Green	Positive

3
4 Trees with symptoms only on the fruits and no symptoms on leaves were tested and these
5 trees tested positive for the fruits and negative for the leaves. This is usually observed when
6 healthy avocado trees were pollinated by infected pollen; in this case only the fruits exhibit
7 symptoms and the rest of the tree remains disease-free (Dodds, 2001). However, this should
8 not be a big problem since a very low pollen transmission rate between 1.8% and 3.125% was
9 documented (Desjardins *et al*., 1979). It was suggested that symptomless carrier trees may be
10 the source of pollen transmission in the field since they maintain higher concentrations of
11 ASBVd (Mathews, 2011). It is therefore crucial to index all trees before they are used for
12 propagative material, regardless of whether they bear symptoms or not. Symptomless trees
13 are misleading since they appear as normal trees with no signs of infections. With the uneven
14 distribution of ASBVd between branches, presented here, the increase in the number of
15 leaves per sample will increase the reliability of ASBVd detection.

16 Results indicated that some of the trees that previously tested positive now tested negative.
17 The explanation for this is unknown, however similar results as reported by Tondo *et al*.
18 (2010) when indexed trees that were previously indexed that were found to be infected in
19 previous surveys. They discussed that the failure to detect ASBVd in accessions previously
20 found to be positive may be due to accuracy of the assay, In the Tondo *et al*. (2010) survey,
21 fourteen of these trees previously tested as positive trees, have tested negative for ASBVd.
22 There is no current explanation for this and it highly recommended that these trees be treated
23 as positive trees and be removed immediately from the fields.

24 **2.5 Conclusion**
25 Current ASBVd indexing techniques are sensitive enough to detect even the lowest viroid
26 concentrations in the infected avocado trees. However, it is evident that ASBVd is unevenly

distributed in the symptomatic trees. Therefore, care should be taken during sampling to ensure that all the branches of the tree are represented in the sample to reduce the false negative results. When the numbers of ASBVd negative leaves in a sample containing one ASBVd infected leaf were increased, the sensitivity of detection was not affected. Thus, there is a possibility of increasing the number of leaves per sample for better representation of each tree per sample. Correct sampling and quality assured indexing methods will contribute to improved ASBV management and production of quality fruits in South Africa.

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1 **CHAPTER 3**

2 **Survey for Avocado sunblotch disease and identification of different *Avocado sunblotch*
3 *viroid* (ASBVd) sequence variants in the two major South African avocado growing
4 provinces**

5 **Abstract**

6 Since the introduction of avocado (*Persia americana* Mill.) into RSA in the 1930s, the
7 production area of avocado has been steadily increasing. Today avocado production occupies
8 over 15 thousand hectares in South Africa. All commercial avocado cultivars are susceptible
9 to *Avocado sunblotch viroid* (ASBVd) and infected trees are lower yielding, the quality of the
10 fruits is downgraded and fruits with severe symptoms are discarded. It is therefore crucial to
11 determine the factors involved in disease occurrence to ensure that Sunblotch disease is
12 properly managed in South Africa. The objectives of this study were to investigate the
13 occurrence of Sunblotch disease in two provinces of South Africa and to determine the
14 molecular differences between the genomes of ASBVd variants. The latter is important in
15 order to verify if the current primer set used for detection is optimal to detect all known
16 ASBVd variants. In addition to the field survey, an online survey was created using the
17 Google sheets and submitted to the South African Subtrop website. The aim of the online
18 survey was to determine the knowledge level of avocado growers and nurserymen on
19 Sunblotch disease. From the online responses, it was discovered that not all avocado growers
20 are familiar with Sunblotch disease symptoms; it was also discovered that some farmers do
21 not take precautions with their cutting tools and removal of infected trees from the field
22 which could pose a serious threat in disease spread. The field survey included visits to farms
23 in the Limpopo and Mpumalanga provinces and avocado samples were randomly collected.
24 In total, 310 trees were randomly sampled from commercial orchards in the Mpumalanga and
25 Limpopo provinces and 10.3% of the trees tested positive for ASBVd using the real-time
26 reverse transcription polymerase chain reaction (RT-PCR) assay. Most infected trees were
27 detected in the Mpumalanga province with 11.6% infection compared to the Limpopo
28 province with 9.05% infection. Most of the infected trees were symptomless carrier trees.
29 Symptomless carrier trees are very common and are a threat to the avocado industry and
30 could be the main reason of new infections in the field. PCR amplicons of samples that tested
31 positive from the survey were sent for sequencing and the sequences were analysed and
32 aligned to known sequences in GenBank®. Phylogenetic comparisons between sequences

obtained from the South African study were identified as different ASBVd variants resulting from minor nucleotide changes in the sequences, one variant was found associated with bleach carrier tissue variant. Alignment of the primers, currently used in the routine diagnostic detection protocol at ARC-TSC, showed a perfect match with the sequences obtained from this study, supporting the reliability of ASBVd detection for the South African avocado industry.

3.1 Introduction

Since the introduction of avocado (*Persia americana* Mill.) into South Africa in the 1930s, the production area of avocado has been steadily increasing. Today avocado production occupies over 15 thousand hectares (Sippel, 2001; Nortje, 2012). The nutritional value of avocado is one of the most attractive to the consumers thus increasing its value (Dreher and Davenport, 2013). South Africa is amongst the top five avocado exporters in the world, with the main market being Europe, which competes against other countries such as Mexico, Chile, Spain, and Israel (Ntombela *et al.*, 2013). For that reason, South Africa should keep producing high quality fruit to maintain its good export reputation. Major avocado production in South Africa is situated in the Limpopo and Mpumalanga provinces with 61% and 30% of total production, respectively. Smaller production areas are found in the KwaZulu-Natal and the Eastern Cape provinces with 8% and 1% of total production, respectively (Nortje, 2012). Most of the jobs created by the avocado industry are in the rural areas and therefore the industry has a great impact on rural upliftment and poverty alleviation in South Africa (DAFF, 2012).

Avocado sunblotch disease is an economically important disease of avocado caused by *Avocado sunblotch viroid* (ASBVd) (Wolstenholme and Whiley, 1999; Tondo *et al.*, 2010). ASBVd is a single stranded RNA molecule with a rod-like secondary structure between 239 and 251 base pairs long (Hammond and Owens, 2006). ASBVd has a total of 108 different variants where three of the specific variants are associated with symptom expression in the leaves. The variants are; ASBVd –B, which produces bleached symptoms, ASBVd-V, which produces variegated symptom, and ASBVd-Sc, which is associated with symptomless carrier trees (Semancik and Szychowski, 1994). However, all the variants have been found to express very similar biological properties and cannot be distinguished as different strains (Semancik and Szychowski, 1994). Leaf symptoms are said to be very rare in the field (Semancik and Szychowski, 1994). The most consistent ASBV symptoms in the field are bark streaking and spotting of avocado tree twigs and limbs, symptoms appear as yellow or

colourless sunken streaks (Wallace, 1958). Fruit develop similar symptoms to stem symptoms with yellow or pink/red sunken streaks (Vallejo-Perez *et al*., 2014).

All commercial avocado cultivars are susceptible to ASBVd and infected trees are lower yielding; the quality of the fruits is downgraded and fruits with severe symptoms are discarded (Wallace, 1958; Randles, 2003). Symptomless carrier trees produce low yields with most of the fruits being downgraded based on quality standards (Randles, 2003). Other direct costs associated with ASBV include indexing costs during the selection of parent material for propagation and eradication of infected trees in the field (Wallace, 1958; Randles, 2003). Furthermore, previously conducted studies indicated an increase in the disease occurrence in the past 15 years in South Africa (Ncango *et al*., 2014). A study conducted by Korsten *et al.,* (1986) showed that out of a total of 3 125 trees indexed for ASBVd, only 2% of the trees tested positive using a dot-blot hybridization technique. More recently, a similar study was conducted by Ncango *et al.* (2014) using mother trees from nurseries. In this study, 24 685 avocado mother trees were indexed using a reverse transcription polymerase chain reaction (RT-PCR) assay and 15.6% of the trees tested positive for ASBVd.

It is therefore crucial to determine the factors involved in disease spread and ensure that Sunblotch disease is properly managed in South Africa. Against this background, the aim of this study were to conduct an online survey to determine the basic knowledge farmers have about Sunblotch disease and to determine the spread of Sunblotch disease in the major production areas in RSA. Positive samples identified during the surveys would also determine which ASBVd variants predominantly occur in RSA. Detection of ASBVd variants was done using a sensitive molecular technique in order to increase the efficiency of detection. The molecular characterization of ASBVd variants was crucial in order to validate if the current ASBVd-specific primers are optimal for detecting ASBVd in RSA.

3.2 Materials and methods

3.2.1 Online survey

An online survey was created using Google sheets and the survey link was submitted to the Subtrop website for famers and nurserymen to complete. The online survey can be accessed from the link below:

https://docs.google.com/forms/d/1igDW_ihOaFp4wYf4uj06nhlg5WAL5dbVLDs9ikUiow/edit#responses

The online survey included the following questions:

A- Can you identify sunblotch symptoms in the field?
B- Are you familiar with Avocado sunblotch disease?
C-Do you have trained workers who know about the disease and can identify disease symptoms?
D- Can you spot the diseased trees?
E- What are the most common symptoms you come across?
F- What do you do with the infected trees?
G- If you remove them do you experience new infections?
H- If yes do you know why?
I-Where do you get your planting material?
J-Do you think you need to be concerned about the disease (is it an important disease)?
K- Do you see any spread of the disease in rows and adjacent plants?
L-Do you clean your tools between cutting trees?
Would you like to know more about the disease and what information would you be interested in?
How many trees have ever been infected with the disease and which cultivars are most likely to be infected?

3.2.2 Field sampling

3.2.2.1 Sample collection

The samples were collected from Mpumalanga and Limpopo provinces of South Africa. Eight farms were sampled in Mpumalanga province from Nelspruit, White river, Hazyview and Kiepersol regions. In Limpopo province, six farms were selected from the Levubu region and six from the Tzaneen region. Trees were selected from all different blocks available on

each farm. Tree selection was random and was not based on any symptoms or indication of ASBV. No specific cultivar was targeted since ASBV infects all avocado commercial cultivars (da Graca and Mason, 1983). In a single block ten trees were selected, the total number of trees per farm depended on the number of blocks present. New and old leaves were sampled from each tree and fruit samples were included if available. Samples were kept in sealed plastic bags in cooler boxes and transported to the ARC-TSC Pathology laboratories in Mbombela, Mpumalanga for processing.

3.2.2.2 ASBVd RNA extraction

All the samples were extracted using the cellulose column chromatography method as described in chapter 2, section 2.2.1

3.2.2.3 Amplification of ASBVd using a one-step real time reaction

Detection was done as explained in chapter 2, section 2.2.2

3.2.2.4 Amplification of ASBVd for sequencing of positive plants

Samples were amplified according to the two-step RT-PCR method, using the Go Script™ Reverse Transcription system (Promega) followed by the GoTaq® Flexi DNA Polymerase system (Promega) described in Chapter 2 using the ASBVd-specific primer pair described by Bar- Joseph *et al.* (1985). The PCR products were sent to Inqaba Biotech for sequencing. The sequences were edited using Chromas 2.6.4 (Technelysium DNA sequencing software) and BioEdit 7.2.5 (Hall 1999) for base calling creating consensus sequences and sequence alignments followed by alignment of sequences using MAFFT version 6 (Katoh, 2007). The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor, 1969) and are in the units of the number of base substitutions per site. The analysis involved 50 nucleotide sequences (Table 3.4). All ambiguous positions were removed for each sequence pair. There were a total of 462 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.*, 2013).

3.3 Results

3.3.1 Online survey

The results presented are a representative of only 20 responses captured so far in this survey. The purpose of the survey was to determine how the growers handle and manage ASBV in their fields. From the responses recorded, 80% of the participants could identify ASBV infected trees from the field and the remaining 20% were not sure (Fig. 3.1 A). About 15.8% of the participants were not familiar with the disease at all and the remaining 84.2 % were familiar with the disease (Fig. 3.1 B). Half (50%) of the participants trained workers to

identify ASBV and the rest did not (Fig. 3.1 C). Twenty five per cent of the participants have never spotted ASBV in their fields before, 55% have spotted it a few times and 20% reported to have the disease occurring regularly in their fields (Fig. 3.1 D).

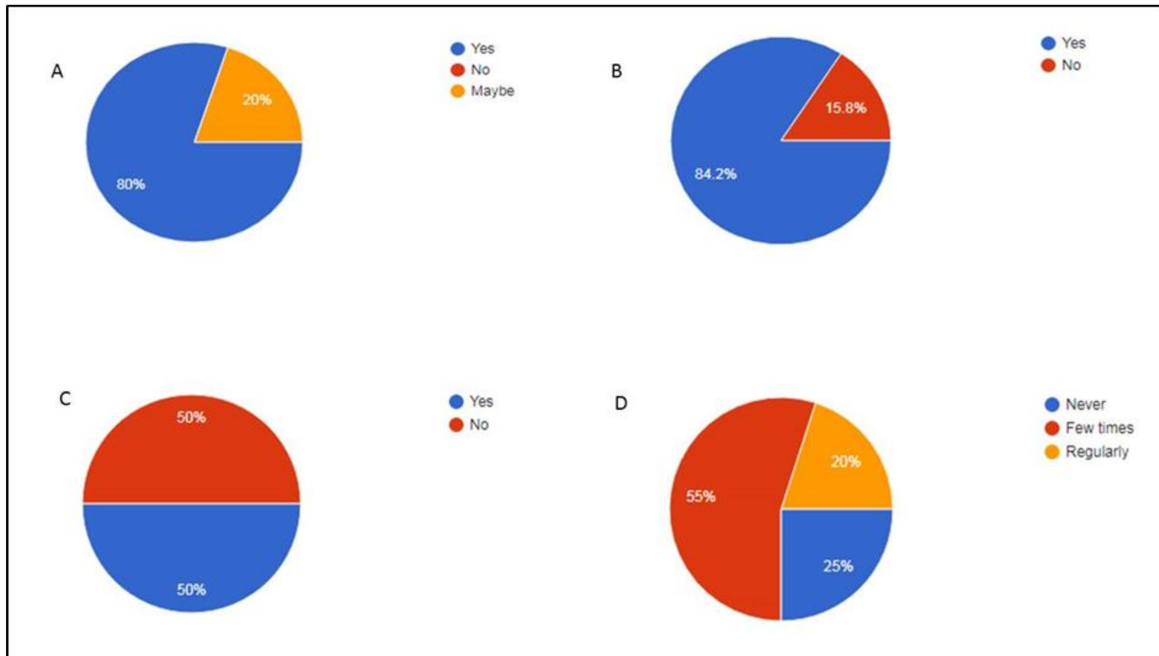
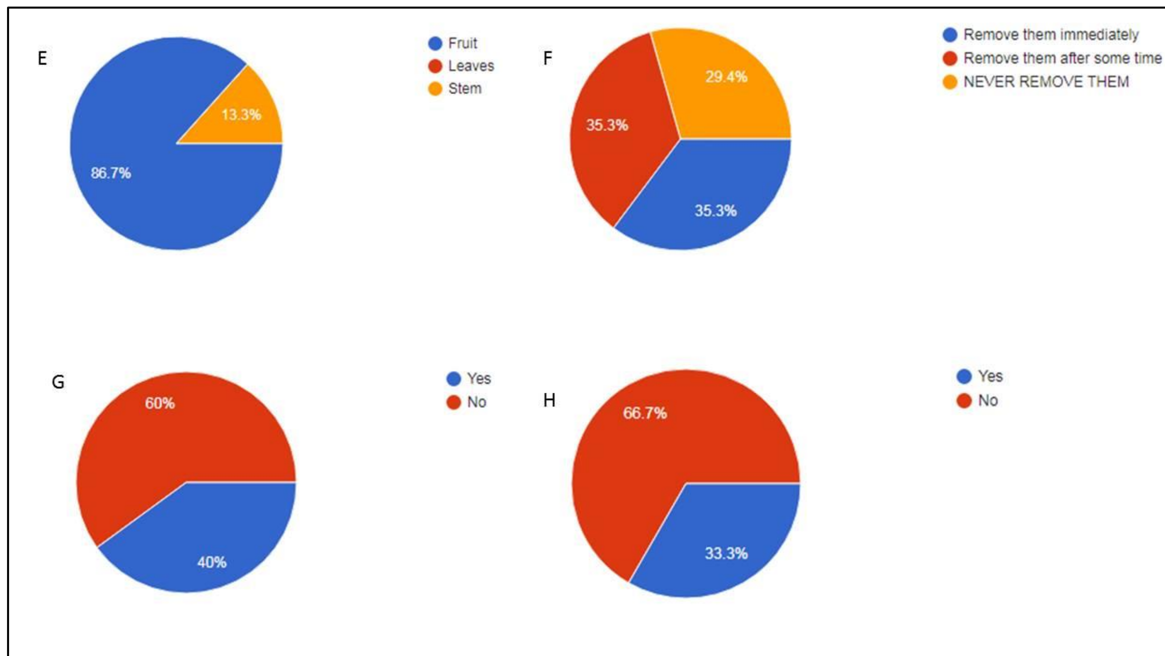


Figure 3.1 Responses from the online survey questions A to D

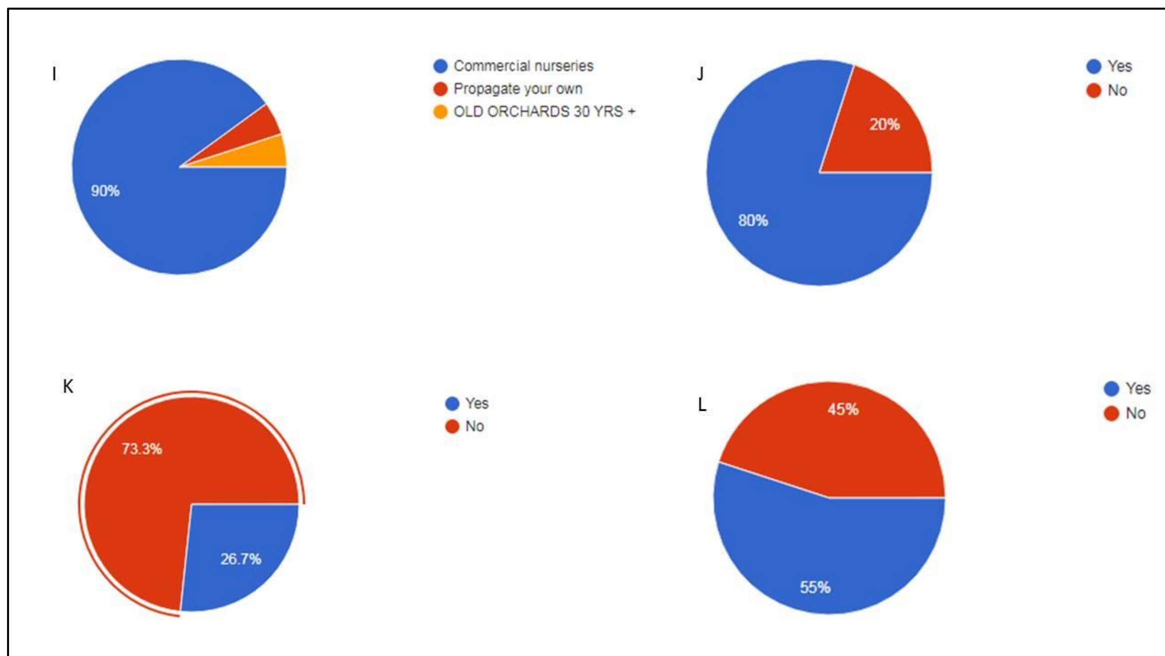
Fruit symptoms are the most common sign of ASBV in the field and most participants (86.7%) could identify symptoms and 13.3% identified stem symptoms before and no participant spotted leaf symptoms before (Fig. 3.2 E). Only 35.3% of participants immediately removed the infected trees from the orchard after detecting the symptoms. This was similar to the percentage of participants who removed the infected trees after some time while 29.4% never removed the infected trees from the field (Fig. 3.2 F). Where trees were removed from the orchard, 40% of participants still experienced new infections and 60% never experienced any new infections (Fig. 3.2 G). About 33% of growers who experience new infections could explain the reason for new infections and the rest could not (Fig. 3.2 H). Most farmers (90%) acquired their planting material from the commercial nurseries, 5% propagated their own and the remaining 5% collected it from old orchards (Fig. 3.3 I). The number of infected trees ranged from none to covering areas of about 10 ha. Ryan, Hass, Fuerte and Pinkerton were the most infected cultivars. When asked if they needed to be concerned about the disease, 80% of participants said yes and 20% think it is not a disease to be concerned about (Fig. 3.3 J). Most of the farmers would like to know more about the symptoms, disease management, spread and the epidemiology of ASBV. A few participants (26.7%) experienced the spread of the disease in rows and adjacent plants and the majority

1 (73.3%) never experienced any spread of the disease (Fig. 3.3 K). There were 45% of
2 participants who did not clean their tools between cutting trees (Fig. 3.3 L).



3
4 **Figure 3.2** Responses from the online survey questions E to H

5



6
7 **Figure 3.3** Responses from the online survey questions I to L

8 3.3.2 Field sampling

9 A total of 310 trees were randomly sampled from commercial orchards in the Mpumalanga
10 and Limpopo provinces and only 10.3% of the trees tested positive for ASBVd in a real-time
11 reverse transcription polymerase chain reaction (RT-PCR) assay (Table 3.1). In the Limpopo

province, a total of 198 trees were collected from twelve farms with six farms being sampled from the Tzaneen growing region and six from the Levubu region (Table 3.1). From the total of 198 trees, only 9.05% (19) tested positive for ASBVd with 16 of the total infected trees being detected in the Tzaneen region and three in the Levubu region (Table 3.1). In the Mpumalanga province, 112 trees were collected from eight farms, and 11.6% (13) tested positive for ASBVd (Table 3. 1). The most infected trees from the Mpumalanga province were obtained from two farms and the rest of the farms had none or very few infections.

3.3.3 Molecular analyses of field material

From a total of 32 positive trees, only four displayed visible ASBV symptoms and the rest were symptomless carriers. Three of trees with symptoms were sampled in Limpopo (Table 3.2; trees 86, 87 and 90) and one was from Mpumalanga province (Table 3.2; tree 18). The two trees from Limpopo expressed both the bleaching- and variegation symptoms on new and old leaves and on the fruits (Table 3.2; trees 86 and 87). The three trees from Mpumalanga (Table 3.2) expressed severe symptoms on the fruit only (Fig. 3.4 D). Tree 18 from Mpumalanga (Table 3.2) also expressed both the bleaching- and variegation symptoms on new and old leaves and on fruits. However, the symptoms were severe and the new leaves and small fruits displayed symptoms (Figure 3. 4 A, B and C) and the old leaves appeared normal. The ASBVd concentration copy number of the symptom-bearing trees was lower when compared to most symptomless carrier trees. In general, trees from the Tzaneen region in the Limpopo province (Table 3.3) had a higher ASBVd concentration compared to the trees from Mpumalanga province (Table 3.3) and the ASBVd concentration detected between different trees ranged from 18 copies/µl to 50 000 copies/µl. The trees in the Tzaneen region had higher concentrations compared to trees from Levubu and Mpumalanga regions.

3.3.4 ASBVd positive samples sequence analysis

Phylogenetic analysis of the sequences obtained from this study shared a high identity with the known ASBVd variants from GenBank® and with each other (Figure 3.5). The low percentage identities detected within this group between isolates S74687.1 and ASBVd 02 (50% identity), ASBVd 05 and ASBVd 39 (57% identity), ASBVd 09, ASBVd 26 and ASBVd 10 (64% identity) indicated a high genetic diversity between these isolates. ASBVd 02 obtained from the Levubu region was found associated with a bleached carrier tissue variant (S74687.1) with a 50% identity as mentioned above. This tree however was a symptomless carrier tree meaning the tree recovered from the bleached symptoms and the symptomless leaves dominated the tree. The MAFFT alignment of all nucleotide sequences

showed similarities between sequences indicating that they belong to the same ASBVd variant group. These sequences included ASBVd13, 14 and 16 from Mpumalanga; ASBVd 06 and 12 from Mpumalanga; ASBVd 22, 28 and 36 from Tzaneen; ASBVd 30, 40, 31, 25, 45 and 29 from Limpopo. Similarities between sequences from the two provinces were observed in samples ASBVd 9 and 10 from Mpumalanga and ASBVd 28 from Limpopo; ASBVd 05 from Mpumalanga and ASBVd 39 from Limpopo. Sequence variations were observed in isolates ASBVd 06 and ASBVd 07; ASBVd 11 and ASBVd 12. Nucleotide differences between sequences obtained from the leaves (ASBVd 32) and the fruits (ASBVd 42 and ASBVd 43) of the same tree were observed (Figure 3.6). Sequences of ASBVd 13 and ASBVd 14 were obtained from two branches of the same tree, these sequences were very similar and regarded as one variant (Figure 3.6).

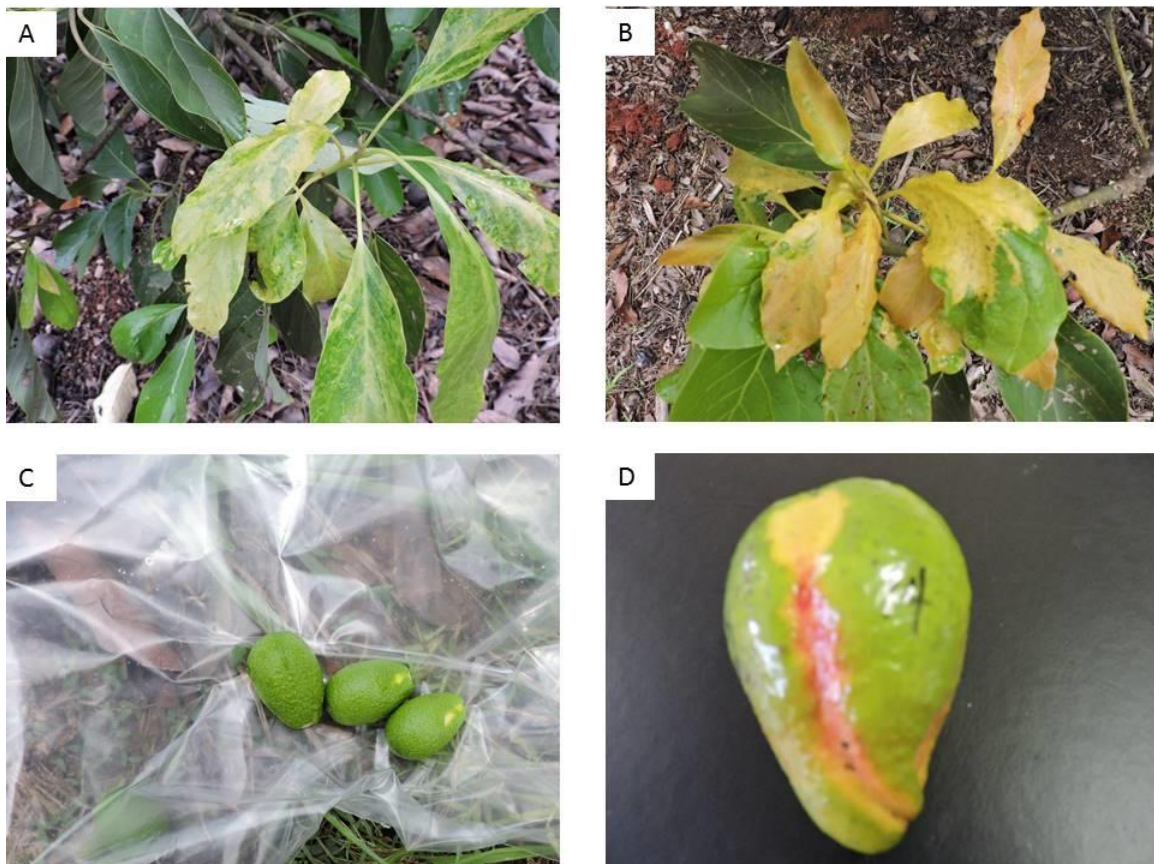


Figure 3.4 Symptoms of Avocado sunblotch disease (ASBV) observed during field surveys in Limpopo and Mpumalanga provinces: A – Severe leaf bleaching; B- severe leaf variegation; and C– sunken yellow patches on small fruit; D – severe fruit infection showing sunken yellow and pink patches on the green avocado skin

1 **Table 3.1** Summary of results of the ASBV field survey conducted on avocados (*Persia*
2 *americana* Mill.) in Mpumalanga and Limpopo provinces

Province	Growing region	Total number of farms sampled	Total no. of trees indexed	Infected trees	(%)
Mpumalanga		8	112	13	11.6%
Limpopo	**Tzaneen**	6	108	16	14.8%
	Levubu	6	90	3	3,30%
Total		20	310	32	10.3%

3

4 **Table 3.2** Real-time RT-PCR results of avocado samples collected from the Limpopo
5 province that tested positive for ASBVd

Sampling area	Tree number	Cyclic threshold (Ct) value	Calculated concentration (copies/µl)
Tzaneen	32	8.71	26,269
	41	10.13	11,354
	42	10.11	11,447
	49	7.60	50,493
	51	8.55	28,869
	52	9.08	21,039
	53	8.76	25,466
	54	9.73	14,381
	55	10.84	7,434
	57	9.94	12,669
	59	10.03	18,684
	85	12.81	3,661
	86	16.82	348
	87	19.36	79
	89	7.84	52,891
	90	15.03	711
	91	14.16	1,203
	113	12.26	3,747
Levubu	11	11.20	2,027
	69	11.40	783
	71	12.67	382

6 Presented in the table are the real-time RT-PCR results for all the trees that tested positive for
7 ASBVd in Limpopo province. These trees had cyclic threshold values below the 1:2000
8 standard. The standards were also used to calculate the concentration of the samples; these
9 results showed concentration variation of ASBVd between the field trees.
10

11

1 **Table 3.3** Real-time RT-PCR results of avocado samples collected from the Mpumalanga
2 province that tested positive for ASBVd

Sampling area	Tree number	Cyclic threshold (Ct) value	Calculated concentration (copies/µl)
Mpumalanga	13	13.69	1,869
	15	16.87	255
	17	14.77	951
	18	12.15	4,908
	19	11.95	5,568
	67	16.50	384
	68	10.22	7,975
	74	21.33	47
	75	20.95	45
	80	19.08	110
	85	20.43	57
	87	22.77	37
	89	19.93	73
	97	7.47	20,288

3

4 **Table 3.4** ASBVd samples used in this study to determine the phylogenetic relationships
5 between South African ASBVd isolates and known isolates

Sample	Cultivar	Locality	GenBank® accession no.
ASBVd 02	Hass	Levubu	Not deposited
ASBVd 03	Hass	Levubu	Not deposited
ASBVd 04	Fuerte	Mpumalanga	Not deposited
ASBVd 05	Fuerte	Mpumalanga	Not deposited
ASBVd 06	Fuerte	Mpumalanga	Not deposited
ASBVd 07	Fuerte	Mpumalanga	Not deposited
ASBVd 08	Fuerte	Mpumalanga	Not deposited
ASBVd 09	Gem	Mpumalanga	Not deposited
ASBVd 10	Gem	Mpumalanga	Not deposited
ASBVd 11	Gem	Mpumalanga	Not deposited
ASBVd 12	Gem	Mpumalanga	Not deposited
ASBVd 13	Gem	Mpumalanga	Not deposited
ASBVd 14	Gem	Mpumalanga	Not deposited
ASBVd 15	Gem	Mpumalanga	Not deposited
ASBVd 16	Edranol	Mpumalanga	Not deposited
ASBVd 17	Fuerte	Mpumalanga	Not deposited
ASBVd 18	Edranol	Mpumalanga	Not deposited
ASBVd 19	Hass	Mpumalanga	Not deposited
ASBVd 21	Hass	Tzaneen	Not deposited
ASBVd 22	Edranol	Tzaneen	Not deposited
ASBVd 23	Hass	Tzaneen	Not deposited

ASBVd 24	Edranol	Tzaneen	Not deposited
ASBVd 25	Edranol	Tzaneen	Not deposited
ASBVd 26	Edranol	Tzaneen	Not deposited
ASBVd 27	Edranol	Tzaneen	Not deposited
ASBVd 28	Edranol	Tzaneen	Not deposited
ASBVd 29	Edranol	Tzaneen	Not deposited
ASBVd 30	Edranol	Tzaneen	Not deposited
ASBVd 31	Hass	Tzaneen	Not deposited
ASBVd 32	Hass	Tzaneen	Not deposited
ASBVd 33	Hass	Tzaneen	Not deposited
ASBVd 34	Hass	Tzaneen	Not deposited
ASBVd 35	Hass	Tzaneen	Not deposited
ASBVd 36	Hass	Tzaneen	Not deposited
ASBVd 38	Fuerte	Tzaneen	Not deposited
ASBVd 39	Fuerte	Tzaneen	Not deposited
ASBVd 40	Edranol	Tzaneen	Not deposited
ASBVd 41	Hass	Tzaneen	Not deposited
ASBVd 42	Hass	Tzaneen	Not deposited
ASBVd 43	Hass	Tzaneen	Not deposited
ASBVd 45	Hass	Tzaneen	Not deposited
ASBVd 46	Hass	Tzaneen	Not deposited
ASBVd 47	Hass	Tzaneen	Not deposited
ASBVd 48	Hass	Tzaneen	Not deposited
Avocado sunblotch viroid complete sequence variant C-9	Rakowski and Symons (1989)		M31099.1
Symptomless carrier tissue associated	Semancik and Szychowski (1994)		S73860.1
Variegated tissue associated	Semancik and Szychowski (1994)		S73861.1
Bleached carrier tissue associated	Semancik and Szychowski (1994)		S74687.1
Peach latent mosaic viroid isolate Sumi complete sequence	Jo *et al*. (2016)		KT005802.1
Eggplant viroid clone ELVd2-94 complete genome	Lopez-Carrasco *et al*. (2015)		KT901928.1

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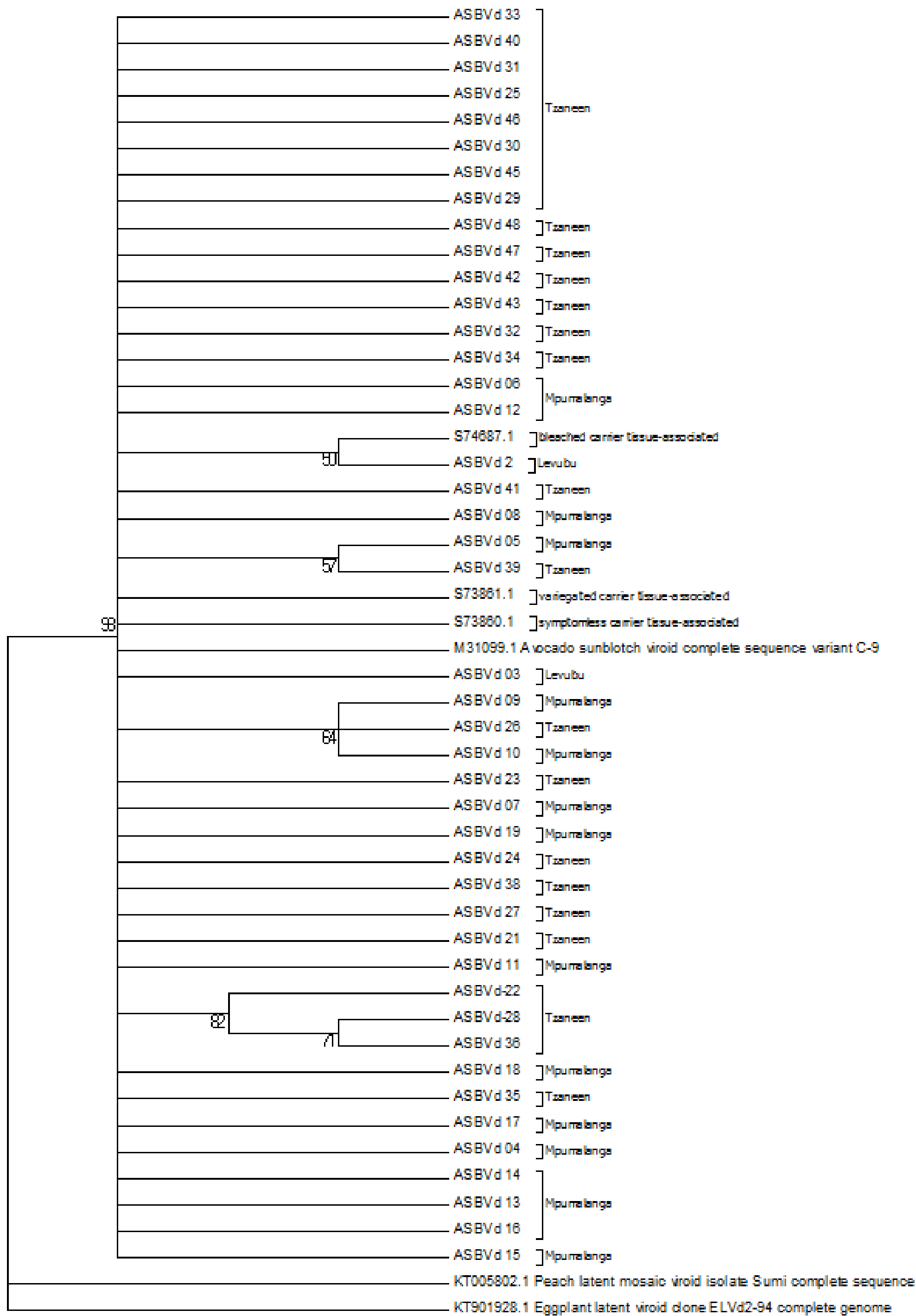

Figure 3.5 Evolutionary relationships of ASBVd variants detected in two major South African avocado growing provinces. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 2.52518398 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). *Peach latent mosaic viroid* (KT005802.1) and *Egg latent mosaic viroid* (KT901928.1) from the family Avsunviroidae were used as out-group.

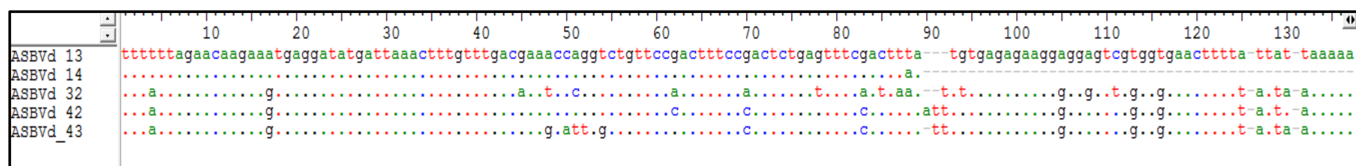

Figure 3.6 BioEdit alignments for ASBVd sequences 13, 14, 32, 42 and 43 showing nucleotide differences from position 1 to 137 of 260 bases.

3.4 Discussion

From results of the online survey, it was shown that 80% of the farmers can identify Sunblotch disease and 50% have trained workers to identify Sunblotch disease symptoms in the field. To establish proper management strategies in orchards, farmers should train workers who can identify the infected trees in the field that will allow for early detection and better management of Sunblotch disease. The survey showed that only 20% of the farmers regularly identified Sunblotch disease symptoms in their orchards with the most common symptoms being on fruit. None of the farmers who participated in the survey had identified leaf symptoms before. This is supported by previous research, which showed that Sunblotch disease symptoms are rare under field conditions (Semancik and Szychowski, 1994). Few farmers (35.3%) removed infected trees immediately after spotting them, 35.3% removed the trees after some time and the remaining 29.4% never removed the infected trees. It is highly recommended that infected trees be removed immediately from the field to avoid further infections (Schnell *et al.*, 1997). In certain cases, all the trees within a 15m radius from the infected trees should be removed (Schnell *et al.*, 1997). This practice is not always feasible for farmers, but care should be taken to remove infected plants and monitor the neighbouring plants. Not removing infected trees is a poor disease management practice and can lead to Sunblotch disease spreading in South African avocado production regions. It is important to create awareness of disease management to all the growers, to establish a culture of disease management and control. Awareness campaigns will be valuable for growers to learn more about Sunblotch disease, the causal organism (ASBVd) and effective management strategies. To avoid infections, the symptomatic trees and trees indexed as positive should be eradicated.

The fact that 40% of the farmers who removed the infected trees from their fields still experienced new infections emphasize the importance to identify the source of new infections in the field. A study conducted by Tondo *et al* (2010) determined the sources of new infections by looking at the proximity of newly infected trees to infected trees and contaminated plots (plots from which infected plants have been removed/died). The study found 24 of the 50 newly infected trees to be adjacent to the previously infected trees. In all

1 these cases, they found that root grafting was the most obvious explanation for the
2 transmission of the viroid. They could not discern any pattern for the appearance of new
3 infections for the other 26 newly infected trees and the distribution of these non-adjacent new
4 infections appeared to be random; they discussed that these plants were probably infected
5 through contaminated pollen or pruning, although strict phytosanitary procedures have been
6 in place for more than 20 years.

7 Online survey results indicated that 90% of the farmers acquire their planting material from
8 the commercial nurseries, 5% propagate their own and the other 5% use material from old
9 orchards. It is important to stress the importance of indexing of all propagation material for
10 all the nurseries before they are sold to the farmers. Moreover, there is still a high number
11 (45%) of farmers who do not clean their cutting tools between the trees. The tools used for
12 cutting and injecting plants should be cleaned between the trees, the point of cleaning the
13 injection material, harvesting clippers and pruning blade is crucial and this could be easily
14 achieved using 5% commercial bleach (*sodium hypochloride* Desjardins *et al*., 1980).
15 ASBVd can easily be transmitted by sap through contaminated injection material, harvesting
16 clippers and pruning blades which were found to have an 8-30% transmission rate (Dodds,
17 2001; Semancik, 2003).

18 From the 310 trees tested during the field survey, 11% tested positive for ASBVd.
19 Mpumalanga province had the highest number of infected plants (11.6%) compared to the
20 Limpopo province (9.05%). Limpopo province was divided into two growing regions;
21 Tzaneen and Levubu. Tzaneen region had a higher number of infected plants (14.8%)
22 compared to the Levubu region which had 3.3% infected plants. From the positive plants,
23 only 4 (12.5%) had visible symptoms and the other 28 (87.5%) were all symptomless carriers
24 of Sunblotch disease. It is evident from the current study that the Sunblotch disease
25 symptomless carrier trees are common in the field and they carry higher concentrations of
26 ASBVd compared to the symptom bearing trees. Sunblotch disease can be present in
27 symptomless carrier trees. Research demonstrated that symptomless carrier trees could arise
28 from an infected symptomatic tree by producing new shoots that appear healthy to replace all
29 the symptomatic leaves (Wallace and Drake, 1962). Since it is impossible to spot
30 symptomless carriers in the field this makes the detection of Sunblotch disease very difficult
31 (Schnell *et al*., 1997).

Moreover, field surveys revealed that farmers are more familiar with fruit symptoms. Fruit symptoms arise when a healthy avocado tree is pollinated by infected pollen (Dodds, 2001). It is possible there is a symptomless carrier in the field spreading the disease to healthy trees, however, the spread to the neighbouring trees could be slow since ASBVd has no vector (Shnell *et al*., 1997; Luttig and Manicom, 1999), and avocado being the only natural host (da Graca and Van Vuuren, 1980). Transmission of ASBVd through pollen occurs at a very low rate of between 1.8% and 3.125% (Desjardins *et al*., 1979). It is evident from the field survey that symptomless trees are common in the field and they pose a threat because results have shown that they carry higher concentration ASBVd copy number compared to the symptom bearing trees. Mathews (2011) reported similar findings that symptomless carrier trees maintain higher viroid concentration. From the study it was discovered that concentrations vary significantly between the trees, this is expected since ASBVd concentration can vary widely even between the branches, leaves and flowers within a single avocado tree (Running *et al*., 1996; Bruening *et al*., 1982). From this study we observed high ASBVd concentration variation between trees from the same province.

Differences observed between the ASBVd sequences were the result of minor nucleotide changes distinguishing them as different ASBVd variants. The differences were observed from the sequences obtained from the same trees showing the high mutation rate of ASBVd in within the same host. ASBVd is a hammerhead viroid, the hammerhead viroid have been proven to have higher mutation rates and thus high sequence diversity (Gago *et al*., 2009. Previous studies indicated that different ASBVd variants arose from slight sequence variations on the ASBVd sequence (Running *et al*., 1996). Most of the changes occurred between U-A bases leading to the sequence variations (Shnell *et al*., 1997). Therefore, the sequences obtained are different but still regarded as the same ASBVd strain. Similar findings were observed in studies conducted on citrus viroids (Lin *et al*., 2015) where it was shown that in a single citrus host, viroid populations were preserved as a genetic pool. It was pointed out that geographic factors might be the cause of high mutation rates or, leading to more difficult, unreliable and unstable detection conditions for viroids than for other pathogens.

3.5 Conclusions

Avocado sunblotch disease is an important disease of avocado and is common in South African avocado orchards. Currently, the disease is not properly managed and more effort is

required from the growers, industry and research institutes. Symptomless carrier trees are very common and could be the main source of new infections in the field. The ASBVd variants found in South Africa are detectable with the current primers and a few of them are similar to the existing variants in GenBank®. Infected trees should be removed from the field immediately when the symptoms are spotted. Raising awareness about the importance of ASBV as an economical important disease, disease symptoms and management strategies are crucial in successfully managing the disease in South Africa. All this can be achieved through the collaboration efforts between the South African avocado growers association (SAAGA), research institutes and the growers. This will lead to constant production of quality avocado, more jobs creation and an increase in the gross value of the crop. Different ASBVd variants were detected in phylogenetic comparisons of the sequences from this study. Small nucleotide changes were observed between sequences. Alignment of the primers, currently used in the routine diagnostic detection protocol at ARC-TSC, showed a perfect match with the sequences obtained from this study, supporting the reliability of ASBVd detection for the South African avocado industry.

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CHAPTER 4

General overview

4.1 Major findings

Detection using the fluorescent based real-time RT-PCR and the ASBVd specific primers for indexing is currently the best option to use in routine ASBVd diagnosis. Real-time applications are more sensitive and faster than the conventional PCR. Results are detected in the early stages of the reaction and the variations between the samples can be detected. The best results were obtained using the Jooste (unpublished) primer pair from RNA extracted with the cellulose column chromatography method, currently making it the most sensitive and reliable method for ASBVd large scale indexing. ASBVd is unevenly distributed between the branches of symptom-bearing trees, however, the following variations were observed in terms of distribution: trees that displayed only fruit symptoms tested positive for fruits and the leaves tested negative; trees displaying symptoms both on fruits and leaves tested positive for some branches and negative for other branches. Moreover, differences were observed between the slightly infected fruits and severely infected fruits. Slightly infected fruits displayed an uneven distribution of ASBVd between the infected yellow part of the skin, which tested positive, and the green part where it tested negative for ASBVd. Severely infected fruits displayed an even distribution of ASBVd where both the infected and the uninfected parts of the skin both tested positive. ASBVd was found evenly distributed between the branches of the symptomless trees; similar results were obtained in the symptomless fruits skin.

From the online survey it was discovered that not all farmers are familiar with Sunblotch disease symptoms in the field, and that fruit symptoms are the most commonly observed in the field for the farmers who are familiar with Sunblotch disease. It was also discovered that not all farmers practice precaution measures when cutting or injecting the trees. The field survey results indicated that symptomless carrier trees are more common in the field than symptom-bearing trees. The indexing results showed that the symptomless trees carry higher ASBVd concentrations (copies µl) compared to symptom-bearing trees. Different ASBVd variants were detected in phylogenetic comparisons of the sequences from this study. Small nucleotide changes were observed between sequences.

4.2 Implications of findings

1 The fact that ASBVd is unevenly distributed between the branches of the same tree could
2 lead to false negative results since the symptoms are very rare and some of the farmers are
3 not familiar with the signs of Sunblotch disease. These findings will improve the sampling
4 method thus increase the reliability of ASBVd indexing. This will also lead to improved
5 management of Sunblotch disease in South Africa. From the responses it was discovered that
6 not all avocado growers are familiar with ASBV disease symptoms; some farmers do not take
7 precautions with their cutting tools and removal of infected trees from the field, which could
8 pose a serious threat in disease spread. From these findings we discovered that Sunblotch
9 disease awareness is necessary in South Africa to familiarise farmers with symptoms and
10 management strategies for Sunblotch disease. The fact that symptomless carrier trees are
11 common in the field and remain undetectable could be problematic in that the disease could
12 be spreading without being recognized which can lead to widespread infections resulting in
13 huge financial loses to the farmers. The detection method currently used in the ARC-TSC
14 laboratory detected all the ASBVd variants and precise indexing of propagation material is
15 crucial to ensure a healthy avocado industry in South Africa. Alignment of the primers,
16 currently used in the routine diagnostic detection protocol at ARC-TSC, showed a perfect
17 match with the sequences obtained from this study, supporting the reliability of ASBVd
18 detection for the South African avocado industry. Therefore, indexing of all propagating
19 material and precise sampling is important for Sunblotch disease management.

20

21 **4.2 Way forward**

22 There is a need to raise disease awareness among farmers by organising study groups that
23 will establish a platform to interact and present important information. Sanitisation of
24 injection tools and pruning tools is very important to avoid the spread of Sunblotch disease in
25 the orchards. It is highly recommended that farmers remove all trees that show any symptoms
26 of Sunblotch disease in the field. Suspected symptomless trees should be sent for indexing to
27 confirm whether they are ASBV positive or negative. Nurserymen should send the
28 propagative material both used as rootstocks and scions for indexing. Sampling of trees in the
29 field should be done by trained personnel to avoid sampling errors which could lead to false
30 negatives. The latter could lead to the spread of Sunblotch disease to other healthy trees in the
31 field and other neighbouring fields. Screening for the symptomless carrier trees should start
32 in the field. Information on the disease should be extended to new farmers and the small-scale
33 farmers. More surveys in Limpopo and Mpumalanga provinces and in KwaZulu-Natal should

be conducted. All samples must be sent for sequencing to enable comparison of all the ASBVd variants in South Africa.

As the trees grow older the roots intersect, and this has been suspected to be one of the methods Sunblotch disease can be transmitted from an infected tree to a healthy one (Wallace, 1958). However, the frequency of root grafting in the field is unknown and could be of minor importance (Semancik and Szychowski, 1994). More work needs to be done to investigate the role of root grafting in transmission; this can be achieved using young avocado trees. Young Sunblotch disease infected trees can be grown together with Sunblotch disease -free trees, thus allowing the roots to intersect. The ASBVd transmission frequency over the years as the trees grow together could then be determined.

The trees that test negative and positive need to be studied further starting from detecting Sunblotch disease from the roots of these trees. Since Sunblotch disease can be unevenly distributed in a tree, it is possible that ASBV is present in the roots and not in the rest of the plant and only moves to the upper part of the trees if the environment is not favourable in the soil for survival. This hypothesis should be studied.

4.4 References

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Conferences to date

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Zwane, Z., Gubba, A., Jooste, A.E.C., Distribution, detection and management strategies for Avocado sunblotch disease (ASBV) in South Africa. Professional Development Programme Conference, ARC-VOP, 4-6 September 2017

1 **Research outputs**

2 Non-peer reviewed article:

3 Z.R. Zwane., Gubba, A. and Jooste, A.E.C. Review of avocado sunblotch disease. Subtrop
4 Journal, Third edition 2017, Vol 19, p32-36

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