A study on avocado sunblotch viroid (ASBVd) with a focus on symptomless carrier trees

By

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Dissertation summary

The avocado (*Persea americana* Mill.) is an important subtropical fruit worldwide (Blakey *et al*, 2014). South Africa is among the top five avocado exporters and the exports are primarily aimed at the European market (DAFF, 2019). According to the profile of the South African avocado market value chain by DAFF (2019), approximately 54% of total avocados produced in South Africa are exported, 14% are sold through the National Fresh Produce Markets (NFPMs), 12% are sold to the informal markets (bakkies and hawkers), 10% are processed, while the remaining 9% are delivered directly to retailers. Avocado sunblotch disease (ASBD), caused by avocado sunblotch viroid *(*ASBVd), is one of the smallest pathogens inciting economical losses in the avocado crop worldwide (Palukaitis *et al*. 1979). The infected trees can either express ASBD symptoms phenotypically or can show no symptoms or signs of infection but still be carriers of ASBVd; these trees are known as symptomless carrier trees (Acheampong *et al*., 2008). Characteristic ASBD symptoms include the appearance of irregular, sunken areas of white, yellow, or reddish colour in infected fruit; white/yellow sunken streaks on green stems and variegated or bleached symptoms on the leaves (Vallejo-Perez *et al*., 2014).

The ASBVd symptomless carrier trees have been described as the primary sources of infection by spreading the disease through budding and grafting practices thus playing an essential role in the epidemiology of ASBVd (Saucedo Carabez *et al*., 2019). Therefore, the main aim of the study was to investigate ASBVd in avocado symptomless carrier trees. The specific objectives of the current study were to (1) identify and monitor ASBVd-infected trees from the flowering stage until harvest for any visible physical indications of the disease compared with healthy trees, (2) determine the maturity of ASBVd - infected and healthy fruits over time from early development stages until harvest, (3) determine the productivity of ASBVd - infected fruits of the symptomless carrier trees to healthy ones by counting the number of fruit per tree, (4) investigate direct ripening, storage effects and internal quality of ASBVd - infected fruits, (5) investigate honeybees as possible ASBVd vectors for tree-to-tree transmission during pollination, (6) determine the occurrence of root graft transmission of ASBVd from root systems of infected trees to healthy trees, (7) investigate the impact of cutting tools on the plant to plant transmission, (8) study the movement of ASBVd from initially infected cells to the rest of the tree through the grafting of infected

scions on healthy rootstocks and (9) investigate the genetic differences between ASBVd variants within a clonal symptomless carrier ‘Fuerte’ tree population.

Differences were observed in the orchard between infected and healthy trees; medium and highly infected trees excessively produced flowers, lost leaves during flowering and ultimately bore few to no fruit at the end of the season. The dry matter content results showed that ASBVd did not affect the maturity of the fruit; fruit from infected and healthy trees matured at the same time. Yield measurements indicated that medium and highly infected trees produced between 83% and 96% lower yields compared to healthy trees. Postharvest studies showed that medium and highly infected fruit significantly lost firmness and developed colour more rapidly than healthy fruit. Infected fruit also developed external rots and shrivels for non-stored fruit, however, these injuries were reduced for fruit stored at 5°C for 28 days. Therefore, flower overbearing with the shedding of leaves and lower yields can be used as an indication of ASBVd infection in ‘Hass’ orchards; however, confirmation with molecular testing is required. These observations can be incorporated as an ASBD management strategy in 'Hass' orchards.

Graft transmission (top-work) was demonstrated by grafting 30 healthy and 30 infected ‘Hass’ scions onto a healthy ‘Bounty’ rootstock. There was a 53% infection success rate for the infected symptomless carrier trees compared to the 43.3% for the healthy trees. The statistical analysis showed that there were no significant differences between grafting the healthy or infected scions to the rootstocks ($p \leq 0.05$), implying that ASBVd spread through the infected scions is unavoidable unless viroid-free scions are used. Two separate root graft experiments were conducted, one in the tunnels where a single infected tree was planted in the 10L plastic bag to force root contact with four healthy trees; the healthy trees outcompeted the infected trees in all six experiments and they died. In the field, the healthy young ‘Fuerte’ trees were planted one meter closer to the old infected orchard trees with confirmed ASBVd positive roots to force root contact. Eventually, the trees died from the lack of sunlight and water stress and the experiment was discontinued. Seventy-five ‘Fuerte’ clonal trees were used in the mechanical transmission experiment using pruning shears (secateurs). The trees were infected by cutting the infected branch and then using the same shear to prune the healthy trees. The shears were treated with four different percentages of sodium hypochlorite (3.5% M/V) and the untreated shears were used

as controls. ASBVd was not successfully transmitted mechanically using pruning shears, however, when the stability of ASBVd was tested on different surfaces, it showed to survive up to 24 hours. Therefore, if equipment used on an infected tree is used for the next tree, there is a chance that ASBVd will be transmitted to the next tree and lead to ASBVd spread in an orchard. Pollen and bees were sampled from the beehives at four different sites in KwaZulu-Natal. ASBVd was successfully detected in the pollen from all four site sites, however, only detected in the bees from three sites. The samples were sequenced and the blast results confirmed the sequences to be ASBVd and the phylogenetic analysis gave a 93% identity between the detected sequences and the existing ASBVd variants retrieved from the GenBank® database. From the current study, it was confirmed that graft transmission is the most prevalent mode of transmission for ASBVd and that honey bees carry pollen from infected trees to healthy trees in the field and can play an important role in pollen transmission.

A total of 103 positive symptomless carrier trees were detected with a real-time qRT-PCR assay from a population of 453 young ‘Fuerte’ trees. Results showed that 22% of this population was infected with ASBVd without showing signs of infection. In a further investigation, complete ASBVd genomes were obtained by using a conventional PCR with primer sequences that yielded a 250 bp product, here only 76 samples tested positive for ASBVd, the remaining 27 samples tested negative. The 76 samples were sequenced and the sequences obtained had lengths that varied between 248 and 253 nucleotides. From the original 76 sequences, 42 ASBVd variants were identified and several sequences were repeatedly detected in the population. The variants were deposited to the NCBI GenBank® and were assigned ON135462 to ON135503. The phylogenetic analysis of the variants obtained from this study showed a high sequence identity of 97% with the reference ASBVd variants obtained from GenBank®. The current study is crucial for the development of accurate detection techniques for ASBVd and contributes to the ASBVd action plan goals that promote the removal of all infected material from avocado orchards to prevent the further spread of the viroid in avocado orchards.

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Introduction to Dissertation

The avocado (*Persea americana* Mill.) is an important subtropical fruit worldwide (Blakey *et al*, 2014). The high nutritional value and health benefits associated with avocado have increased its consumption and thus its popularity amongst consumers (Magwaza and Tesfay, 2015). Additionally, avocado consists of bioactive compounds that are known to decrease blood cholesterol levels in consumers (Duarte *et al*., 2016). Some countries produce small volumes of avocado oil, which is used in its raw form by the pharmaceutical and cosmetics industries (Duarte *et al*., 2016). Avocado leaves are a pharmaceutical ingredient widely used in extracts for therapeutic purposes, and also as teas in folk medicine (Duarte *et al*., 2016).

Even though the true origin of the avocado is vague, wild avocados are found in Mexico, it is thus suspected that they originated from either Mexico or nearby countries of Southern America, Central America and Mexico (Storey *et al*, 1986; Sippel, 2001). The Americas (North and South) are the leading avocado producers, contributing 76% of global avocado production. Africa accounts for 11% of the total production followed by Asia and Europe with 9% and 2%, respectively (DAFF, 2012). South Africa is amongst the top five avocado exporters with exports primarily aimed at the European market (DAFF, 2019; Ntombela *et al*, 2013). According to the profile of the South African avocado market value chain by DAFF (2019), approximately 54% of total avocados produced in South Africa are exported, 14% are sold through the National Fresh Produce Markets (NFPMs), 12% are sold to the informal markets (bakkies and hawkers), 10% are processed, while the remaining 9% was delivered directly to retailers.

Pests and diseases play a major role in the productivity and marketability of crops (Grove, 2001). Common pests that are important in avocado production include; fruit flies, mealybugs, adult coconut bugs, and heart-shaped scale insects. These pests can be detrimental to avocado production as their activity within orchards can cause lesions on fruit, early fruit drop (Grove, 2001; Joubert, 2001), as well as cause the formation of sooty mould from honeydew production which disturbs photosynthesis activity (De Villiers 2001). Diseases commonly affecting avocado include; Phytophthora root rot caused by *Phytophthora cinnamomi*, Stem canker, caused by *Phytophthora citricola*, *P. cactorum P. cinnamomi*, and Cercospora spot caused by

Pseudocercospora purpurea and Anthracnose, which is a pre- and post-harvest problem that causes economic losses on production (Marais, 2007).

Avocado sunblotch disease (ASBD) is caused by avocado sunblotch viroid *(*ASBVd), which is the smallest viroids causing economical losses in the avocado worldwide (Palukaitis *et al*. 1979). ASBVd is an infective single-stranded, covalently closed, circular RNA molecule (Palukaitis *et al*., 1979; Symons, 1981; Saucedo-Carabez *et al*., 2015). The infected trees can either express ASBD symptoms or can show no symptoms or signs of infection but still be carriers of ASBVd; these trees are known as symptomless carrier trees (Acheampong *et al*., 2008). Characteristic symptoms of ASBD include the appearance of irregular, sunken areas of white, yellow, or reddish colour in infected fruit; white/yellow sunken streaks on green stems and variegated or bleached symptoms on the leaves (Vallejo-Perez *et al*., 2014).

Significance of research

The ASBVd symptomless carrier trees have been described as the primary sources of infection by spreading the disease through budding and grafting practices thus playing an essential role in the epidemiology of ASBVd (Saucedo Carabez *et al*., 2019). This implies that the symptomless ASBVd infected trees can potentially be used as sources for propagation material as a result of the lack of information on the management and control of these symptomless carrier trees and because they remain unremoved in the orchards. The only way to detect if a the tree is an infected symptomless carrier tree is through molecular indexing, without visible symptoms the infected trees are mistaken for healthy trees. The common existence of symptomless carrier trees in South African orchards implies that 1) indexing of propagation material was not always conducted systematically or that 2) field transmission of ASBVd occurs frequently. This also implies that the importance of symptomless carrier trees in the epidemiology of ASBVd is not well understood by avocado growers. In addition, the mechanisms of spread of the disease is not well understood and producers are not familiar with applying optimal preventative measures for disease spread in orchards. The lack of clear signs or symptoms associated with symptomless carrier trees makes ASBD very difficult to control. It was therefore necessary to undertake an intensive study that focused on the symptomless carrier trees to identify answers that could assist the avocado industry in understanding the role played by symptomless carrier trees in the epidemiology of ASBD.

Research aims and objectives

Aim

The main aim of the study was to investigate avocado sunblotch viroid (ASBVd) in avocado symptomless carrier trees

Study objectives

1. Identify and monitor ASBVd-infected trees from the flowering stage until harvest for any visible physical indications of the disease compared with healthy trees.
2. Determine the maturity of ASBVd - infected and healthy fruits over time from early development stages until harvest.
3. Determine the productivity of ASBVd - infected fruits of the symptomless carrier trees to healthy ones by measuring yield (kg/tree) and fruit size.
4. Investigate direct ripening, storage effects and internal quality of ASBVd - infected fruits.
5. Investigate the involvement of honeybees for tree-to-tree transmission during pollination.
6. Determine the occurrence of root graft transmission of ASBVd from root systems of infected trees to healthy trees.
7. Investigate the impact of cutting tools on the plant to plant transmission
8. Study the movement of ASBVd from initially infected cells to the rest of the tree through the grafting of infected tissues on healthy trees.
9. Investigate the genetic differences between ASBVd variants within a clonal symptomless carrier ‘Fuerte’ tree population

Dissertation structure

The dissertation is divided into five chapters. The first chapter reviews the literature on the importance of avocado (*Persia americana* mill.) in South Africa, the importance of ASBD in the avocado industry and some of the prevention strategies used to manage the disease. Chapter 2 investigates the effect of ASBD on tree morphology, fruit maturity; yield and quality of ‘Hass’ avocado in South Africa. Chapter 3 investigates the occurrence of four transmission mechanisms for ASBVd from infected to healthy avocado plants. Chapter 4 investigates the genetic differences between avocado sunblotch viroid (ASBVd variants in a clonal population of young, symptomless ‘Fuerte’

trees. Chapter 5 is the general overview of the study and includes the major findings, the implication of the findings, the future research and research outputs.

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CHAPTER 1

Literature review

1.1 The host - Avocado (*Persea americana* mill.)

1.1.1 Origin and distribution

The true origin of avocado is vague; however, as wild avocados are found in Mexico (Storey *et al*, 1986), it is suspected that they originate from either Mexico or nearby countries of Southern America, and Central America, Guatemala (Storey *et al*, 1986; Sippel, 2001). Americas have the significant avocado production worldwide, however, countries lie Peru, Indonesia, Colombia, and Kenya grow more avocados compared to the United states (FAOStats, 2022). Countries such as Venezuela and Israel are also in the top ten of the most avocado producing countries (FAOStats, 2022). Africa accounts for 11% of the total production followed by Asia and Europe with 9% and 2% respectively (DAFF, 2012). Avocado was only introduced to South Africa in 1933 in the province of KwaZulu-Natal, the first orchard consisted of 10 000 West Indian seedlings (Sippel, 2001). To date, avocados are produced in four South African provinces namely Limpopo, Mpumalanga, KwaZulu-Natal and Eastern Cape (Nortje, 2012; SAAGA, 2020).

1.1.2 Production and economic importance

The avocado (*Persea americana* Mill.) is an important subtropical fruit (Magwaza and Tesfay, 2015). The high nutritional value and health benefits associated with avocado, have increased its consumption and thus its popularity amongst consumers (Magwaza and Tesfay, 2015). Additionally, avocados consist of bioactive compounds that are known to decrease blood cholesterol levels in consumers (Duarte *et al*., 2016). Some countries produce small volumes of avocado oil, which is used in its raw form by the pharmaceutical and cosmetics industries (Duarte *et al*., 2016). Avocado leaves are a pharmaceutical ingredient widely used in extracts for therapeutic purposes, and also as teas in folk medicine (Duarte *et al*., 2016).

The total land utilised for avocado production in South Africa is 19,000 hectares (ha), with an estimated 1500 new plantings being added annually (SAAGA, 2020). The majority of South African production is based in the Limpopo and Mpumalanga provinces with the lowest production being in KwaZulu-Natal and Eastern Cape provinces (Figure 1.1). Limpopo leads with 60% of total production, followed by Mpumalanga province with 29% of the total production (SAAGA, 2020). KwaZulu-

Natal and Eastern Cape follow with 8% and 2% of the total avocado production, respectively (SAAGA, 2020). The industry has a 5-year average annual production of 125,600 tons, and it is playing a role in rural upliftment as the majority of the jobs created from this industry are within rural areas. For every 2.6 ha of avocado production, one worker is permanently employed (SAAGA, 2020). Between March and September, the peak periods for avocado, the industry alone creates approximately 6,000 permanent jobs and an additional two thousand jobs in the form of casual labour (Van Zyl and Ferreira, 1995; DAFF, 2019). Avocado production additionally provides a livelihood to 36,000 individuals who generate income and support their households by selling avocados (DAFF, 2012).

The South African avocado industry is export-oriented and the exports are primarily aimed at the European market (DAFF, 2020). According to the Statista Research Department (2022), South African export value was 77.09 million US dollars rating number eight among the leading avocado exporters worldwide. According to the profile of the South African avocado market value chain by DAFF (2020), approximately 54% of total avocados produced in South Africa are exported, 14% are sold through the National Fresh Produce Markets (NFPMs), 12% are sold to the informal markets (bakkies and hawkers), 10% are processed, while the remaining 9% was delivered directly to retailers. In 2018, South Africa exported 89,343 tons of avocados. The total fruit exported during 2018 was 106.43% higher than what was exported in 2017 (43,492 tons) (DAFF, 2020).

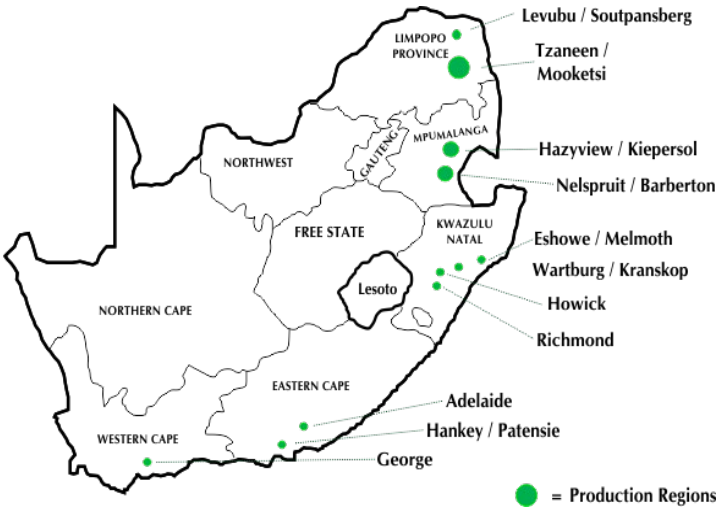


Figure 1.1 Avocado production regions in South Africa (SAAGA, 2020) https://www.avocado.co.za/avocado-production-regions/

1.1.3 Physiology and cultivation

Avocado is 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, the Guatemalan type (var. *guatemalensis*) from Guatemala and the West Indian type (var. *americana*) from America (Bergh and Ellstrand, 1986). Avocados are grown in tropical, subtropical and semi-tropical conditions; the three types are all adapted to different climatic conditions and possess different traits that are specific to each type as demonstrated in Table 1.1 . In South Africa, the production area is divided into cool and warm subtropical, where KwaZulu-Natal and Eastern Cape are the cool and Limpopo and Mpumalanga are the warm regions. The cultivars grown under these temperatures belong to Mexican and Guatemalan types (DAFF, 2012; Nortje, 2012).

Table 1.1 Different tree traits expressed by cultivars from the three different avocado 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 colour	medium	often redder	paler

Avocado production is mostly favoured in regions with temperatures between 20-25˚C, with sufficient annual rainfall, as these trees are sensitive to water stress (Kotzé, 1979; DAFF, 2012). The average annual rainfall required is >1000 mm per annum (p.a.), however, semi-arid regions with >400 mm p.a. can be sufficient for avocado production (Vorster, 2001). Avocados can only tolerate light frost after flowering and before fruit 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). Available commercial cultivars and their characteristics are presented in Table 1.2 . A normal, healthy avocado tree produces millions of flowers (Ish-Am,

2005), however, the majority of the flowers die, leaving <0.1% of flowers for fruit set (Garner and Lovatt, 2016). Research shows that avocado trees are not very good attractors of pollinators, and therefore they enter into a mass bearing phase. By producing vast volumes of flowers, the tree increases its chances of attracting pollinators (Ish-Am, 2005). This would affect the productivity of the trees, although when this happens, a tree would usually bear a few hundred fruits (Garner and Lovatt, 2016).

Table 1.2 South African avocado cultivars, availability and their characteristics (SAAGA, 2020)

Cultivar	Availability	Characteristics
'Edranol'	June -September	Thick green skin with small light brown speckles (lenticels). It stays green when ripe and the flesh has a rich, nutty taste.
'Fuerte'	March to July	Pear or egg-shaped. It has a thin waxy skin, which remains green on ripening and separates easily from the flesh. The flesh has a rich creamy texture.
'Hass'	May to October	Egg-shaped with a rough green skin, which usually turns purple-black when ripe. The flesh is firm with a creamy texture and a slightly nutty taste. The thick skin is an advantage when serving half an avocado as the flesh can be easily scooped out.
'Lamb Hass'	August to November	Shiny pebbly skin that turns purple-black, and is squarish at the stem end. Unlike Hass, it may turn dark before ripening.
'Maluma Hass'	March to July	Has a thick, pebbly dark green, shiny skin that turns purple-black once ripe. The flesh has a rich nutty taste.

‘Pinkerton’	April to July	A pronounced thickish neck compared to most other cultivars. It has a thick, rough, green skin that does not yield easily to pressure and remains green when ripe. The flesh is sometimes slightly sweet.
‘Reed’	September to November	Has a more rounded shape and is usually quite large. Ripe fruit remains green and is soft to the touch. It has a distinctive taste with a creamy, smooth, buttery texture.
‘Ryan’	July to October	Egg-shaped with a green skin that does not change colour on ripening. It is popular towards the end of the avocado season.

1.1.4 Maturity and Harvesting

There are several factors affecting the fruit quality of avocado, these factors include the production of disease-free fruit; harvesting of fruit at proper maturity; correct harvesting of fruit and handling procedures (Barmore, 1976). These factors are all equally important for the quality, however, from a postharvest perspective, quality begins at harvest with physiological maturity (Barmore, 1976). This makes fruit maturity an important factor in determining the avocado fruit quality (Magwaza and Tesfay, 2015). Before discovering the methods to measure fruit maturity, most of the incurable troubles, which affected the consumer, were from the selling of immature fruit (Barmore, 1976).

The physiological maturity of avocado fruit is simply defined as the stage of development where most growth has already occurred, at which fruit will ripen after harvest resulting in an edible product (Young and Lee, 1978; Lee *et al*., 1983). It is difficult to determine if avocado fruit has matured while still on the tree because there are no distinguishable physiological changes visible at this stage. Changes become visible after the fruit has been harvested during the ripening process (Magwaza and Tesfay, 2015). To address this challenge, some techniques have been developed. The most well-known method to estimate fruit maturity is to determine the degree of ripeness of avocado fruit by measuring dry matter content (DM) before harvest (Woolf

et al., 2003). According to this method, fruit is collected from trees several weeks before harvest. The percentage dry matter content (% DM) of the fruit is then determined and a certain threshold, which is different for each cultivar, is required for the fruit to be considered mature enough for harvest.

Fruit ripening is described by the processes involving both catabolic and anabolic changes together with large amounts of energy resulting in changes in colour, texture and taste, making the fruit acceptable for consumption (Bower, 1988). Ripening of 'Hass' avocado fruit is determined by the firmness of the fruit flesh and the colour change of the fruit rind, as the fruit becomes softer and the skin colour changes from green to purple/black during ripening (Cox *et al*., 2004). However, colour change can be misleading because some fruit can change colour before harvest. When this occurs, the fruit colour changes late in the harvest season but the fruit flesh remains hard (Cox *et al*., 2004). This becomes more complicated in cultivars that do not change colour during ripening; therefore, fruit firmness is the most reliable method for measuring fruit ripeness (White *et al*., 1999). Avocado fruit is very delicate and tends to develop rot and other internal disorders during the later stages of ripening. In addition, external stresses such as fungal infections caused by diseases are also a major problem contributing to yield reduction and poor fruit quality.

1.1.5 Pests and Diseases

Pests and diseases play a major role in the productivity and marketability of crops. Common pests that are important in avocado production include; fruit flies, mealybugs, adult coconut bugs, and heart-shaped scale insects. These pests can be detrimental to avocado production as their activity within orchards can cause lesions on fruit, early fruit drop (Joubert 2001, Grové 2001), as well as cause the formation of sooty mould from honeydew production which disturbs photosynthetic activity (De Villiers 2001). Diseases commonly affecting avocado include; Phytophthora root rot caused by *Phytophthora cinnamomi*, Stem canker, caused by *Phytophthora citricola*, *P. cactorum P. cinnamomi*, and Cercospora spot caused by *Pseudocercospora purpurea* and Anthracnose (*Colletotrichum gloeosporioides*) which is a pre-and post-harvest problem that causes economic losses on production (Marais, 2007). The current study focused on avocado sunblotch disease (ASBD) caused by avocado sunblotch viroid (ASBVd). The main concern with the disease is that it is incurable. Once infected; the trees must be removed from the orchards to prevent further spread. The mechanisms

for the spread of the disease are not well understood and producers are not familiar with applying optimal preventative measures for disease spread in orchards. The lack of clear signs or symptoms associated with symptomless carrier trees makes ASBD very difficult to control.

1.2 Avocado sunblotch disease (ASBD)

1.2.1 History

The ASBD was first discovered in Southern California in 1914 (Whitsell, 1952; Horne and Parker, 1932). The disease is characterised by the formation of yellow sunken areas on fruit, which later turned brown (Whitsell, 1952), and was therefore initially confused for a physiological disorder as it resembled sunburn (Coit, 1928). Later, the disease was formerly described as being graft transmissible which led to ASBD being mistaken for a viral disease (Horne and Parker, 1932). Due to the failed attempts to detect ASBVd using common virus detection methods, Thomas and Mohammed (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 viroids 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).

Despite the efforts of Thomas and Mohammed (1979), Palukaitis *et al.* (1979) were the first to report the causal agent of ASBD as a viroid by hybridization analysis with ^{32}P-complementary DNA; they named it avocado sunblotch viroid (ASBVd). The primary and secondary structures of ASBVd were proposed to be 247 base pairs (bp) long (Figure 1.2) (Symons, 1981). Symons (1981) compared ASBVd to the structures of the viroids 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 sequence homology of only 18% with the two viroids whereas PSTVd and CSVd shared a sequence homology of 69% with each other (Symons, 1981). In South Africa, the disease was only identified in 1954, however, it was believed to have been present long before its discovery (Loest and Stofberg, 1954; Da Graça and van Vuuren, 2003). In 1983, ASBVd was reported to be present in all commercial cultivars in South Africa (Da Graça and Mason, 1983).

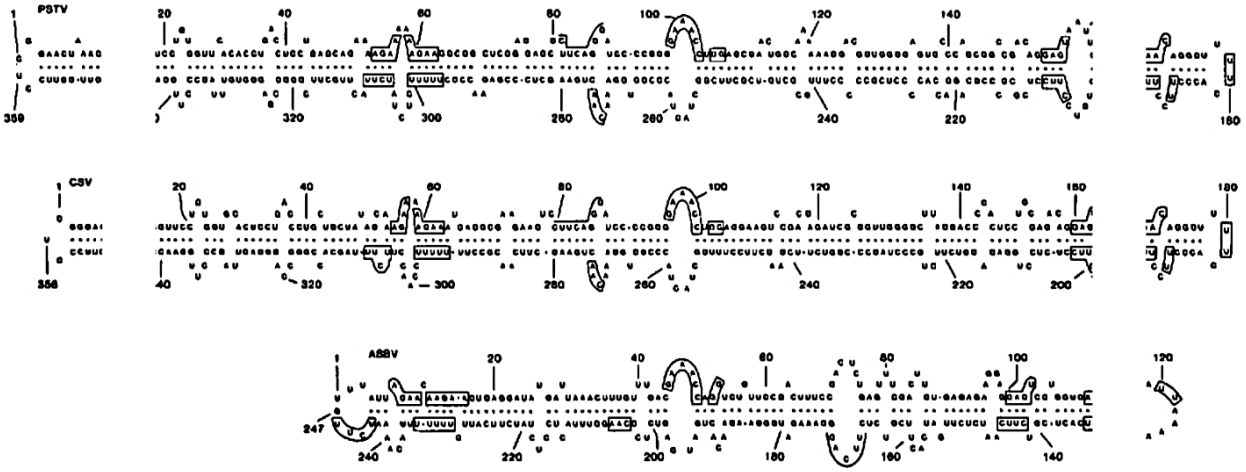


Figure 1.2 Proposed Avocado sunblotch viroid (ASBVd) secondary structure compared to that of potato spindle tuber viroid (PStVd) and chrysanthemum stunt viroid (CSVd) (Symons, 1981).

1.2.2 Causal agent – Avocado sunblotch viroid (ASBVd)

The ASBVd is an infective single-stranded, covalently closed, circular RNA molecule (Palukaitis *et al*., 1979; Symons, 1981; Saucedo-Carabez *et al*., 2014). ASBVd forms a small rod-like secondary structure between 239 and 251bp in length (Hammond and Owens, 2006). ASBVd is the classified under family *Avsunviroidae* together with peach latent mosaic viroid (PLMVd), chrysanthemum chlorotic mottle viroid (CChMVd) and eggplant latent viroid (ELVd). This viroid family does not consist of a central conserved region and the members of this family have the self-cleavage ability (Delan-Forino, 2014). ASBVd is structurally and functionally distinct from other family members, and differences between these viroids are demonstrated in Table 1.3. ASBVd is the single member of the genus *Avsunviroid* and 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).

There are 108 known ASBVd variants deposited in the GenBank® database. The variants are associated with different symptoms they induce in the avocado host. The symptom-associated variants include a symptomless carrier (ASBVd-Sc), a bleached symptom associated (ASBVd-B) and variegated symptom associated (ASBVd-V) variants (Semancik and Szychowski, 1994). Different ASBVd variants arise from slight sequence variations on the ASBVd sequence (Running *et al*., 1996). Most of the changes occur between U-A bases leading to the sequence variations (Shnell *et al*., 1997), however, because the variants share similar biological properties, they are

therefore not identified as different strains (Semancik and Szychowski, 1994). The variation found between ASBVd variants is a crucial factor to consider when developing molecular detection techniques for ASBVd. Symptomless carrier trees play an essential role in the epidemiology of ASBVd, as they have been described as the primary sources of infection by spreading the disease through budding and grafting practices (Saucedo Carabez *et al*., 2019).

Table 1.3 Species classified under the family *Avsunviroidae* together with ASBVd (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

1.2.3 Epidemiology

ASBVd is a systemic pathogen, but its concentration can vary widely between branches, leaves and flowers within a single avocado tree (Running *et al*., 1996; Bruening *et al*., 1982). This variation can be influenced by temperature and growing seasons (Running *et al*., 1996). Damage of viroid diseases is known to be more severe in hotter climates compared to cooler climatic regions (Singh, 1983). Increasing the temperature (28-30˚C) all day and night can accelerate symptom development in ASBD indicator avocado trees (Da Graça and Van Vuuren, 1981). However, a combination of higher temperatures with consistent light, and day and night length is more effective in symptom development (Desjardins, 1987). Cutting back the trees to force new growth can also effectively accelerate symptom development in the infected avocado seedlings (Da Graça and Van Vuuren, 1980). Schnell et al (2011) reported a threefold increase in infection on the old plantings with ASBVd infected trees over a 13 year span. The location of new positive trees were both defined as adjacent or non-

adjacent. They further reported that the pattern of infection in new plantings are a result of the reintroduction of ASBVd rather than a natural spread, and found that the increase of infections occurred despite the strict sanitization procedures both in field and the greenhouse operations. The study emphasises the importance of removing all ASBVd infected trees' was added at the end of the sentence

1.2.3.1 Transmission

The use of infected propagative material is the most important mode of spreading ASBVd, however, there is no sufficient evidence of the natural infection from an infected- to a healthy tree (Wallace, 1958). ASBVd can be transmitted via seed from infected trees, from infected propagative rootstocks, infected scions used for grafting and via root grafts and pollen. To date, no vector has been reported for the transmission of ASBVd, however, honeybees were experimentally proven to carry ASBVd from an infected to a healthy tree and thus have been suspected as a possible vector (Desjardins *et al.,* 1979). Avocado is the only known natural host for ASBVd (Shnell *et al*., 1997). The following are the modes of ASBVd transmission:

1.2.3.1.1 Graft transmission (Top-work)

Grafting is one of the plant propagation techniques used to join two or more plants to grow as a single plant (Bilderback *et al*., 2014). Transmission of ASBD can occur through the grafting of unhealthy scion onto a healthy rootstock or vice versa. This type of transmission is regarded as the most important for the spread of ASBVd (Bar-Joseph *et al.*, 1987). ASBVd was first discovered as graft transmissible by Horne and Parker in 1931 and described ASBD as graft transmissible. They also discovered that disease symptoms manifests two to three years after grafting.

1.2.3.1.2 Root graft transmission

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

1.2.3.1.3 Seed transmission

Seed transmission was described by Zentmyer (1946) and confirmed by Wallace (1950). There are two types of seed transmission; symptom-bearing trees and symptomless carriers (Wallace, 1950). In 1953, Wallace and Drake indicated that

seeds obtained from symptomless carriers could transmit between 86 and 100% of the disease to the seedlings. The progeny of the symptomless carrier trees all result as symptomless carriers (Wallace, 1958). Mathews (2011) mentioned that symptomless carrier trees have been found to maintain high concentrations of ASBVd in leaves, fruit and seed which could explain the high transmission raterates. Seeds obtained from plants showing symptoms can only transmit 0-5% of the disease to the new trees, which also develop visible ASBD symptoms (Wallace, 1958).

1.2.3.1.4 Pollen transmission

Pollen transmission occurs when a healthy avocado tree is pollinated by pollen from a diseased tree. In such a case, only the fruits exhibit symptoms and the rest of the tree remain disease-free (Dodds, 2001). This could be detrimental if the fruit was infected with symptomless carrier associated variants because the fruit would not show any symptoms and when the seed of that particular tree is used for propagation, it will play a direct role in the transmission of ASBVd. It has also been suggested that symptomless carrier trees may be the main sources of pollen transmission in the field because they maintain higher concentrations of ASBVd (Mathews, 2011). Desjardins *et al.* (1979) experimentally demonstrated pollen transmission on avocado plants and found a low transmission rate between 1.8% and 3.125%.

1.2.3.1.5 Transmission by vectors

To date, no vector has been reported to transmit ASBVd from one tree to the 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.,* 1980; Dodds, 2001).

1.2.3.1.6 Mechanical transmission

The ASBVd can be transmitted on pruning blades, injection material and harvesting clippers (Semancik, 2003). An 8% to 30% mechanical transmission rate by cutting blades has been reported (Dodds, 2001). Slash inoculations and leaf rub with extracts from ASBVd infected tissue have also been proven to successfully transmit ASBVd (Semancik, 2003).

1.2.3.2 Host range

ASBVd has a narrow host range restricted to the family Lauraceae with *Persia americana* species being the only known natural host (da Graça and Van Vuuren, 1980). Cinnamon was the first alternative experimental host to be described for ASBVd

(da Graça and Van Vuuren, 1980). Da Graça 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 ASBD symptoms following graft inoculation with ASBD diseased material, however, no natural infection has been reported for all the alternative hosts and low disease transmission rates were reported (Da Graça and Van Vuuren, 1981).

1.2.4 Economic significance

Viroid diseases lead both to direct and indirect losses in crop production (Randles, 2003). However, there is difficulty in stating the exact percentages of yield losses due to the lack of essential quantitative data on diseases caused by viroids (Singh *et al.*, 2003). Generally, ASBD infected trees are lower yielding and the infected fruits are discarded as consumers reject the lesions associated with ASBD (Randles, 2003). Similar to symptomless carrier trees, they produce lower yields, with most of the fruits being downgraded on quality standards. No cultivar is tolerant to ASBVd although symptom development is delayed on a 'Zutano' cultivar (Wallace, 1958). Indexing costs during the selection of parent material for propagation, and eradication of infected trees in the field account for some of the costs that are directly lost to avocado production as a result of ASBVd infection (Wallace, 1958; Randles, 2003).

Several studies have been conducted worldwide to evaluate yield losses associated with ASBVd infections (Da Graça, 1985; Running *et al*., 1996; Randles, 2003; Tondo *et al* 2010; Saucedo-Carabez *et al*., 2014). ASBVd affects the postharvest quality of symptomatic fruits but asymptomatic fruits satisfy the international quality standards (Saucedo-Carabez *et al*., 2014). However, the latter could have implications for quarantine restrictions since the majority of South African avocados are exported. In terms of yield reduction, Da Graça (1985) conducted a three-year trial testing the effect of ASBVd in 'Fuerte' cultivars, both in symptomless carrier trees and symptomatic trees. Symptomatic fruits showed a yield reduction of 14% while symptomless carrier trees' yield was reduced by 82% (Da Graça, 1985).

Running *et al*., (1996) conducted a study in Miami based on the detection of ASBVd and estimated infection rates among accessions in the national germplasm. They indexed 429 trees using a reverse transcription-polymerase chain reaction (RT-PCR) to determine the incidence of infection and found that 18.9% of the trees were infected

with ASBVd. They also discovered that infection rates between different races were similar; except for West Indian races which had higher ASBVd infection rates (Running *et al*., 1996). A similar study was conducted in 2009 on the same accessions using ASBVd specific RT-PCR (Tondo *et al.,* 2010). In this study, they discovered an increase in the number of infected trees from 19% in 1996 and 2000 to 24% in 2009. The newly infected plants were either adjacent to previously infected plants, adjacent to plots from which infected plants had been removed, or adjacent to other newly infected plants that are adjacent to previously infected plants or contaminated plots. They attributed these infections to root grafting. Other new infections were random and these were related to pollen transmission and contamination during pruning (Tondo *et al*., 2010). Korsten *et al.* (1987) conducted a study on the occurrence of ASBVd in South African nursery trees using a dot-blot hybridization technique. A total of 3,125 trees were tested and only 2% of those trees tested positive for ASBVd.

A study based on indexing records was conducted at the ARC-TSC by Ncango *et al.* (2014) using a qRT-PCR assay. This study was conducted using data compiled over a five year period which 24,685 avocado mother trees from 14 different commercial nurseries in Limpopo and Mpumalanga, South Africa. The results showed that 15.6% of indexed trees tested positive for ASBVd. ASBVd infection is not only increasing in South Africa but also other avocado producing regions worldwide. ASBVd may be more widespread because sunblotch symptoms vary and symptomless carriers of ASBVd are common, leading to the unintentional introduction of ASBVd into orchards (Luttig and Manicom, 1999).

1.2.5 Geographical distribution

ASBD has been reported in all avocado growing areas worldwide and infects all commercial cultivars (Saucedo-Carabez *et al*., 2014). The disease has been reported in Europe (Spain), North - (Mexico, USA, California, Florida) and South America (Peru, Venezuela), Australia, Asia (Israel), and Africa (South Africa (Da Graça, 1980) and Ghana (Acheampong *et al*., 2008) (Figure 1.3).

Figure 1.3 Avocado sunblotch disease global distribution (Carabez *et al*., 2019)

1.2.6 Signs and Symptoms

Some of the ASBVd-infected tree signs may include: stunting with branches spreading unevenly to the sides (Dodds, 2001) and sprawling of the lateral branches, exposing the tree to sunburn (Acheampong *et al*., 2008). The trees may develop abnormal growth, growing in a flattened shape with limbs bending towards the ground (Wallace, 1958). Thinning of the tree canopy has also been described as a sign of ASBD (Dodds, 2001). The trees displaying the above-mentioned signs must be sent for indexing to confirm the presence of infection.

The symptoms of ASBD manifest 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 form is where no symptoms are displayed and these trees are termed symptomless carriers (Thomas and Mohammed, 1979). Mohammed and Thomas (1979) described ASBVd to be more confined on the lesions in the symptom bearing trees thus rendering it undetected to the rest of the tree, and the ASBVd in the symptomless carrier trees to be more localised and easy to detect throughout the infected, such trees must be sent for indexing to confirm the presence of an infection. tree. The symptoms are described below.

1.2.6.1 Stem symptoms

Symptoms of ASBD symptoms on infected young stems appear as yellow or colourless, sometimes reddish sunken longitudinal streaks on young growth (Figure 1.4) (Parker and Horne, 1932). The trunk usually develops rectangular cracking on

older trees, also referred to as alligator bark. This has been described as a common ASBD symptom in fields and is of value for the diagnosis of ASBD from orchards (Wallace, 1958).

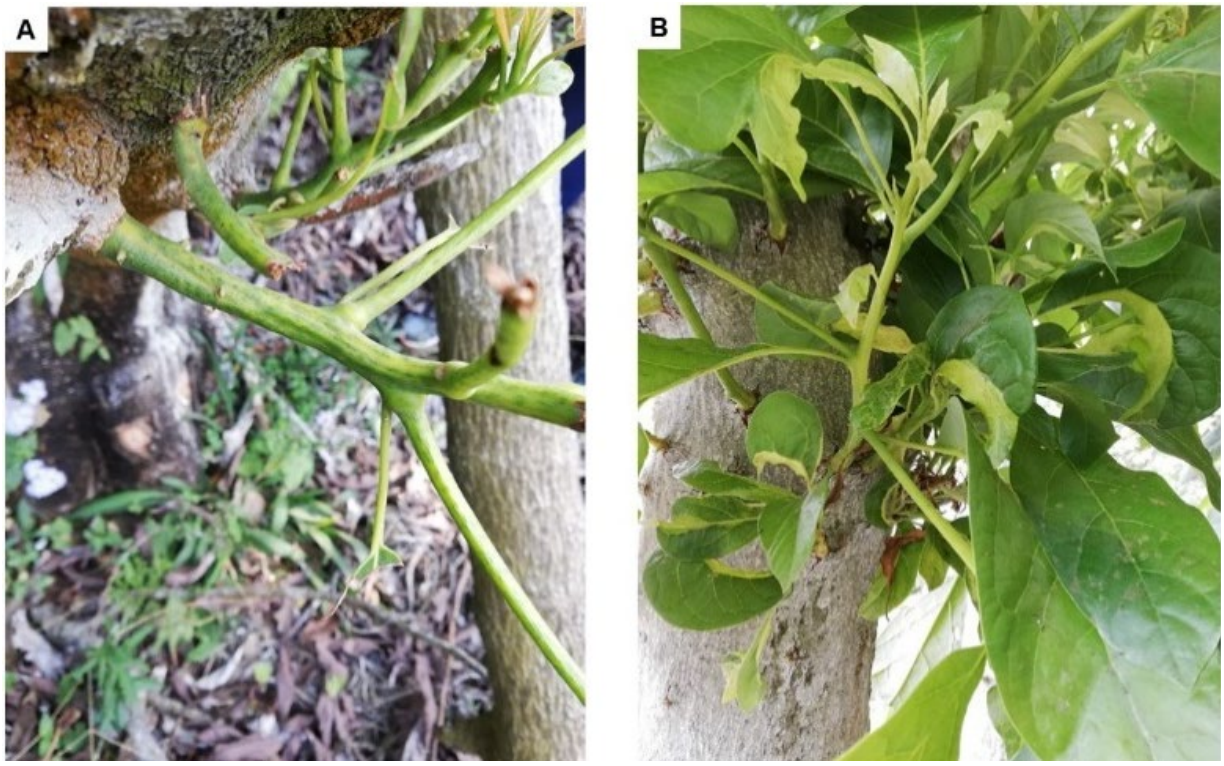


Figure 1.4 Symptoms ASBD on young emerging growth from an old infected 'Fuerte' branch, **A**- showing yellow sunken streaks on stem and **B**- variegation of young leaves.

1.2.6.2 Fruit symptoms

Fruit infected by ASBVd 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, sometimes fruits appear small and misshapen (Wallace, 1958). ASBD fruit symptoms are caused by anatomical and chemical changes in the structure of the exocarp and mesocarp cells. This 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). A recent study by Vallejo-Perez *et al*., (2014) showed an increase of up to 62% in phenolic compounds of symptomatic fruits compared to asymptomatic fruit, 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 1.5.

Figure 1.5 Three types of avocado fruit symptoms found in the orchards. **A**- represents symptomless carrier infection without visible symptoms. **B** - represents symptom-bearing fruit at the early stages of infection with yellow sunken streaks, and **C**- represents severely ASBVd infected fruit showing yellow and reddish sunken streaks.

1.2.6.3 Leaf symptoms

Leaf symptoms are rare in the field, they are expressed as white/yellow variegation and bleaching of the leaves (Semancik and Szychowski, 1994, Figure 1.6). 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). The differences in symptoms arise from the minor nucleotide changes in the ASBVd genome (Semancik and Szychowski, 1994).

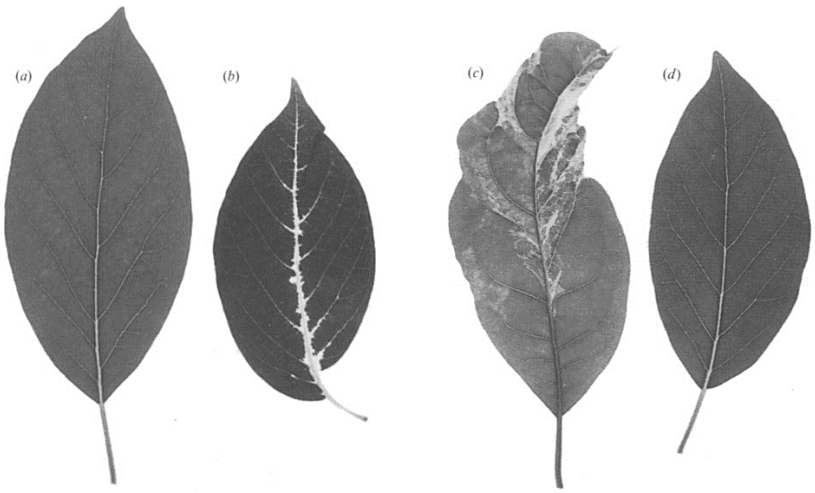


Figure 1.6 Avocado sunblotch leaf symptoms associated with different variants, (**a**) a healthy avocado leaf, (**b**) Variegated symptom associated with the ASBVd-V variant, (**c**) a bleached symptom associated with the ASBVd-B variant and (**d**) a symptomless

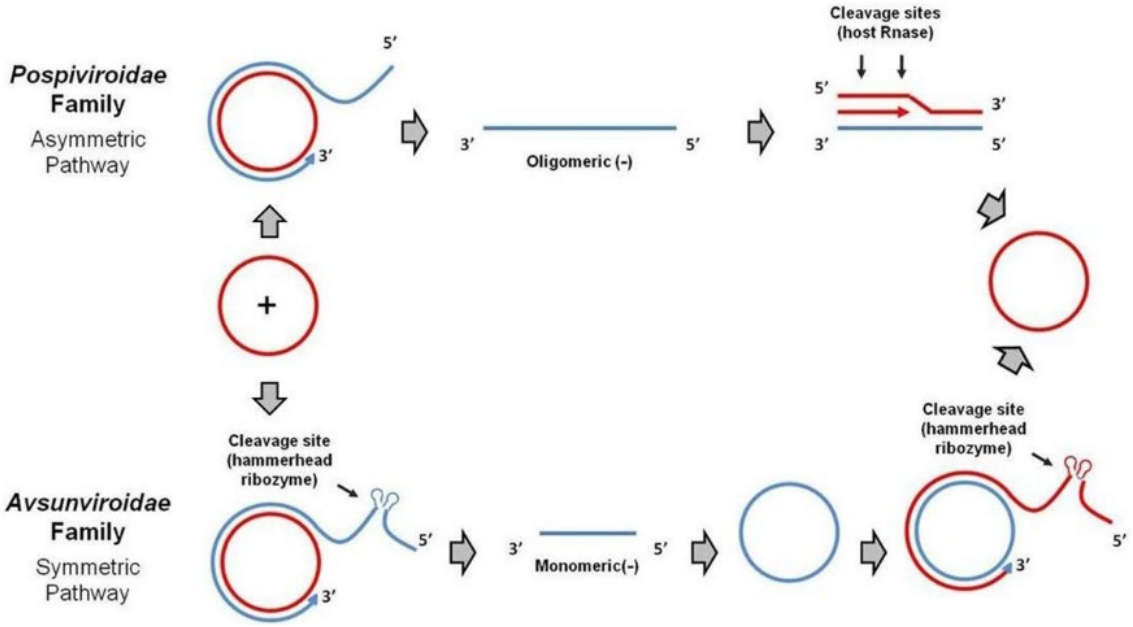
carrier symptom associated with ASBVd-SC variant. (Semancik and Szychowski, 1994).

1.2.6.4 Symptomless carriers

A symptomless carrier generally refers to a tree that is infected with ASBVd but does not show any visible symptoms of the disease. Symptomless carrier trees appear healthy, except for producing lower yields. These trees can arise in two different ways, symptomless carriers can either arise from parent symptomless carrier trees that underwent recovery during an early greenhouse stage, allowing time for the healthy appearance to become dominant (Wallace and Drake 1962; Da Graça, 1980). However, these trees can exhibit symptoms when they are exposed to stress, e.g. fire, when the trees are cut back, or when a healthy scion is grafted onto a symptomless carrier rootstock (Dodds, 2001). Or, symptomless carrier trees can be produced directly from see of an infected symptomless carrier tree, the progeny that arises from the seed will be symptomless and never show any symptoms even when exposed to stress (Wallace, 1958).

1.2.7 Replication

The ASBVd self cleaves at both polarities on a hammerhead ribozyme and replicates autonomously when inoculated in an avocado tree (Delan-Forino, 2014). The replication cycle of ASBVd is extremely dependent on host enzymatic activities (Navarro *et al*., 1999). ASBVd replicates through an RNA-RNA symmetrical rolling circle mechanism in the chloroplast (Figure 1.7 ; Delan-Forino *et al*., 2011; Saucedo-Carabez *et al*., 2014). Both the negative and positive dimeric ASBVd RNA molecules fold in different directions to reach their active self-cleaving structures (Delan-Forino *et al*., 2011). The negative strand is easily cleaved using the double-hammered structure during *in vitro* transcription and by a single–hammerhead structure after purification of dimeric RNA. A positive strand requires more stable double-hammerhead structures for self-cleavage during and after *in vitro* transcription (Delan-Forino *et al*., 2014). Positive strands are more dominant in the infected tissues compared to the negative strands (Delan-Forino *et al*., 2014). Delan-Forino *et al* (2014) presented new ASBD structures for both strands (Figure 1.8).



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856 **Figure 1.7** Replication cycle of Pospivioidae and Avsunviroidae family via a rolling
857 circle mechanism (Delan-Forino *et al.*, 2011)

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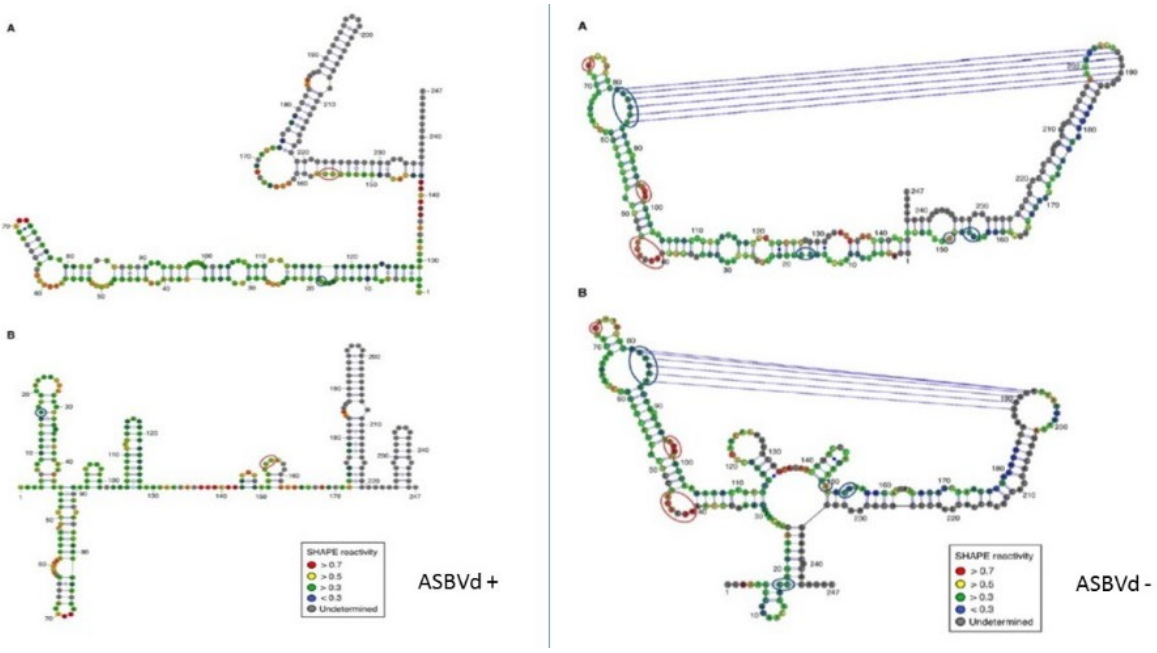

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860 **Figure 1.8** Proposed secondary structure models of both negative and positive
861 strands of ASBVd (Delan-Forino *et al.*, 2014). A and B are both proposed ASBVd
862 different structures of both positive and negative strands.

1.2.8 The role of diagnostics in the management and control of ASBD

The most important control measure for ASBD is a careful selection of pathogen-free bud wood and seed that are used for propagation, therefore, indexing plays an important role to ensure that propagation material is viroid-free (Wallace, 1958). Diagnosis of ASBVd is divided into two indexing techniques, these are biological indexing and molecular indexing. Different techniques were used in the past and will be described below. Although not used as current/modern detection strategies, it is worthwhile to take notice of the advancement in detection using different approaches.

1.2.8.1Biological indexing

Biological indexing aims at the planting of ASBVd disease-free trees, by ensuring that both the scion and the rootstock are disease-free (Wallace, 1958). When undertaking biological indexing at least ten seedlings are desirable which takes about 3-5 months from germination. Indexing involves grafting a diseased bud or bark patches into a healthy indicator tree, which is either 'Hass' or 'Collison' cultivar (Da Graça 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 in the greenhouse and if the experiment is undertaken in the field, the trees must be shaded to prevent sunburn which could mask early ASBD symptoms (Wallace, 1958). This method is effective for viroid indexing, however, due to delay in the infection and symptom development, which can take up to 18 months, makes this method is unsuitable for commercial indexing (Burns *et al*., 1969). However, 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, currently, the methods that have been rapidly used for the indexing of the viroids are the nucleic hybridization methods and the reverse transcriptase coupled with polymerase chain reaction (RT-PCR) (Morey-Leon *et al*., 2018).

1.2.8.2 Molecular techniques

1.2.8.2.1 Standard polyacrylamide gel electrophoresis (PAGE)

Standard polyacrylamide gel electrophoresis (sPAGE) is based on the separation of the RNAs in 5% polyacrylamide gels (da Graça 1981). The method requires the use of intercalating dyes, one of which is 0.01% toluidine blue. This dye requires distaining with several changes of 5% acetic acid and viewed UV light. Another commonly used dye is ethidium bromide. Ethidium bromide proved to be the most suitable compared

to toluidine due to its quick reaction. However, using this method for indexing had limitations; as lower concentrations of ASBVd could not be detected making the method unreliable (da Graça and Trench, 1985; Running *et al*., 1996). Moreover, the method failed to detect ASBVd from symptomless carrier plants (Shnell *et al*., 1997).

1.2.8.2.2 Hybridization analysis with a ^{32}P-labelled cDNA probe

Research conducted by Running *et al*., (1996) proved that hybridization with a ^{32}P-labeled 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.

1.2.8.2.3 Dot-blot hybridization with ^{32}P-labelled ASBVd

This method works by spotting healthy and infected leaf extracts on nitrocellulose paper followed by hybridization with a ^{32}P labelled synthetic probe (Bar-Josephs *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 positives (Acheampong *et al*., 2008).

1.2.8.2.4 DNA hybridization with digoxigenin (DIG) labelled probes

Manicom and Luttig (1996) conducted an 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).

1.2.8.2.5 Reverse transcription-polymerase chain reaction (RT-PCR)

A test that was sensitive, safe and can be used on a large commercial scale was required for the indexing of ASBVd. An RT-PCR assay for the detection of ASBVd was adapted from assays developed for apple scar viroid (ASSVd), dapple apple viroid (DAVd) citrus exocortis viroid (CEVd) and cachexia viroid (CCaVd) (Schnell, 1997), using ASBVd specific primers developed by Hadidi and Yang, (1990). This method allowed a decrease in the time required for indexing and no safety hazards were reported (Shnell *et al*., 1997). The method allowed for the detection of ASBVd from both purified and unpurified RNA samples (Shnell *et al*., 1997). To date, RT-PCR is the most used method for ASBVd indexing. There are currently two variations of RT-PCR used for ASBVd, indexing; these are conventional RT-PCR and quantitative real-time PCR (qRT-PCR).

The conventional RT-PCR amplifies a nucleic acid target within the ASBVd genome using two ASBVd-specific primers and after amplification, the products are run on an agarose gel to check for the correct size of the band, indicating the presence of the viroid using a molecular weight marker. The conventional RT-PCR is sensitive enough, however; it takes a long to get results. In addition, the chances of contamination from target RNA are possible when loading samples. Moreover, the variations between the samples are difficult to detect based on the band size only. The conventional method is not as sensitive when ASBVd concentrations are too low, the results can be false-negative with the formation of primer-dimers (Morey-Leon *et al*., 2018).

Real-time PCR is a more sensitive and faster method than conventional PCR. Results are detected in the early stages of the reaction (Bar-Josephs *et al*, 1986). There are two types of RT-qPCR based on the chemistry used, one method uses specific probes (probe-based detection), whereas the second uses intercalating dyes which emit fluorescence, should amplification occur. Probe technologies are expensive and thus not favoured for the use of commercial indexing. The most commonly used method is therefore fluorescent-based qRT-PCR; with the commonly used dye being SYBR Green 1. 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. The high sensitivity of the qRT-PCR has made it possible for the detection of ASBVd in the early infections of the avocado trees (Morey-Leon *et al*., 2018).

1.2.8.2.6 Detection of ASBVD using filter paper capture and RT-PCR

Recently, Mathews *et al*. (2022) published a protocol for ‘Detection of avocado sunblotch and other viroids using RNA filter paper capture and RT-PCR’. The protocol was developed for leaves and fruit tissue, they used Whatman No. 1 filter paper to absorb RNA which is subsequently eluted to standard RT-PCR tubes after washing and drying. They concluded that the method was stable and the disks can be stored over a year without a decline in quality or quantity.

1.2.8.3 Control

1.2.8.3.1 Inactivation of viroids by Sanitisation

The use of 5% commercial bleach (sodium hypochlorite) has been proven to be effective in the 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 was effective in the inactivation of the viroid on pruning tools, harvesting clippers and injection equipment (Desjardins *et al*. (1987). Inactivating of viroids using 20% skim milk has been demonstrated for potato spindle tuber viroid (PSTVd) on tomatoes (Mackie *et al*., 2015). The skim milk was found to deactivate PSTVd effectively as commercial bleach, which could be more beneficial than sodium hypochlorite as it corrodes metal, degrades when in contact with organic matter and also is an irritant to humans (Mackie *et al*., 2015).

1.2.8.3.2 Eradication

All the suspected positive trees should be indexed and all the infected trees and those within a 15 m radius of the infected trees should be tested and positive trees should be removed from orchards. Symptomless carriers are not easy to spot in the field thus making detection of the disease very difficult (Schnell *et al*., 1997). Healthy propagation material is the key to healthy orchards and this material should be selected based on sound detection techniques.

1.3 Conclusions

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. ASBD affects the quality and the yield of the fruits. Ensuring sensitive detection of ASBVd is crucial to avoid losses and restrictions that could arise when infected material is exported. According to the findings of most studies conducted for ASBD worldwide, an increase in the occurrence of ASBD was reported over the years. Shipping of avocados is affected by factors such as storage temperatures with a minimum of 28 days of cold storage (Blakey, 2011). Therefore, unnecessary shipping delays and quarantine procedures could affect the fruits thus leading to economic losses for the avocado industry. Therefore, constant research on ASBVd generating knew and updated knowledge is important for keeping up with disease advancement. The lack of information about the management of the disease can be a hindrance in future production. Propagation of disease-free trees is considered the first step in establishing healthy plants (Saucedo-Carabez, 2014). This is achieved through regular and precise indexing of propagation material to prevent unnecessary ASBD outbreaks.

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CHAPTER 2

The effect of Avocado sunblotch disease (ASBD) on tree morphology, fruit maturity, yield and quality of 'Hass' avocado in South Africa.

Abstract

Avocado sunblotch disease (ASBD) is a chronic infection that is induced by avocado sunblotch viroid (ASBVd) in avocado worldwide affecting yield and fruit quality. The trees develop two types of infections: symptom-bearing and symptomless infections. Symptoms are expressed as sunken yellow streaks on leaves, fruit and green stems of symptom-bearing trees and symptomless carrier trees do not display any symptoms. The latter is described as the primary source of disease transmission in orchards. Hence, this study investigated the impact of ASBVd-infected symptomless carrier trees on tree morphology, fruit maturity, yield and quality of 'Hass' avocado from 2019 until 2021. Differences were observed in the orchard between infected and healthy trees; medium and highly infected trees excessively produced flowers, lost leaves during flowering and ultimately bore few to no fruit at the end of the season. The dry matter content results showed that ASBVd did not affect the maturity of the fruit; fruit from infected and healthy trees matured at the same time. Yield measurements indicated that medium and highly infected trees produced between 83% and 96% lower yields compared to healthy trees. Postharvest studies showed that medium and highly infected fruit significantly lost firmness and developed colour more rapidly than healthy fruit. Infected fruit also developed external rots and shrivels for non-stored fruit, however, these symptoms were reduced for fruit stored at 5 degrees for 28 days. Therefore, flower overbearing with the shedding of leaves and lower yields can be used as an indication of ASBVd infection in 'Hass' orchards; however, confirmation with molecular testing is required. These observations can be incorporated as an ASBD management strategy in 'Hass' orchards.

Keywords: *avocado sunblotch disease/viroid, avocado* (*Persea americana* Mill.)*, maturity, yield, quality, 'Hass' cultivar*

2.1 Introduction

Avocado sunblotch disease (ASBD) is an economically important disease affecting avocados' productivity and fruit quality globally (Palukaitis *et al*. 1979). ASBD has previously been considered a quarantine disease in Australia (Geering, 2018). It was initially discovered in Southern California in 1914 and only described in South Africa in 1954 (Da Graça and Van Vuuren, 2003). The causal agent of ASBD is avocado sunblotch viroid (ASBVd); which is a small, infective, single-stranded circular RNA molecule of 246–251 nucleotides in size (Kuhn *et al*, 2017). Avocado is the only known natural host for ASBVd, and all avocado cultivars are susceptible (Saucedo Carabez *et al*., 2019). The infected trees can either express ASBD symptoms phenotypically or can show no symptoms or signs of infection but still be carriers of ASBVd; these trees are known as symptomless carrier trees (Acheampong *et al*., 2008). Characteristic symptoms of ASBD include the appearance of irregular, sunken areas of white, yellow, or reddish colour in infected fruit; white/yellow sunken streaks on green stems and variegated or bleached symptoms on the leaves (Vallejo-Perez *et al*., 2014).

Symptomless carrier trees play an essential role in the epidemiology of ASBVd, as they have been described as the primary sources of infection spreading the disease through budding and grafting practices (Saucedo Carabez *et al*., 2019). Studies have shown that ASBD can significantly reduce the yield and quality of avocado fruit (Da Graça, 1985, Saucedo-Carabez *et al*., 2014). The ASBVd–infected symptomless carrier trees could lead to between 50 % to 80 % yield reductions compared withto the symptom bearing trees, which cause a yield reduction between 15 to 30% (Saucedo-Carabez *et al*., 2014).

The high nutritional value and health benefits associated with avocado fruit have increased the consumption and its popularity among consumers worldwide (Magwaza and Tesfay, 2015). Advanced postharvest technologies and reduced trade barriers have contributed to a steady increase in avocado production worldwide (Shahbandeh, 2022). The leading avocado producers include Mexico, Dominican Republic, Peru, Columbia, Indonesia and Brazil, respectively (Shahbandeh, 2022). South Africa is ranked as one of the top avocado exporters with the market primarily aimed at the European market (SAAGA, 2020).

Research has shown that avocado trees are not very good attractors of pollinators, therefore the trees enter into a mass bearing phase to attract pollinators (Ish-Am, 2005). A typical, healthy avocado tree produces millions of flowers (Ish-Am, 2005); followed by a lot of abscissions, leaving less than 0.1 % of flowers for fruit set (Garner and Lovatt, 2016). Fruit acceptability by consumers depends on the fruit quality, which is determined by several factors such as the cultivation of healthy avocado trees, harvesting of fruit at the appropriate maturity stage and the correct harvesting and handling procedures of fruit (Barmore, 1976). These factors are all equally important to ensure quality; however, avocado only starts showing physical changes after harvest, during the ripening stage (Barmore, 1976). Fruit maturity is therefore an essential factor in determining avocado fruit quality to prevent harvesting of immature fruit that does not ripen properly (Magwaza and Tesfay, 2015).

The physiological maturity of avocado fruit is simply defined as the stage of development where most growth has already occurred, at which fruit will ripen after harvest resulting in an edible product (Young and Lee, 1978; Lee *et al*., 1983). It is difficult to determine if avocado fruit has matured while still on the tree because there are no distinguishable physiological changes visible at this stage. Changes become visible after the fruit has been harvested during the ripening process (Magwaza and Tesfay, 2015). To address this challenge, some techniques have been developed. The most well-known method to estimate fruit maturity is to determine the degree of ripeness of avocado fruit by measuring dry matter content (DM) before harvest (Woolf *et al*., 2003). According to this method, fruit is collected from trees several weeks before harvest. The percentage dry matter content (% DM) of the fruit is then determined and a certain threshold, which is different for each cultivar, is required for the fruit to be considered mature enough for harvest.

Fruit ripening is described by the processes involving both catabolic and anabolic changes together with large amounts of energy resulting in changes in colour, texture and taste, making the fruit acceptable for consumption (Bower, 1988). Ripening of ‘Hass’ avocado fruit is determined by the firmness of the fruit flesh and the colour change of the fruit rind, as the fruit becomes softer and the skin colour changes from green to purple/black during ripening (Cox *et al*., 2004). However, colour change can be misleading because some fruit can change colour before harvest. When this occurs, the fruit colour changes late in the harvest season but the fruit flesh remains

hard (Cox *et al*., 2004). This becomes more complicated in cultivars that do not change colour during ripening; therefore, fruit firmness is the most reliable method for measuring fruit ripeness (White *et al*., 1999). Avocado fruit is very delicate and tends to develop rot and other internal disorders during the later stages of ripening. In addition, external stresses such as fungal infections are also a major problem contributing to yield reduction and poor fruit quality.

The purpose of this study was to investigate the effect of avocado sunblotch viroid (ASBVd) on tree morphology, fruit maturity, yield and quality of 'Hass' avocado in South Africa. The study consisted of four objectives, i) to identify and monitor ASBVd-infected trees from the flowering stage until harvest for any visible physical indications of the disease compared with healthy trees, ii) to determine the maturity of ASBVd-infected fruit obtained from symptomless carrier trees by measuring the fruit dry matter content (% DM), iii) to determine the impact of ASBVd-infected symptomless carrier trees on yield compared to healthy trees by counting the number of fruit per tree for two consecutive growing seasons, and iv) to investigate the physical quality parameters of ASBVd-infected fruit of symptomless 'Hass' carrier trees compared to fruit from healthy trees.

2.2 Material and methods

2.2.1 Orchard description

The experimental field was located at a commercial farm in Mbombela, Mpumalanga province, South Africa. The experimental 'Hass' trees were obtained from an orchard, established in 2009 (longitude: 30.928410887745482; latitude: -25.4292923547822). Thirty trees were utilised for all the investigations of the current study including 15 infected symptomless carrier trees (selected by molecular screening) and 15 healthy trees (confirmed by molecular screening).

2.2.2 Field selection of symptomless carrier trees

Since ASBVd infected symptomless carrier trees are difficult to identify in the field, infected trees showing fruit symptoms were first identified. In their immediate vicinity, trees were screened using molecular testing. Further, the screening was conducted in the immediate areas where the confirmed positive trees had been removed.

2.2.2.1 dsRNA extraction

Leaf samples were collected from avocado trees. Double-stranded RNA (ds-RNA) was extracted from 400 mg leaf samples using a cellulose column-chromatography technique developed at the Agricultural Research Council – Tropical and Subtropical Crops (ARC-TSC) (Luttig and Manicom, 1999). Leaf samples were macerated in 5 ml avocado extraction buffer (500 mM sodium acetate.$3H_2O$, 10 mM magnesium chloride, 3% Sodium Dodecyl Sulfate (SDS), 20% ethanol (molecular grade), 1.2% sodium sulphite, pH 6 and 2.5 ml chloroform/iso-amyl alcohol (CL/Iso) (24:1). The supernatant from macerated samples was transferred to 2 ml reaction tubes containing 200 µl Chloroform/Isoamyl alcohol (CL/Iso) (24:1) and spun for 3 minutes (min) at 13 500 rpm. An aliquot of 700 µl of the supernatant was removed and mixed with 300 µl of alcohol (EtOH) in new 2 ml reaction tubes. Samples were loaded onto a column prepared with Cellulosepulver (MACHEREY-NAGEL MN 2100); 450-µl Cellulosepulver MN 2100 slurry packed in 1 ml Avacare syringes and allowed to drain. Columns were washed with 1xSTE_{35} (50 mM Tris-HCl, 0.1 M Sodium chloride (NaCl), 1 mM Ethylenediaminetetraacetic acid (EDTA) pH 6.8 and 35 % alcohol), allowed to drain and then were eluted with 500 µl of 1x STE into a new 2 ml tube. Double-stranded RNA was precipitated by adding 3 volumes of EtOH and 50 µl of 3 M Sodium acetate (NaAc) (pH 5.2) and stored overnight at -20 °C. Samples were centrifuged for 10 min, the supernatant was discarded and the pellet was dried for 30 min, suspended in 50 µl of nuclease-free water, and stored at -20 °C until use.

2.2.2.2 Amplification of ASBVd nucleic acid

All the trees used in the study were tested and confirmed for the presence or absence of ASBVd using a quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assay. A fluorescent-based real-time one-step qRT-PCR assay was optimised to amplify ASBVd using the qPCRBIO SyGreen 1-Step Go Lo-ROX kit (PCRBIOSYSTEMS, UK), according to the manufacturer's instruction in a Rotor-Gene Q instrument (Qiagen, Germany). The Rotor-gene Q software 2.3.1 (Qiagen) was utilized to calculate PCR efficiency, quantification cycle (Cq)-, and quantification values for establishing the standard curve and for determining the concentration of samples. The efficiency and sensitivity of the real-time qRT-PCR assay were validated using a 10 ng/µl g-block® Gene Fragment (GenBank® S73860-Sc) in a ten-fold, seven point dilution, standard curve. An ASBVd-specific primer set, (5’-

AGAGAAGGAGGAGTCGTGGTGAAC -3'; 5'- TTCCCATCTTTCCCTGAAGAGAC -3'), amplifying a 99 bp product (Jooste, unpublished), was used at a final concentration of 400 nM in a 12.5 µl reaction volume. A standardised volume, 1.2 µl of the dsRNA template, was added to the reaction. The cycling conditions included reverse transcription at 50°C for 10 min, followed by polymerase activation at 95°C for 2 min. The PCR step included 35 cycles with a denaturation step at 95°C for 5 sec and an annealing step at 56°C for 30 sec.

2.2.2.3 Cyclic threshold (Ct value) scoring

The positive trees were separated into three infection levels, *viz.* low, medium and high infection based on Ct values. All trees with Ct values equal to or less than 10 were rated as highly infected, Ct values from 10.1 to 18 were rated as medium and Ct values from 18.1 to 25.4 were rated as low/slightly infected.

2.2.3 Seasonal monitoring of trees

Infected and healthy trees were monitored for three consecutive seasons from 2019 until 2021, from the flowering stage until harvest. Trees were monitored for differences in tree morphology (branches, leaves and reproductive structures), flowering patterns and fruit sets that could be physical indicators of ASBVd symptomless carrier trees in avocado orchards.

2.2.4 Determination of the Dry matter content (% DM) of fruit

The degree of ripeness of avocado fruit was determined by measuring the % DM content of fruit at four different intervals before harvest in the years 2019 and 2020 (Woolf *et al*., 2003). The % DM measurements were conducted by randomly selecting a single fruit from each of the 30 experimental trees (15 infected and 15 healthy trees) at each interval. For each fruit, the skin was removed by peeling; the flesh was grated into a Petri dish and measured to a standard weight of 10 g. The Petri dishes containing the fresh flesh were then oven-dried at 30 °C for 48 hours. After 48 hours, the dry mass of each sample was recorded. The percentage of dry mass was calculated as follows: Dry mass (%) = (dry flesh weight/fresh flesh weight) X 100. The fruit was considered to reach full maturity once a moisture content of 77% was reached, as recommended by the South African Avocado Growers' Association minimum maturity requirements expressed as maximum allowable moisture content (Kruger and Claassens, 2001).

2.2.5 Yield

The yield per tree was determined by counting the number of fruit on each tree in the years 2020 and 2021. Two individuals conducted the counting of fruit, each repeating the count on each tree three times. The average count was used to determine the number of fruit on each tree.

2.2.6 Determination of fruit ripening and quality

In each of the selected trees, infected and healthy trees, 30 fruit were harvested, except in the case of highly infected trees because they produced less than 30 fruit and in this case, all available fruit was harvested. The fruit was transported to the ARC-TSC postharvest laboratory on the day of harvest. The fruit was divided equally into two parts, non-stored and stored fruit. Non-stored fruit was immediately taken to the darkroom at 21 ± 2 °C for ripening. The stored fruit was taken to the cold storage at 5 °C and kept for 28 days and then moved to the darkroom for ripening under the same conditions as mentioned above. Ripening was determined by measuring firmness and colour every second day during postharvest/storage. Fruit quality was determined when the fruit had ripened.

2.2.6.1 Fruit firmness

Fruit firmness was measured using an automated Sinclaire IQ instrument (51DFTB, International Ltd, Jorrold, Bowthorpe Norwich, NR5, 9.D, England). The fruit was rated using the IQ score,; the fruit receiving an IQ score of 70 was considered hard and an IQ of 10 was considered soft (Howarth, *et al*., 2003). The fruit was considered ripe after reaching an IQ score of ≤ 25.

2.2.6.2 Colour rating

The colour change was monitored visually using a six-point rating where: 1, emerald; 2, forest green; 3, approximately 25% coloured; 4, approximately 75% coloured; 5, purple; and 6, black (White *et al*., 2009).

2.2.6.3 Determination of fruit quality

External rot, flesh bruising, diffuse flesh discolouration, vascular browning, stem-end and body rot were rated according to the International Avocado Quality Manual (White *et al*., 2009) using a scale of zero (0%) to three (≥ 50% of the fruit surface affected). Shrivel was determined using a rating scale between zero (no shrivel) to three (fruits appear shrivelled) (White *et al*., 2009).

2.2.7 Data analysis

Statistical analysis of variance was obtained using STATISTICA 8 and the means were separated using the Post-hoc Tukey HSD test at a significance level of $p \leq 0.05$.

2.3 Results

2.3.1 Field screening of symptomless carrier trees

Detection was confirmed in the qRT-PCR assay using the seven-point, ten-fold dilution, and a standard curve that showed an amplification efficiency of 1.03 with a linearity correlation coefficient (R^2) of 0.999. The qRT-PCR result confirmed 15 trees as ASBVd positive symptomless carriers. The ASBVd titre from the trees varied considerably between trees. Based on the cycling threshold cyclingthreshold (Ct) values, the infected trees were divided into three infection levels *viz* low, medium and high (Table 2.1). Seven of 15 trees had a high ASBVd infection, five trees had a medium infection and only three trees had a low infection level.

Table 2.1 Fluorescent based real-time qRT-PCR results of the infected experimental trees showing cyclic threshold (Ct) and level of infection of each tree.

	Infection levels		
	Low +	**Medium ++**	**High +++**
Ct values	19.89	10.77	7.22
	20.32	11.04	7.35
	21.13	11.05	8.05
		11.56	8.22
		11.61	9.25
			9.56
			10.00
Total	**3**	**5**	**7**

2.3.2 Seasonal monitoring of trees

Observations of the infected symptomless carrier trees in the orchard were conducted over three years (2019-2021). Morphological differences were found between infected symptomless carrier trees with medium and high infection levels and healthy trees in the orchard. The first observation was the remarkably huge number of flowers produced by the infected trees both in ‘on’ and ‘off-seasons; the flowers appeared

clustered and were exposed to the sun, therefore triggering trees to remain longer in the flowering stage and delaying the fruit set (Figure 2.1 A). Secondly, a substantial amount of leaves was lost, leaving trees looking unhealthy and completely dominated by flowers (Figure 2.2). Lastly, the trees lost the majority of flowers (Figure 2.1 B), started recovering the lost leaves instead of producing fruit, and ultimately recovered most of the leaves, looking healthier but with very little to no fruit. Additionally, the bud-wood of symptomless carrier trees was bigger with larger buds compared to that of healthy trees; this was consistently observed with more infected trees in the field over the three years (Figure 2.3). However, trees with low infection levels produced a mixture of bigger bud-wood with large buds and normal bud-wood.

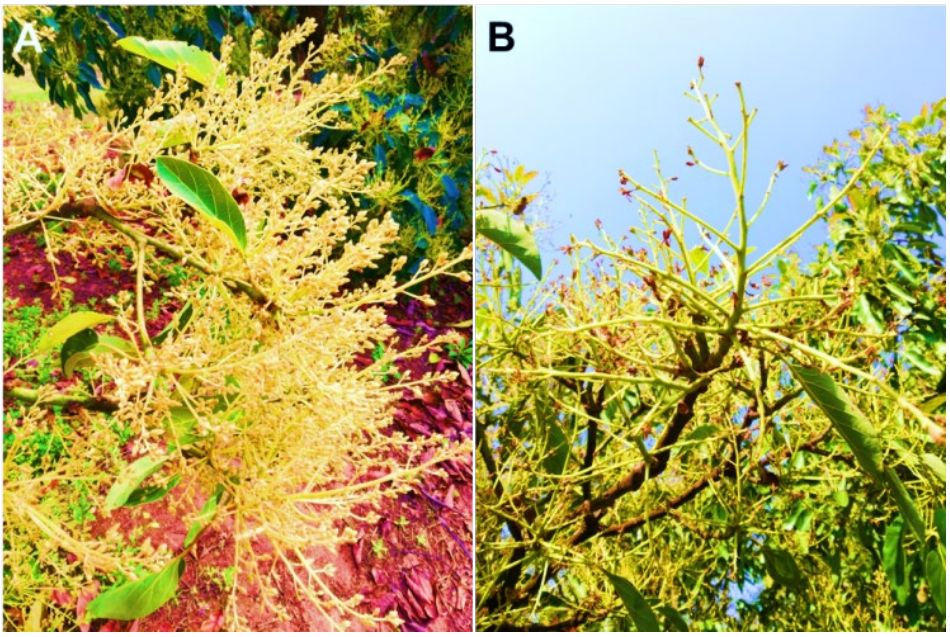

Figure 2.1 Abnormal heavy flowering of ASBVd infected symptomless carrier trees (**A**) and a large amount of flower abscission at the end of the flowering stage (**B**)

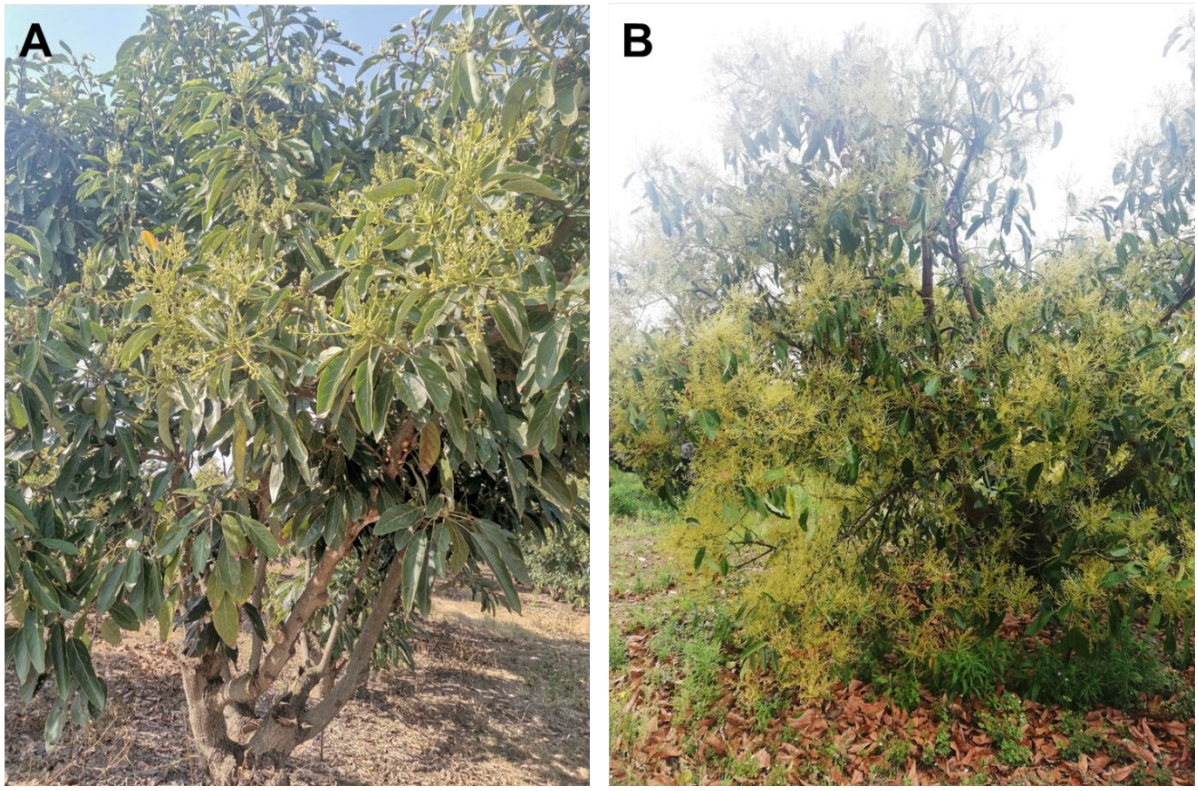

Figure 2.2 A comparison between (A) a healthy avocado tree and (B) symptomless carrier tree with a high ASBVd infection level completely dominated by flowers and lost the majority of its leaf canopy.

Figure 2.3 Example of 'Hass' cultivar infected ASBVd symptomless carrier and healthy avocado trees bud-wood, the infected bud-wood is larger with bigger buds compared to the healthy trees.

2.3.3 Determining fruit dry matter content (DM)

The dry matter content of the fruit was determined to measure the fruit's maturity. The % DM measurements were conducted for the 2019 and 2020 harvests. In 2019, the % DM measurements were taken at four intervals between 15 April and 06 May, there. There were no significant differences between the dry matter content of the healthy and infected trees in all four intervals (Table 2.2). The measurements were repeated in 2020 from 23 April until 13 May. The % DM ratings of highly infected fruit were significantly higher than that of healthy fruit for the first two intervals ($p \leq 0.05$, Table 2.2). In the first interval, the % DM of the highly infected fruit was rated 30.9 ± 3.3%, 4.2% higher than that of healthy fruit which was rated 26.7 ± 2.0%. Again, in the second interval, the DM of highly infected fruit rated 30.1 ± 3.6 %, 3.7% higher than that of healthy fruit, which rated 26.4 ± 1.7%. There were, however, no significant differences recorded for the % DM of all fruit between the third and the fourth intervals.

Table 2.2 Dry Matter content (% DM) was measured for avocado fruit for four consecutive four different intervals in the years 2019 and 2020.

Percentage Dry Matter (% DM) 2019					
Intervals		**One**	**Two**	**Three**	**Four**
Infection level	**Healthy**	22.7 ± 3.4 a	25.1 ± 3.7 a	24.5 ± 3.2 a	26.3 ± 3.4 a
	Low	24 ± 1 a	23.7 ± 1.5 a	21.3 ± 2.1 a	28.7 ± 2.5 a
	Medium	24 ± 1.9 a	23.6 ± 3.0 a	23 ± 3,7 a	28.4 ± 2.7 a
	High	23.2 ± 3.2 a	24.8 ± 3.6 a	26.7 ± 2.7 a	25.5 ± 3.8 a
	P value	**0.798**	**0.794**	**0.092**	**0.359**
	F value	**0.345**	**0.343**	**2.392**	**1.123**
Percentage Dry Matter (% DM) 2020					
Infection level	**Healthy**	26.7 ± 2.0 a	26.4 ± 1.7 a	26.8 ± 2.8 a	28.7 ± 3.1 a
	Low	30 ± 6.1 ab	25.7 ± 1.2 a	27.7 ± 0.6 a	26.7 ± 1.5 a
	Medium	29.8 ± 2.0 ab	28 ± 3.0 ab	29 ± 3.1 a	30.6 ± 1.3 a
	High	30.9 ± 3.3 b	30.1 ± 3.6 b	28.9 ± 4.8 a	27.6 ± 6.5 a
	P value	**0.013**	**0.013**	**0.448**	**0.486**
	F value	**4.334**	**4.321**	**0.914**	**0.837**

Values represent means ± standard deviations (SD). Means followed by the same letters do not differ significantly at $P \leq 0.05$ (Tukey HSD).

2.3.4 Yield

The yield of the infected trees was determined by counting the number of fruit per tree. The values represent the average number of fruit counted in trees from different infection levels (Figure 2.4). Yield counts for 2020 averaged 221-± 55.1 fruit per tree for the healthy trees, significantly different from the medium and highly infected trees, which averaged between 38 ± 8.6 and 12 ± 6.9 fruit per tree, respectively ($p \leq 0.05$, Figure 2.4). This indicates that infected trees with medium and high infection levels produced between 83% and 95% lower yields compared to the healthy trees, while trees with low infection levels produced an average yield of 177 ± 28.5 fruit per tree; not significantly different from the yield of healthy trees (Figure 2.4). Yield counts were repeated in the year 2021, healthy trees produced an average of 437 ± 117.3 fruit per tree, which was significantly higher compared to 41 ± 19.1 and 19 ± 11.8 fruit per tree produced by medium and highly infected trees, respectively ($p \leq 0.05$, Figure 2.4). This time, the high and medium infected trees produced yields between 91% and 96% lower than healthy trees. Yet again, the low infected trees produced an average of 395 ± 22.5 fruit per tree, not different from the yield of healthy trees (Figure 2.4).

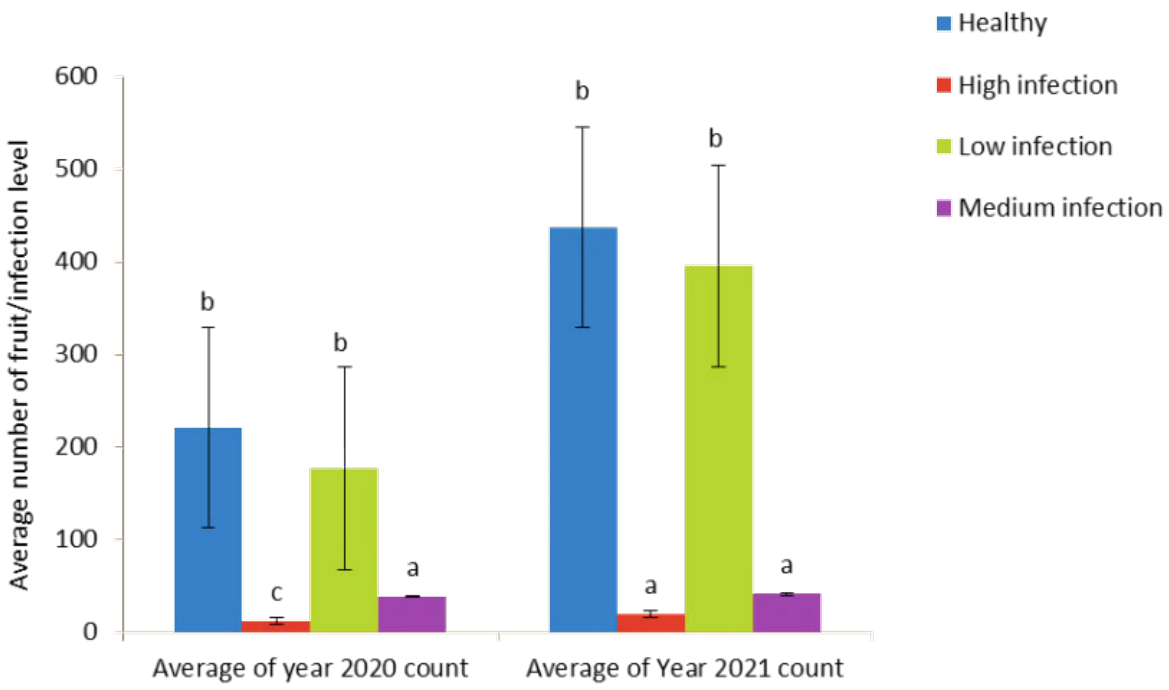

Figure 2.4 Average fruit yield counts of ASBVd infected symptomless 'Hass' carrier trees and healthy avocado trees for the 2020 and 2021 seasons. Values represent means ± standard errors (SE). Bars with the same letters did not differ significantly at $P \leq 0.05$ (Tukey HSD).

2.3.5 Effect of ASBVd on avocado fruit ripening and postharvest quality

2.3.5.1 Fruit firmness

2.3.5.1.1 Non-stored fruit

Fruit firmness was measured using Sinclairs IQ score to determine fruit ripening from day of harvest. The average IQ scores on the day of harvest were all over 70, implying the fruit was still very hard. Healthy fruit had an IQ score of 75.7 ± 6.97, low infected fruit had an IQ score of 76.0 ± 7.2, medium infected fruit had an IQ score of 74.4 ± 6.4 and high infected fruit an IQ score of 72.9 ± 10. There were no significant differences between the fruit on the day of harvest. The IQ scores had decreased on day five suggesting that the fruit started losing firmness, the IQ score for healthy fruit decreased to 49.8 ± 60, medium infected fruit decreased to 45.0 ± 9.6 and highly infected fruit decreased to 36.1 ± 14.5, both significantly different from the firmness of healthy fruit ($p \leq 0.05$, Figure 2.5 A). Further firmness was lost on day seven; the highly infected fruit had an IQ score of 25.4 ± 12.7 suggesting that the fruit had ripened. The IQ score of medium infected fruit was reduced to 33.9 ± 10.1 and was significantly different from the firmness of the healthy fruit with the IQ score of 42.2 ± 6.5 ($p \leq 0.05$). The IQ score of medium infected fruit had decreased to 20.3 ± 8.8 on day nine suggesting that the fruit were ripe, significantly different from the healthy IQ score of medium infected fruit (26.3 ± 7.06) and healthy fruit (28.0 ± 7.7) which had not yet reached the minimum IQ score of ≤ 25 to be considered ripe ($p \leq 0.05$, Figure 2.5A).

2.3.5.1.2 Stored fruit

The fruit was removed from the 5°C storage after 28 days, their firmness was determined on the first day of removal from the storage. The fruit had already lost firmness during storage. Highly infected fruit had an IQ score of 26.1 ± 6.81, medium infected fruit a firmness of 33.6 ± 10.12 IQ, and had significantly lower IQ scores compared to the firmness of low infection fruit at 45.0± 6.5 IQ and healthy fruit firmness at 46.9 ± 8.3 IQ ($p \leq 0.05$, Figure 2.5 B). The high and medium infected fruit had IQ scores of 14.7± 3.2 and 16.8 ± 4, respectively, on day three of ripening suggesting that they had ripened. The healthy and low infected fruit remained with IQ scores of 29.2 ± 9.2 and 26.3 ± 6.60, respectively, not yet reaching the IQ score of ≤ 25 to be considered ripe. Similarly to the non-stored fruit, the stored fruit from the highly and moderately infected group ripened before the healthy and medium infected fruit.

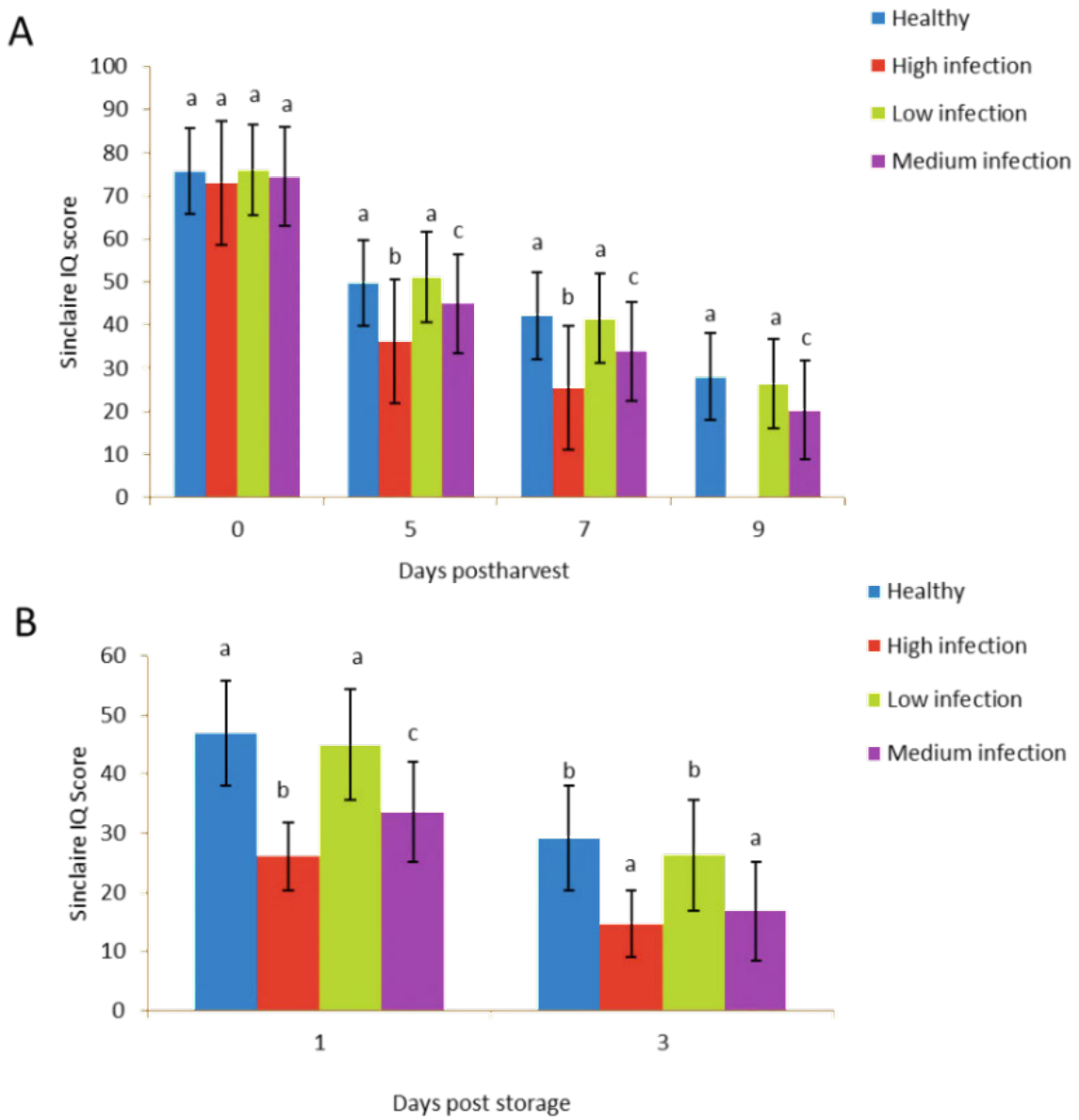

Figure 2.5 Loss of firmness for non-stored (**A**) and cold-stored fruit at 5 °C for 28 days (**B**) 'Hass' avocado fruit was ripened in the dark at 21°C and the IQ scores were recorded from the Sinclair instrument. Values represent means ± standard errors (SE). Bars with the same letters did not differ significantly at P ≤ 0.05 (Tukey HSD).

2.3.5.2 Colour rating

2.3.5.2.1 Non-stored fruit

The colour change was monitored visually and rated using a six-point rating scale. The first colour ratings on the day of harvest showed that all fruit had the same emerald colour with a rating of one (Figure 2.6 A). The fruit started to develop colour on day five post-harvest changing from emerald to forest green, however, highly infected fruit

significantly differed from other fruit as they were already developing a purple colour ($p \leq 0.05$). By day seven, all highly infected fruit had already developed a 25% colour rating 3 ± 0.91, and some of the moderately infected fruit had also started to develop a purple colour with an average rating of 2.5 ± 0.72, all significantly different from the healthy fruit which remained forest green with a rating of 2.1 ± 0.28 ($p \leq 0.05$, Figure 2.6 A). The healthy and low infected fruit only developed a purple colour on day nine with a rating of 2.6 ± 0.62 and 3.0 ± 0.67, respectively (Figure 2.6 A).

2.3.5.2.2 Stored fruit

The fruit was removed from the cold storage after 28 days and moved to the dark ripening room. Upon removal, colour was rated and fruit had already changed colour from emerald (harvest day) to forest green with healthy and low infected fruit rating 2.1± 0.13 and 2.1 ± 0.32, respectively. This is significantly different from medium and highly infected fruit with a rating of 2.5 ± 0.56 and 2.6 ± 0.49, respectively, which had already started to develop a purple colour ($p \leq 0.05$). By day three, almost all the fruit had developed 25% of the colour, all having a rating of approximately three (Figure 2.6 B).

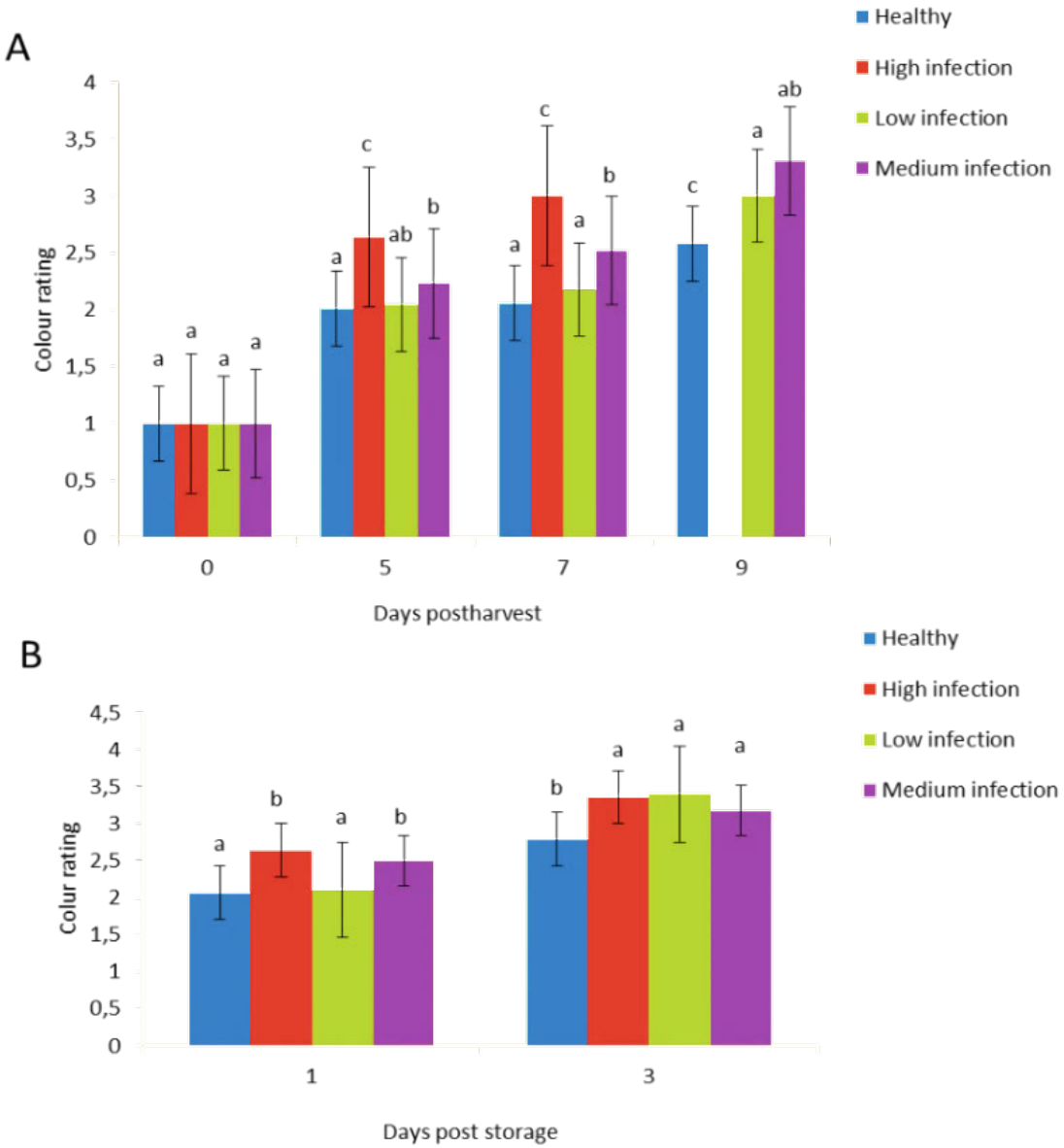

Figure 2.6 Ratings of skin colour development for non-stored (A) and stored (B) avocado fruit using a six-point scale. Values represent means ± standard error (SE). Means followed by the same letters do not differ significantly at P ≤ 0.05 (Tukey HSD).

2.3.5.3. External quality

2.3.5.3.1 Non-stored fruit

After ripening, the fruit was analysed for external damage (Figure 2.7). The fruit was rated for external rots using a rating scale between zero and 50%. High, medium and low infected fruit developed significantly higher incidents of rots between 27% and 30%, significantly higher than healthy fruit with an average incident of 4.2 ± 2.9 % (p ≤ 0.05, Table 2.3). The shrivel was rated using a rating scale between zero and three, there were no significant differences between fruit that developed shrivel.

2.3.5.3.2 Stored fruit

There were lower incident cases for both external rots and shrivel for the stored fruit. Here, the only fruit with a high infection rate developed 5 ± 4.5% of external rots, significantly different from all other fruit that developed no rots. The fruit with high infection rated 1 ± 0.63 for shrivel was the only fruit that developed shrivels significantly different from other fruit that did not develop any shrivels ($p \leq 0.05$, Table 2.3).

Table 2.3 Rates of external rots (%) and shrivel (0-3) for both stored and non-stored fruit

Non-stored fruit		
Infection level	**External rot (%)**	**Shrivel (0-3)**
Healthy	4.2 ± 2.9 b	0.54 ± 0.52 a
Low	28.3 ± 20.2 a	1.3 ± 0.58 a
Medium	27 ± 14.4 a	1.4 ± 1.08 a
High	30.8 15.9 a	1.5 ± 1 a
P value	**<0.001**	**0.06**
F value	**10.2**	**2.938**
Stored fruit		
Infection level	**External rot (%)**	**Shrivel (0-3)**
Healthy	0 ± 0 a	0 ± 0 a
Low	0 ± 0 a	0 ± 0 a
Medium	0 ± 0a	0 ± 0 a
High	5 ± 4.5 b	1 ± 0.63 b
P value	**<0.001**	**<0.001**
F value	**8.944**	**17.89**

Values represent means ± standard deviations (SD). Means followed by the same letters do not differ significantly at $P \leq 0.05$ (Tukey HSD).

Figure 2.7 Examples of external damage between non-stored healthy (**A**) and medium (**B**) infected fruit nine days post-harvest.

2.3.5.4 Internal fruit quality

2.3.5.4.1 Non-stored fruit

Flesh bruising, diffuse flesh discolouration, vascular browning, stem-end rot and body rots were rated using a percentage between zero to 50%. Fruit with a high level of infection was more prone to flesh bruising, the fruit developed an average of 30.8 ± 15.9% bruising, significantly different from the healthy fruit which only developed an average of 8.7 ± 8.1% bruising ($p \leq 0.05$; Table 4). The diffuse discolouration of highly infected fruit was significantly higher (20.5%) than that of healthy fruit ($p \leq 0.05$). No vascular browning developed for highly infected fruit, healthy fruit developed 6 ± 5.1% vascular browning which was still significantly different from the highly infected fruit ($p \leq 0.05$). Stem end rot significantly differed for highly infected fruit with a 17.1% difference from the healthy fruit ($p \leq 0.05$, Table 2.4). Highly infected fruit had the highest rot incidences, averaging 41.7 ± 12.9%, which significantly differed from the healthy fruit with an average of 10.7 ± 9.9% ($p \leq 0.05$; Table 2.4).

2.3.5.4.2 Stored fruit

The fruit's internal injury was reduced at 5°C for 28 days. The fruit only developed flesh bruising and body rots and there were no significant differences between the injuries for all fruit (Table 2.4).

Table 2.4 Rates of flesh bruising; diffuse flesh discolouration; vascular browning; stem-end rot and body rots (%) for stored and non-stored fruit

Non-stored fruit					
Infection level	**FB (%)**	**DFD (%)**	**VB (%)**	**SER (%)**	**BR (%)**
Healthy	8.7 ± 8.1 a	5.3 ± 5.1 a	6 ± 5.1 b	8.7 ± 8.1 a	10.7 ± 9.9 a
Low	10 ± 0 ab	6.7 ± 5.7 a	6.6 ± 5.7 b	10.0 ± 0 a	28.3 ± 20.2 ab
Medium	21 ± 17.5 ab	6.0 ± 5.4 a	0 ± 0 a	8.0 ± 4.5 a	29 ± 20.1 ab
High	30.8 ± 15.9 b	25.8 ± 19.6 b	0 ± 0 a	25.8 ± 19.6 b	41.7 ± 12.9 b
P value	**0.004**	**0.002**	**0.007**	**0.019**	**0.001**
F value	**5.743**	**6.5428**	**5.002**	**4.004**	**8.245**
Stored fruit					
Infection level	**FB (%)**	**DFD (%)**	**VB (%)**	**SER (%)**	**BR (%)**
Healthy	7.3 ± 5.9 a	-	-	-	0 ± 0 b
Low	15 ± 8.6 a	-	-	-	10 ± 0 a
Medium	16 ± 8.2 a	-	-	-	6.6 ± 5.4 a
High	13 ± 9.8 a	-	-	-	6 ± 5.1 a
P value	**0.089**	**N/A**	**N/A**	**N/A**	**0.151**
F value	**2.427**	**N/A**	**N/A**	**N/A**	**1.929**

Values represent means±standard deviations (SD). Means followed by the same letters do not differ significantly at P ≤ 0.05 (Tukey HSD). **FB**- Flesh bruising; **DFD**-Diffuse flesh discolouration; **VB**- Vascular browning; **SER**-Stem end rot; **BR**-Body rots. Dashes represent data with no variance

2.4 Discussion

The quantitative real-time qRT-PCR was used to identify the infected trees in the orchard; the results showed that there was a variation in the concentration between symptomless carrier trees. In this study, we observed a clear difference between flower-bearing of infected and healthy trees. The % DM results showed no significant differences in maturity between the infected and healthy fruit in 2019. However, in 2020, there were significant differences in maturity on the first and the second intervals

of measurements, before harvest. Fruit counting results for two consecutive seasons showed that the number of fruit produced by trees that are moderately and highly infected was significantly lower than that of the healthy trees. The yield of medium and highly infected trees was reduced by 83% and 95%, respectively, in 2020. When the yield counts were repeated in 2021, the yield reductions were 91% and 96% for medium and highly infected fruit compared to the healthy trees. In this study, fruit harvested from infected trees softened and changed skin colour faster than those harvested from healthy trees. Non-stored fruit showed higher external rot than cold-stored fruit, this applied to both healthy and diseased fruit. The stored fruit took only three days to mature, the firmness decreased and the fruit darkened. The stored fruit recorded lesser external and internal injuries compared to non-stored fruit.

Avocado sunblotch viroid (ASBVd) is a systemic pathogen, but its concentration can vary widely between branches, leaves and flowers within a single avocado tree (Bruening et al., 1982; Running et al., 1996). The concentrations can further vary between seasons and leafage for both symptomless and symptom-bearing trees (Running et al., 1996). The trend of avocado trees to produce excessive flowers to attract pollinators was discussed previously (Ish-AM, 2005). Even though massive bearing was observed on the symptomless ‘Hass’ carrier trees in this experiment, the flowers were excessive for trees with medium and high infection levels compared with healthy and trees with low infection levels.

The loss of canopy and excessive flowering, because of disease stress, has been associated with *Phytophthora* disease in avocado (Wolstenholme, 2001). However, the difference with ASBVd infection is that the infected trees recover their canopy completely; unlike with *Phytophthora* disease infection, where canopy recovery is rare and the tree would end up dying from severe infections (Marais, 2007). Given the above-mentioned observations on ASBVd infected symptomless carrier trees, it is possible that ASBVd-infected trees have developed a survival strategy where they first overbear flowers to ensure successful transmission. Subsequently, they save the energy for survival to the following season by producing less fruit while, at the same time, recover the lost leaf canopy as a disguise and appear healthy in the orchard.

Saucedo Carabez et al., (2014) conducted a similar study on the dry matter and oil content of ASBVd asymptomatic fruit and symptomatic fruit and found no significant

differences in dry matter and oil content between these fruits. These findings agreed with the findings obtained in 2019 in this study. The fruit of infected trees would have a higher DM content than those of healthy trees, because the infected trees produce far fewer fruit and therefore don't have to divide carbohydrate across as many carbohydrate sinks (the fruit). Da Graça (1980) conducted a similar study and found that the dry matter for fruit obtained from recovery growth was different to the other infected fruit (infected or healthy 'Edranol' fruit).

The findings of this study were supported by several previous studies to evaluate yield losses associated with ASBVd infections worldwide (Da Graça, 1985; Running et al., 1996; Randles, 2003; Tondo et al., 2010; Saucedo-Carabez et al., 2014). In terms of yield reduction, Da Graça (1985) conducted a three-year trial testing the effect of ASBVd in 'Fuerte' cultivars, both in symptomless carrier trees and symptomatic trees. Symptomatic trees showed a yield reduction of 14%, while the yield of symptomless carrier trees was reduced by 82% (Da Graça, 1985). Cultivar type has no influence on ASBVd severity given that our study gave a similar result of approximately 90% fruit yield reduction from the infected symptomless carrier trees. Symptomless trees had extremely low yields compared to healthy trees and this yield reduction should be regarded as a symptom. Therefore, infected trees can be identified based on the number of fruit they produce. If the fruit yield is extremely low in a tree, the tree should be flagged and submitted for ASBVd indexing.

The study confirmed that ASBVd severity contributes to reduction of yield in symptomless carrier trees regardless of whether the season is 'on' or 'off'. The 'on' and 'off' seasons are triggered by alternate bearing, which results from avocado trees that are alternating between off and on-crop years (Thorp et al., 2011).

The higher the ASBVd concentration, the lower the yield produced for a specific tree. However, this was not true for the trees with low infection levels; these trees produced yields almost similar to healthy trees and, given the fact that these trees have no sign of ASBVd infection, it would be very difficult to convince a farmer to remove good yielding trees that show no sign of infection from the orchard. The question remains with the trees with low infection levels; does this mean that the infection will progress to medium and high titres within time? These are some of the research questions that

are yet to be explored. These findings also emphasise the importance of indexing all propagation material, this is the only way to determine if the tree is truly infected.

Ripening of 'Hass' avocado fruit is determined by the firmness of the fruit flesh and the colour change of the fruit rind, as the fruit becomes softer and the skin colour changes from green to purple/black during ripening (Cox et al., 2004). Avocado fruit tends to develop rots and internal disorders during ripening regardless of whether they are diseased or healthy (White et al., 1999). Possible causes of poor quality fruit could arise from physical damage from abrasion, impact or compression during harvesting, grading, packing and transport (White et al., 2009). It has also been proven previously that directly ripened fruit are more prone to rot compared to cold-stored fruit; this was also evident in the current experiment. This could be explained by the fact that the enzyme activity that is responsible for the maturation and darkening of fruit is not inactivated during the storage and continue to play their role (Vanini et al., 2010).. Blakey et al. (2014) demonstrated that cold storage minimises physiological changes and maintains fruit quality.

Saucedo-Carabez et al. (2014) conducted a similar study in Mexico. The researchers investigated the effect of ASBVd on the postharvest quality of avocado in both symptomatic and asymptomatic fruit. They found that ASBVd affected firmness, weight loss and colour change in symptomatic fruit. Infected symptomless fruit was not affected by the variables evaluated and developed similar conditions to that of fruit from healthy trees and fulfilled quality standards required by national and international markets. In the current study, we determined that ASBVd affected the firmness and colour change of fruit from high and medium infected trees. The only difference was detected with the fruit from the low infection level where firmness and colour change were similar to fruit from healthy trees. In this study, the ASBVd infected symptomless 'Hass' carrier trees were divided into different infection levels which were not done in previous studies.

2.5 Conclusions

Results clearly showed that trees with high and medium ASBVd infection levels significantly had a lower yield than healthy trees. A few signs of infected trees that can assist to identify infected trees in orchards have been observed. Firstly, ASBVd infected symptomless carrier 'Hass' trees produced excessive flowers and shed

leaves in the process. Therefore, the flowering stage of 'Hass' can be useful in identifying the symptomless carrier trees in the field, before trees recover their canopy. Secondly, infected 'Hass' trees also tend to remain in the flowering stage longer than healthy trees that are already at the fruiting stage at the same time interval. Lastly, the infected 'Hass' trees produce very little to no fruit at all and by the end of the season, the trees will have recovered from the loss of canopy appearing greener and healthier just like healthy trees. These observations can be incorporated as part of ASBD management strategies in 'Hass' orchards. Postharvest effects of ASBVd infection were observed and included that the fruit from infected trees ripened faster than normal fruit and was more prone to fungal infections during the ripening process. Because infected trees are not displaying symptoms, regular systematic indexing of orchards, especially trees for bud-wood and seed sources, is required. All the field observations yield and postharvest outcomes from this experiment are only applicable for the 'Hass' cultivar, and similar studies on other important cultivars will benefit the understanding of the relationship between ASBVd infection and yield losses.

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CHAPTER 3

Investigating four transmission mechanisms for avocado sunblotch viroid (ASBVd) from infected to healthy avocado (*Persea americana* mill.) plants.

Abstract

Avocado sunblotch disease (ASBD) is an economically important disease affecting avocado (*Persia americana* mill.). Transmission through infected propagative material has been described as the most important mode for spreading ASBVd in avocado orchards and ASBVd is mainly transmitted through grafting, root grafting, mechanical means and pollen. However, no vector has been reported as a vector for ASBVd. Therefore, the objectives of the study were to investigate four modes of ASBVd transmission and to illustrate the importance of indexing and sanitation protocols when dealing with ASBVd infection in the nursery and in the field. Graft transmission was demonstrated by grafting 30 healthy and 30 infected 'Hass' scions onto a healthy 'Bounty' rootstock. In the current study, there was a 53% success rate of grafting ASBVd infected scions compared to the 43.3% of the heathy scions. This finding implies that there is a 53% transmission success rate of ASBVd through grafting practices. There were no significant differences between grafting the healthy or infected scions to the rootstocks, implying that ASBVd spread through the infected scions is unavoidable in the nurseries unless viroid-free rootstocks and scions are used. Two separate root graft experiments were conducted, one in temperature - controlled tunnels, where a single infected tree was planted in the 10L together with four healthy trees, and in the field where young trees were planted closer to the infected orchard trees. The healthy trees planted in 10L bags with one infected tree outcompeted the infected trees in all six experiments and infected trees died. The trees planted in the orchard died from the lack of sunlight and water stress and further analysis was not possible. The ASBVd was not successfully transmitted mechanically using pruning shears, however, when the stability of ASBVd was tested on different surfaces, it showed to survive up to 24 hours. Pollen and bees were sampled from beehives at four different sites in KwaZulu-Natal and ASBVd was successfully detected in the pollen in the hives and in the bees. Sequence analyses confirmed the sequences to be ASBVd and phylogenetic analysis of the sequences showed a 93% identity between the detected sequences and the existing ASBVd variants retrieved from the GenBank® database. It was concluded that graft transmission is the most

prevalent mode of transmission for ASBVd and that honey bees carry pollen from infected trees to healthy trees in the field and play an important role in pollen transmission.

***Keywords**: Avocado (Persea americana), graft, root graft, mechanical, pollen, transmission*

3.1 Introduction

Avocado sunblotch disease (ASBD) is an economically important disease affecting avocado (*Persea americana* mill.) (Randles, 2003). The disease has a worldwide distribution and affects all known commercial cultivars (Saucedo-Carabez *et al*., 2014). If uncontrolled, the disease can cause up to 80% yield reductions for symptomless carrier trees and the symptoms render the fruit quality making it unmarketable (Da Graça, 1985; Running *et al*., 1996). Symptoms are found on the leaves, green stems and fruit. They are expressed as sunken yellow, whitish to pink streaks (Parker and Horne, 1932; Vallejo-Perez *et al*., 2014). The disease is caused by a small pathogen called avocado sunblotch viroid (ASBVd). ASBVd is an infective single-stranded, covalently closed, circular RNA molecule between 239 and 251 base pairs (Palukaitis *et al*., 1979; Symons, 1981; Saucedo-Carabez *et al*., 2015). ASBVd can be transmitted via seed from infected trees, from infected propagative rootstocks, infected scions used for grafting and via root grafts and pollen. To date, no vector has been reported for the transmission of ASBVd, however, bees are a suspected carrier of infected pollen (Desjardins *et al*., 1979).

Schnell et al (2011) provided evidence on the natural infection of ASBVd in the field from an infected- to a healthy tree, however, spread through infected propagative material has been described as the most important mode of spreading ASBVd in the orchards (Wallace, 1958; Bar-Joseph *et al*., 1986). ASBVd can be transmitted through grafting, first explained by Horne and Parker (1931). They described ASBVd as graft transmissible pathogen and showed that after grafting with infected plant material, the disease will manifest after two to three years given that the disease is transmitted from symptom-bearing trees and/or recovered symptomless carrier trees. This, however, cannot be true for the symptomless carrier trees that originate from infected seeds. A symptomless carrier generally refers to a tree that is infected with ASBVd but does not show any visible symptoms of the disease. Symptomless carrier trees appear healthy but produce significantly lower yields compared to the healthy trees (Da Graça, 1985).

Symptomless carriers are a result of trees that have recovered from the infection from trees that previously showed symptoms but they still carry the viroid in their tissues (Da Graça, 1980). Wallace and Drake (1962) also demonstrated that symptomless carries arise from an infected symptomatic tree. The tree sends up new shoots that appear healthy; these shoots dominate the tree, replacing all symptomatic leaves. Symptomless carriers can also arise from parent symptomless carrier trees that underwent recovery during an early greenhouse stage, allowing time for the healthy appearance to become dominant. However, these trees can exhibit symptoms when they are exposed to stress, e.g. fire, when the trees are cut back, or when a healthy scion is grafted onto a symptomless carrier tree (Dodds, 2001). Symptomless carrier trees that are produced from the seed will pass the viroid to their progeny, and it will remain symptomless even if they are exposed to stress (Wallace, 1958).

Spread by root grafting from an infected tree to an adjacent healthy tree has been reported for ASBD (Wallace, 1958). As the trees grow older the roots intersect, this has been suspected to be one of the methods ASBVd can be transmitted from an infected to a healthy tree (Wallace, 1958). However, the frequency of root grafting in the field is unknown and could be of minor importance (Semancik, 2003). Pollen transmission occurs when a healthy avocado tree is pollinated by pollen from a diseased tree. In such a 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).

To date, no vector has been reported to transmit ASBVd from one tree to the 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). ASBVd can be transmitted on pruning blades, injection material and harvesting clippers (Semancik, 2003). A low transmission rate, between 8% and 30%, was reported for cutting blades (Dodds, 2001). Slash inoculations and leaf rub with extracts from ASBVd infected tissue have also been proven to successfully transmit ASBVd (Semancik, 2003). Against this background, the objectives of the study were to

investigate four modes of ASBVd transmission namely grafting (top-work), root grafting, mechanical transmission and pollen transmission to illustrate the importance of indexing and sanitation protocols when dealing with ASBVd infection in the nursery and in the field.

3.2 Material and methods

3.2.1 Grafting (Top-work)

Sixty healthy 'Bounty' rootstock trees obtained from a commercial nursery (longitude: 30.773893; latitude: - 25.402504), in Mpumalanga, were used in the experiment. Thirty rootstocks were top worked with infected 'Hass' scion and 30 were top worked with healthy 'Hass' scion which was used as control (Figure 3.1). The bud-wood (scion) was collected from the 'Hass' orchard in Mbombela (longitude: 30.928410887745482; latitude: -25.4292923547822). Healthy bud-wood was collected from confirmed healthy trees and infected bud-wood was collected from selected ASBVd symptomless carrier trees earlier identified as positive using qRT-PCR. The trees were grafted by a professional nurseryman from the ARC-TSC in the tunnels (longitude: 30.968347; latitude: - 25.452229) where plants were also maintained and monitored. Before grafting, all bud-wood was treated with a 2g/L dosage of Mancozeb for 5 minutes and allowed to air dry to disinfect fungal pathogens. The rootstocks were top worked using either angle grafting, plain grafting or cleft grafting depending on the size of the rootstock compared to the scion. After grafting, all the graft wood was sealed with grafting tape and monitored and kept at the ARC-TSC pathology tunnels from January 2019 until December 2021. A Chi-square test at $p \leq 0.05$ was performed to compare successful grafts between the healthy and infected trees.

3.2.2 Root grafting

Two separate experiments were conducted, a field experiment and a tunnel experiment. The tunnel experiment was conducted at the ARC-TSC pathology tunnels (longitude: 30.968347; latitude: -25.452229) here four healthy 'Fuerte' seedlings were forced to grow in a 10L bag together with one infected seedling in the middle. The aim was to force the intersection of their roots (Figure 3.2 A). There were six 10L bags in total each with four healthy and one infected seedling (Figure 3.2 B). The healthy seedlings were grown from the healthy 'Fuerte' seeds and infected seedlings were grown from infected 'Fuerte' seeds. A field trial was conducted at a commercial 'Hass' orchard (longitude: 30.928410887745482; latitude: -25.4292923547822), planted in 2009. Two trees, where ASBVd infection in the root system was confirmed, were

identified as well as two trees in the immediate vicinity that were healthy, the latter used as controls. Four young seedlings (15 months old) were planted around each of the four selected trees, one meter away from the tree trunks and monitored for six months (Figure 3.3).

Figure 3.1 (**A**) Healthy and infected 'Hass' bud-wood being air-dried after Mancozeb treatment. (**B**) 'Bounty' rootstock before the cutting and grafting. (**C**) Grafting of 'Hass' on 'Bounty' (**D**) trees immediately after grafting. (**E**) Trees after nine months of grafting.

Figure 3.2 (**A**) A ten-litre bag containing four healthy ‘Fuerte’ seedlings on the edges and one infected seedling in the middle. (**B**) The experiment was repeated six times in a tunnel.

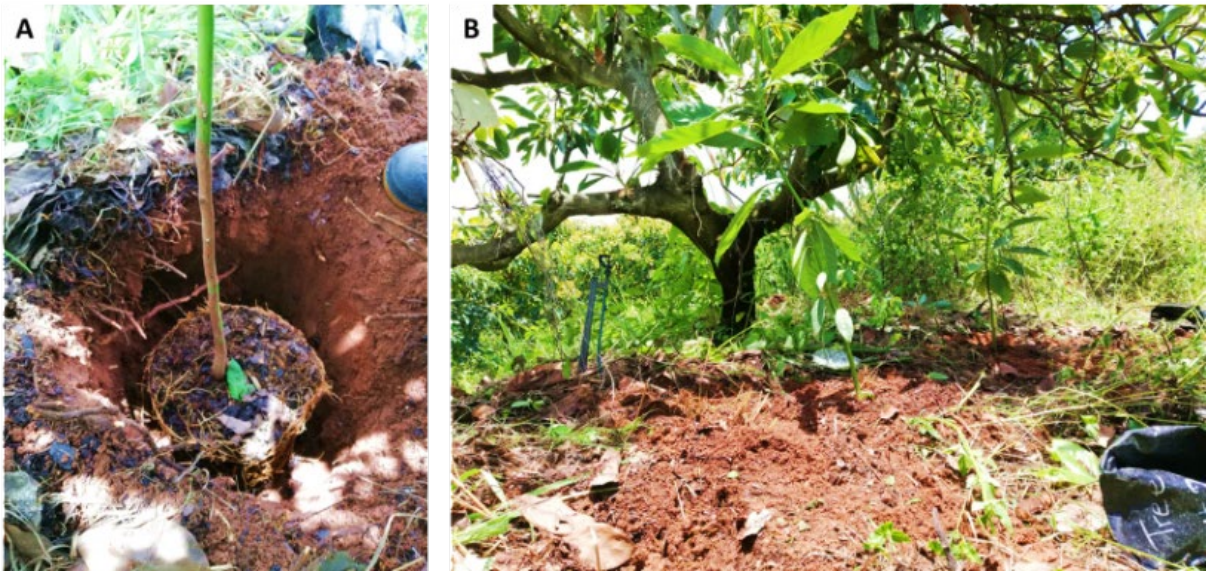

Figure 3.3 (**A**) ‘Fuerte’ seedlings planted in the field around a (**B**) symptomless ASBVd infected ‘Hass’ tree with a confirmed infected root system

3.2.3 Mechanical transmission

Seventy-five ‘Fuerte’ clonal trees were used in the experiment. Mechanical transmission was conducted using pruning shears (secateurs). The infected material was obtained from the ‘Hass’ orchard in Mbombela (longitude: 30.928410887745482; latitude: -25.4292923547822), the branches used for the mechanical transmission experiment were cut from previously identified ASBVd symptomless carrier trees. The trees were infected by cutting the infected branch and then using the same shear to

prune the healthy trees. The shears were treated with four different concentrations of sodium hypochlorite (3.5% M/V) and the untreated shears were used as controls (Table 3.1), each treatment had 15 young trees.

Table 3.1 Household bleach (Sodium Hypochlorite 3.5% M/V) at different percentages of active ingredients used to treat pruning shears

Treatments	Number of trees
0 active ingredient (tap water)	15
1% active ingredient	15
1.5% active ingredient	15
2% active ingredient	15
Controls – untreated shears	15
Total	**75**

3.2.3.1 Stability of ASBVd on different surfaces, *i.e*, metal, cotton and latex gloves

The stability of ASBVd on different surfaces were tested with three treatments including i) extracted RNA, ii) plant sap macerated in avocado extraction buffer (ARC-TSC protocol) and iii) plant sap macerated in water. The surfaces included metal (silver knife), a latex glove and cotton surfaces. The three surfaces were subsequently treated with extracted RNA and the two plant sap treatments, plant sap macerated in deionized water and in avocado extraction buffer (500 mM sodium acetate.$3H_2O$, 10 mM magnesium chloride, 3% Sodium Dodecyl Sulfate (SDS), 20% ethanol (molecular grade), 1.2% sodium sulphite, pH 6 and 2.5 ml chloroform/iso-amyl alcohol (CL/Iso) (24:1). One micro liter of either plant sap or extracted RNA was pipetted on different surfaces. The samples were left to dry for one minute, five minutes, 15 minutes, one hour and 24 hours, respectively. After drying, 20 µl of deionized water was used to re-suspend dry samples into 2 ml reaction tubes, the tubes were kept at -20 °C for further use.

3.2.4 Pollen transmission

3.2.4.1 Pollen collection

Pollen was collected from flowers of three ‘Fuerte’ plants (Block 4A) and one ‘Ryan’ plant (Block C) that previously tested positive for ASBVd at a commercial farm in KZN (-29,5445538; 30,3735709). The pollen was subsequently collected from the positive trees by removing anthers with unshed pollen. Anthers were carefully removed from

the flowers using forceps and transferred to small petri dishes, sealed with parafilm and cold stored until use.

3.2.4.2 Collection of bees and pollen from hives

Within a few meters to hundred meters from positive trees, beehives were identified with the help of professional beekeepers. The pollen was selected by identifying a hive frame with most pollen, pollen was then scooped from the parts of the frame with light yellow to white pollen using a spatula and transferred to 2 ml reaction tubes. From a single hive, ten 2 ml tubes were sampled. Bees were collected from hives by opening the hives and rolling a 50 ml tube over the bees and close the lid (Figure 3.4). The exact protocol was shared by a collaborator and not mentioned in detail in this write-up.

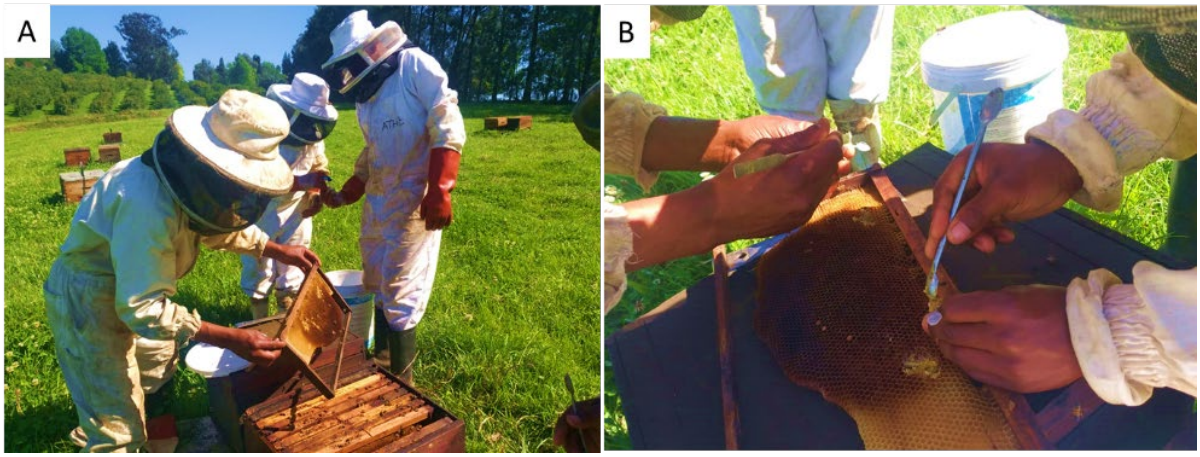

Figure 3.4 Sampling of pollen and Bees from the beehives. (**A**) Frame selection and (**B**) scooping of pollen from the honeycombs

3.2.5 Isolation and confirmation of ASBVd

The presence of ASBVd in the trees was confirmed using qRT-PCR as described in Chapter 2, section 2.2.2.2. ASBVd RNA from ~ 400mg leaf, pollen and bee samples was extracted using a cellulose column chromatography technique developed at the ARC-TSC (Manicom and Luttig, 1999) as described in chapter 2.2.2.1.

Subsequently, primer pair 5'-ATCACTTCGTCTCTTCAGGGAAAGA-3' and 3'-CAAGAGATTGAAGACGAGTGAACTA-5', amplifying 250bp product (Luttig and Manicom, 1999), was used to detect ASBVd in a conventional RT-PCR in a ProFlex PCR system by Life technologies using qPCRBIO SyGreen 1-Step Go Lo-ROX kit (PCRBIOSYSTEMS, UK). 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. PCR products were visualised on a 1% agarose gel using 1x TAE buffer and stained with ethidium bromide.

3.3 Results

3.3.1 Grafting (Top-work)

After grafting 30 infected and 30 healthy scions onto the healthy rootstocks, there were 16 successful grafts from the infected scion compared to the 13 unscuccessful grafts from the healthy scions. This translates to a 53% success rate achieved for grafting the infected symptomless scions compared a 43.3% grafting success rate achieved grafting healthy scions (Table3.2). Some scions (infected or healthy) died as early as three weeks after grafting, some took up to three months to die. Although the number infected scions that survived was more than the healthy scions, according to a Chi-Square test there were no significant differences between the grafting healthy or infected scions. Therefore, the proportion of successful grafts does not depend on whether the scion is infected or healthy (Table 3.2). During the monitoring, all the infected (successful and unsuccessful grafts) trees were tested yearly for the presence of ASBVd for three consecutive years. The results showed that only the grafted, infected scions were ASBVd positive and the rootstocks remained negative. An example of unsuccessful and successful grafting is shown on figure 3.5 below.

Table 3.2 Results of graft transmission of avocado sunblotch disease infected 'Hass' cultivar scion to healthy Bounty rootstock.

Bud-wood	Trees grafted	Successful grafts	RT-qPCR	Success rate %	infected plants	P-Value
Positive	30	16	Positive	53	16	**0.483**
Healthy	30	13	Negative	43,3	0	

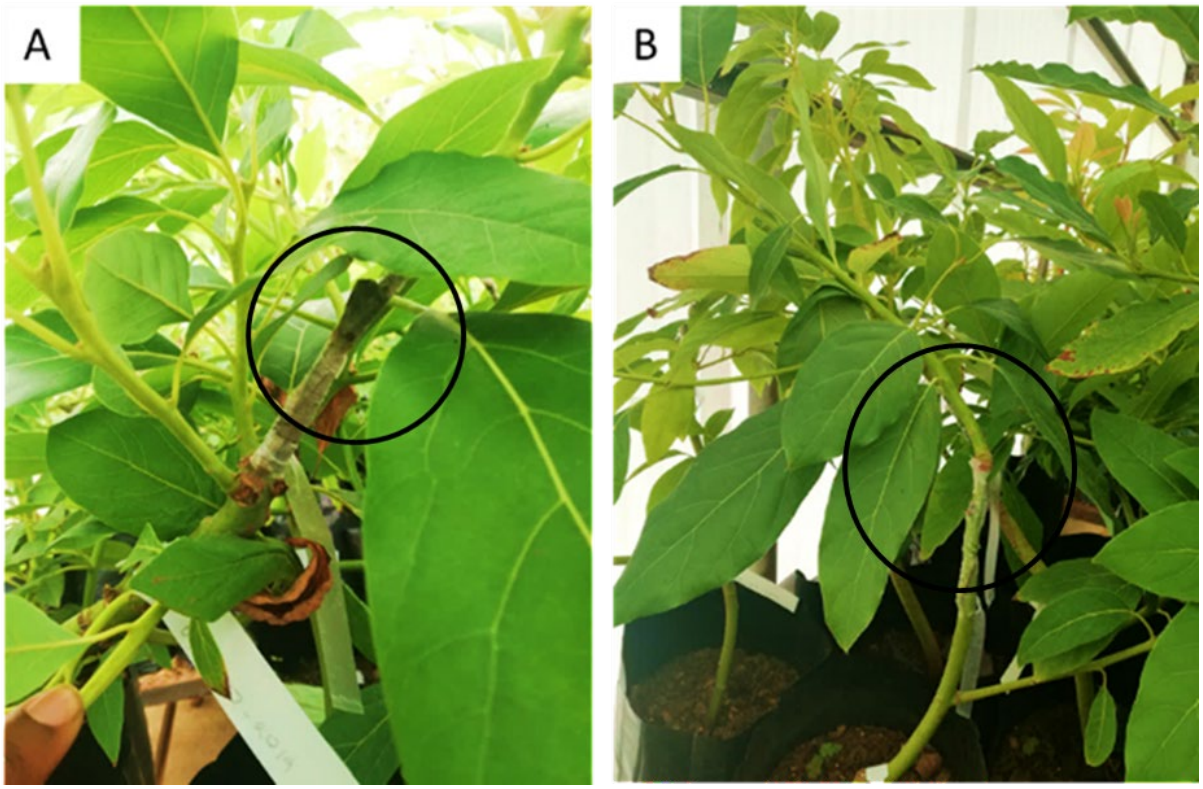

Figure 3.5 An example of (**A**) unsuccessful and (**B**) successful grafts during the grafting of the 'Hass' ASBVd infected symptomless and healthy scions on healthy 'Bounty' rootstocks.

3.3.2 Root grafting

A single infected 'Fuerte' tree was planted in a 10L bag together with four healthy 'Fuerte' seedlings and monitored from July 2018 until October 2021. The experiment was repeated six times. Upon removal of the trees from the bags, the root systems of the trees were entangled (Figure 3.6 A). However, the root system of the healthy trees looked more vigorous (Figure 3.6 B) compared to that of infected trees (Figure 3.6 C). The root system of infected trees was fully dominated and outcompeted by the healthy trees' root system (Figure 3.6 D) and the healthy root system had successfully intersected which was evident by the formation of the root nodes (Figure 3.6 E). Similar observations were made for all six replicates of the experiment. Therefore, in the current experiment, the root system of the infected trees did not intersect with the root system of the healthy trees and could not transmit ASBVd through the root system because it was outcompeted and had started to die. Molecular test results confirmed that the healthy trees remained healthy and therefore no successful ASBVd transmission occurred through the root system. The field experiments were

unsuccessful, the trees were only monitored for six months and they eventually died from the lack of sunlight and water stress.

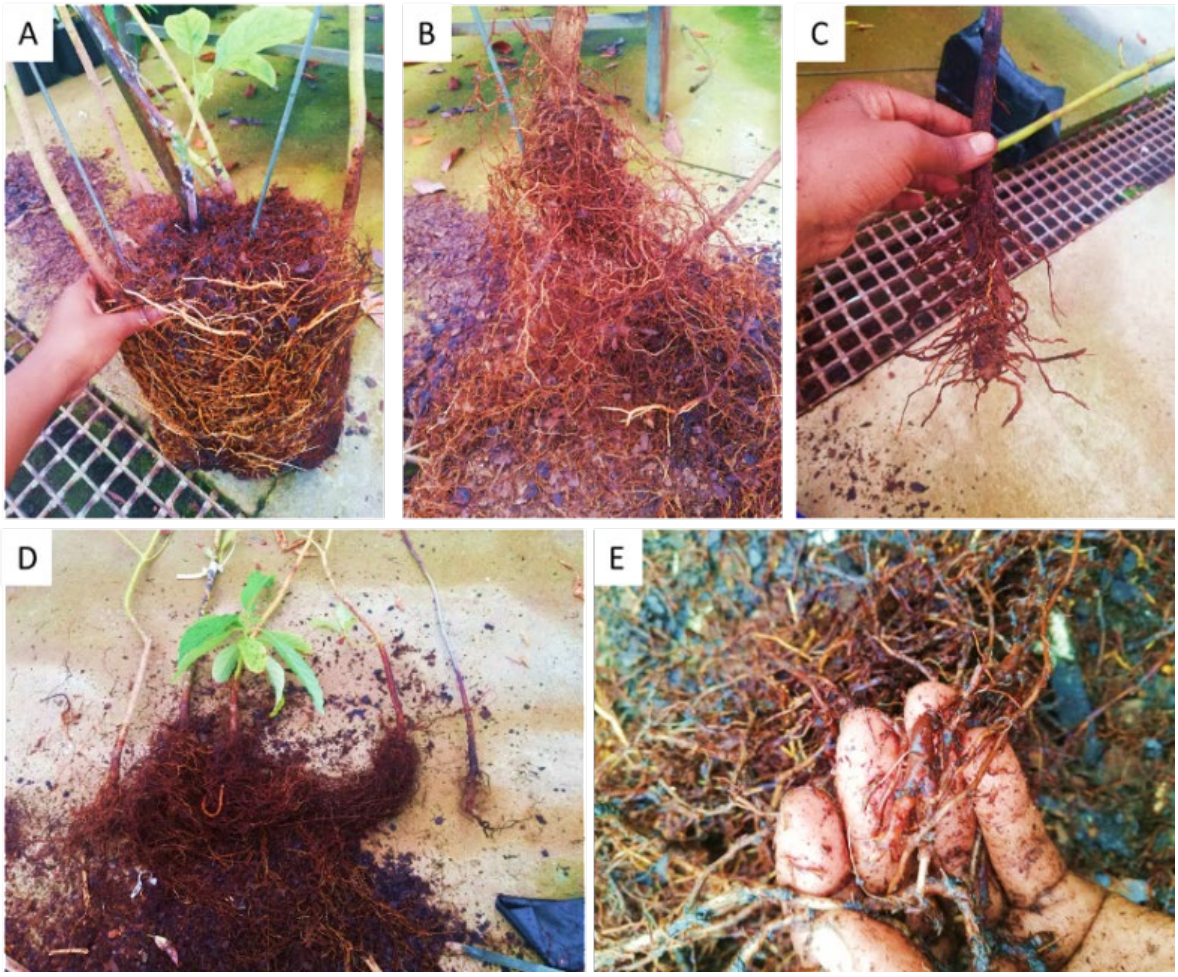

Figure 3.6 An example of the root system of five “Fuerte” seedlings after growing in a 10L bag for over a year. (**A**) The root system of all trees had intertwined together in a bag making one solid mass of roots. (**B**) The root system of healthy trees look vigorous and (**C**) the root system of infected trees was in a bad shape. (**D**) The root system of healthy trees intersected with each other and the root system of infected trees died. (**E**) The root system of heathy trees formed nodes showing a successful intersection.

3.3.3 Mechanical transmission

Seventy-five trees were inoculated using the pruning shears and when the trees were tested for the presence of ASBVd after three years, none of the trees tested positive. Therefore, the transmission of ASBVd from the infected to healthy trees using the pruning shears was unsuccessful.

3.3.3.1 Stability of ASBVd on different surfaces, i.e, metal, cotton and latex gloves

A study was done to test the concept of an experiment to determine the stability of ASBVd on different surfaces. The results of the concept study are summarized in Table 3.3. Few differences were detected between the treatments with the first three time intervals (1 minute to 15 minutes). Binding of ASBVd to different surfaces was demonstrated for all treatments. The treatment of plant sap macerated in water will be the closest to simulating the use of metal pruning equipment in the field. After 24 hours, the ASBVd RNA remained intact on the metal surface. The finding demonstrate the importance of sanitation practices in field and in nurseries.

Table 3.3. Cyclic threshold (Ct) values of qRT-PCR results of ASBVd detection after exposure to different surfaces on different time intervals

Surface	Time exposed	Extracted RNA	Plant sap in buffer	Plant sap in water
Latex glove	1 min	22.57 +	27.14 w	22.23 +
	5 min	23.30 +	-	20.16 +
	15 min	23.43 +	-	23.11 +
	1 hour	18.51 +	26.24 w	21.24 +
	24 hours	13.94 +	24.16 +	25.39 +
Metal	1 min	11.37 +	16.56 +	17.80 +
	5 min	10.76 +	16.52 +	17.16 +
	15 min	10.66 +	16.12 +	16.48 +
	1 hour	8.99 +	17.12 +	16.61 +
	24 hours	8.94 +	16.98 +	16.47 +
Cotton	1 min	13.44 +	14.92 +	17.48 +
	5 min	12.57 +	13.81 +	16.78 +
	15 min	11.22 +	13.38 +	17.86 +
	1 hour	10.75 +	13.95 +	17.84 +
	24 hours	9.72 +	12.87 +	18.17 +

+ indicate a positive reaction (Ct < 25.55),

w indicate a low reaction (Ct > 25.55),

- indicate a negative reaction

3.3.4 Pollen transmission

The infected trees within the 100m radius of the beehives were tested for ASBVd and low Ct values were detected in the trees, indicating a high ASBVd titre. The results showed the presence of ASBVd in pollen derived from symptomless carrier trees with similar levels of infection in leaf and pollen samples (Table 3.4).

Table 3.5. summarizes the result of individual bee samples and pollen samples collected from the four hives. ASBVd was detected in both sample types but prevalently detected in pollen samples, with no ASBVd presence being recorded for bees collected from site 4. Site 4 is located 23 km from the research site and the prevalence to detect ASBVd in pollen in hives was shown to be higher than from bees directly. Ten bee and pollen samples were collected from each beehive, at site 1, nine bee and all ten pollen samples tested positive for ASBVd. Nine bee and five pollen samples tested positive for ASBVd collected from site 2 and five bee and nine pollen samples tested positive for ASBVd collected from site 3. None of the bee samples collected from site four tested positive but six pollen samples tested ASBVd positive. These results confirm the ability of bees to carry pollen in their fur and successfully store the pollen inside their honeycombs. Two positive pollen and one bee sample were selected from each site, except for site four, here all samples were selected from the pollen samples and sent for sequencing at Inqaba Biotech, South Africa. Sequence results showed a 93 % identity with three reference ASBVd sequences from the NCBI GenBank® database. (Figure 3.7)

Table 3.4. Detection of ASBVd in leaf and pollen samples from positive symptomless carrier trees

Block-Plant number	Cultivar	Leaf sample		Pollen	
		Ct value	Copy number	Ct value	Copy number
Block 4A-04	'Fuerte'	11.94 +	1 210 397	14.80 +	155 702
Block 4A-06	'Fuerte'	14.38 +	210 096	12.23 +	964 180
Block 4A-07	'Fuerte'	10.98 +	1 304 7292	21.63 +	1215
Block C- 35	'Ryan'	16.83 +	36 723	15.58 +	182 471

\+ indicate a positive reaction (Ct < 25.55)

2287 **Table 3.5** RT-PCR results from individual bee and pollen samples collected from four beehives

Site number/ Location	Sample type	Sample number	Ct value	RT-PCR result	Site number/ Location	Sample type	Sample number	Ct value	RT-PCR result
Site 1	Bee	1	26.32	w	Site 3	BEE	47	-	-
S 29°32'38.2"		2	22.86	+	S 29°32'37.9"		48	-	-
E 30°22'22,5"					E				
		3	24.83	+			49		-
		4	28.33	w			50	26.84	w
		5	-	-			51	-	-
		6	29.84	w			52	27.70	w
		7	25.41	+			53	29.59	w
		8	26.19	w			54	-	-
		9	22.53	+			55	29.93	w
		10	25.85	w			56	28.52	w
		11	-	-		Pollen	57	28.47	w
		12	24.89	+			58	28.38	w
	Pollen	13	19.58	+			59	27.61	w
		14	20.08	+			60	28.55	w
		15	20.09	+			61	26.14	w
		16	18.27	+			62	27.31	w
		17	18.82	+			63	28.11	w

		18	22.45	+			64	25.26	+
		19	22.88	+			65	29.50	w
		20	21.69	+			66	-	-
		21	22.02	+	Site 4	Bee	69	-	-
		22	26.66	w	S 29°27'13.1" E 30°16'05.7"		70	-	-
Site 2 S 29°32'33.1" E 30°22'15.7"	Bee	25	26.82	w			71	-	-
		26	27.84	w			72	-	-
		27	25.99	w			73	-	-
		28	23.12	+			74	-	-
		29	29.80	w			75	-	-
		30	26.20	w			76	-	-
		31	28.95	w			77	-	-
		32	29.83	w			78	-	-
		33	-	-		Pollen	79	23.91	+
		34	27.94	w			80	21.73	+
	Pollen	35	-	-			81	28.52	w
		36	25.75	w			82	-	-
		37	28.97	w			83	-	-

38	-	-
39	25.27	+
40	29.51	w
41	26.97	w
42	-	-
43	-	-
44	-	-
84	25.55	w
85	-	-
86	25.15	+
87	-	-
88	18.63	+

+ indicate a positive reaction (Ct < 25.55)

w indicate a low reaction (Ct > 25.55)

- indicate a negative reaction

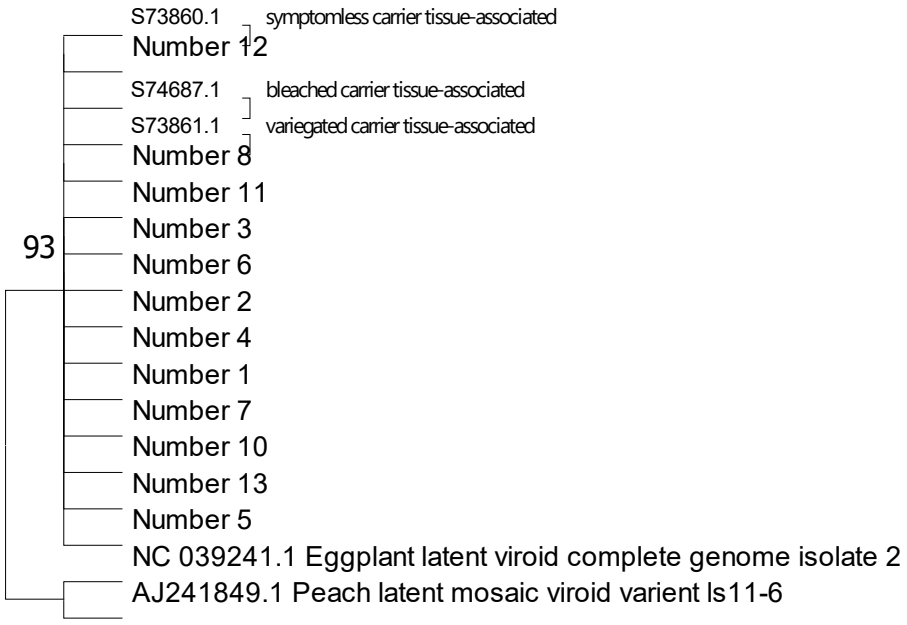

Figure 3.7 Evolutionary relationships of ASBVd sequences isolated from the beehives of KwaZulu-Natal. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 2.5652073 is shown. The percentage of replicate trees 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* (AJ241849) and *Egg latent mosaic viroid* (NC_039241) from the family Avsunviroidae were used as an out-group. Sequences 1-3 were selected from site one, 4-6 from site two, 7,8 and 10 from site three and 11- 13 were selected from site four.

3.4 Discussion

ASBVd was first discovered as graft transmissible by Horne and Parker (1931). These researchers discovered that ASBD can be transmitted through the grafting of unhealthy scion onto a healthy rootstock or vice versa and the symptoms of the disease in an infected tree will only manifest after two to three years from the time of infection. Since then graft transmission is regarded as the most important mode for the spreading ASBVd (Bar-Joseph *et al.*, 1986). In the current study, there was a 53% success rate of grafting ASBVd infected scions compared to the 43.3% of the heathy scions. This finding implies that there is a 53% transmission success rate of ASBVd through grafting practices. It was determined that there were no significant differences between grafting infected and healthy scions to the healthy rootstock. Implying that a

healthy rootstock is able to support the infected scion as much as it does with the healthy scion and since there are no visible symptoms ASBVd, spread is unavoidable. These are some of the reasons ASBVd symptomless carrier trees have been described as the primary sources of infection by spreading the disease through budding and grafting practices thus playing an essential role in the epidemiology of ASBVd (Saucedo Carabez *et al*., 2019). It is thus crucial to index all the propagation material for ASBVd to avoid the further spread of symptomless carrier trees in the orchards.

Spread by root grafting from an infected tree to an adjacent healthy tree has been reported for ASBD (Wallace, 1958). However, the frequency of root grafting in the field is unknown and could be of minor importance (Semancik, 2003). In the current study, a single infected tree was grown in a 10L bag with four healthy trees, the infected trees root system was fully dominated and outcompeted by that of the healthy trees. There was no successful transmission of ASBVd *via* the root system of the infected trees. Therefore, according to the current findings, root graft transmission could be of minor importance as described by Semancik (2003). However, in a study conducted by Tondo *et al.,* 2010, they discovered an increase in the number of infected trees from 19% in 1996 and 2000 to 24% in 2009. The newly infected plants were either adjacent to previously infected plants, adjacent to plots from which infected plants had been removed, or adjacent to other newly infected plants that are adjacent to previously infected plants or contaminated plots and they attributed these infections to root grafting. Therefore, ASBVd can spread through root grafting but the frequency remains unknown.

ASBVd can be transmitted on pruning blades, injection material and harvesting clippers and an 8% to 30% mechanical transmission rate by cutting blades have been reported (Dodds, 2001; Semancik, 2003). When the infected trees were cut and the same blade was used to prune the healthy trees, none of the trees in our experiment tested positive for ASBVd and therefore there was no successful mechanical transmission of ASBVd in the current study. However, since ASBVd can take up to three years to manifest in a tree (Bar-Joseph *et al.*, 1986), it is still possible that the ASBVd in this current study took even longer to manifest. Desjardins *et al*. (1987) successfully transmitted ASBVd mechanically using the slash technique, however, in

the current study the normal pruning shears (secateurs) were used to simulate the orchard conditions.

The stability of ASBVd on different surfaces was tested and binding of ASBVd to different surfaces was demonstrated for all treatments. The treatment with plant sap macerated in water was the closest to simulating the use of metal pruning equipment in the field. After 24 hours, the ASBVd particles remained intact on the metal surface. Therefore, if equipment used on an infected tree is used for the next tree, there is a chance that ASBVd will be transmitted to the next tree and lead to ASBVd spread in an orchard. This finding demonstrates the importance of sanitation practices in the field and in nurseries to mitigate against the risk of the spread of ASBVd. All equipment, including drill bits, syringes, handsaws, hand clippers or loppers, pole pruners, chain saws, should be sterilized between operations. If the ASBVd status of an orchard is unknown, cleaning of equipment between plants and between rows is recommended. In ASBVd-infected orchards, growers should avoid pruning with all cut pruners. It is crucial to remove ASBVd-infected trees from orchards, although it is not always easy to convince growers to remove infected trees when they are still bearing fruit. One should keep in mind that over time, the yield of the symptomless carrier trees will decline and the infected trees will be a source of infection in orchards.

The use of 5% commercial bleach (sodium hypochlorite) and a 1:1 mixture of 2% sodium hydroxide and 2% formaldehyde or 6% solution of hydrogen peroxide effectively inactivate ASBVd (Desjardins *et al*., 1980; Desjardins *et al*.,1987). Inactivating of viroids using 20% skim milk has been demonstrated for potato spindle tuber viroid (PSTVd) on tomatoes (Mackie *et al*., 2015). The method could be adopted for the inactivation of ASBVd in the orchards because it is more environmental friendly, does not corrodes metal, degrades when in contact with organic matter and not an irritant to humans (Mackie *et al*., 2015). However, it still need to be tested in the control of ASBVd on equipment.

The results showed the presence of ASBVd in pollen derived from symptomless carrier trees with similar levels of infection in leaf and pollen samples and in high titre. Pollen and bee samples collected from three beehives, that were located close to infected trees, tested positive for ASBVd. The spatial position of the infected hives in relation to the ASBVd-positive trees (not shown) was determined and ranged between 100 m

from an infected tree to 1.7 km. Results showed that bees can carry ASBVd from pollen of symptomless carrier trees to bee hives. Although transmission of ASBVd via pollen is known to be relatively low (1.8% and 3.125% according to Desjardins *et al*., (1979) this is a concern as it represents yet another avenue for transmission of the disease. Therefore, since bees can carry ASBVd pollen to their beehives, the current findings agree with the findings of Desjardins *et al.* (1979) which showed there is a possibility of the honeybees being the vectors of ASBVD. When analysed using BLASTC ,all the sequences were confirmed as ASBVd variants. Sequences were aligned with three reference sequences from the NCBI Genbank ® database. The sequences had an identity of 93 % with the reference sequences. Therefore the ASBVd sequences that were isolated from the bees and the pollen from the honey combs are the variants of ASBVd.

Mohammed and Thomas (1979) described ASBVd to be more confined on the lesions in the symptom bearing trees thus rendering it undetected to the rest of the tree, and the ASBVd in the symptomless carrier trees to be more localised and easy to detect throughout the infected tree. Therefore, symptomless carrier trees remain a risk to keep in the orchard as they contain a high ASBVd titre in their pollen which could be easily carried by bees for further spread. When a symptomatic fruit is detected on a tree and occurs as single infected fruit, it is most likely that the infection derived from pollen transmission. The rest of the tree will remain negative and only the fruit will be infected. These trees should be marked and monitored over time. Symptomatic fruit that derived from infected pollen should be removed from the tree to ensure that these fruit are not used as seed source.

3.5 Conclusions

To mitigate against the risk of field transmission of ASBVd, a comprehensive suite of management strategies are needed. This includes, firstly, to know the ASBVd status of trees in orchards and to remove infected trees. Secondly, it is important to understand the modes of transmission of ASBVd and to include sanitation as a critical strategy to prevent the spread of ASBVd in orchards and nurseries. Equipment should be sanitised between plants and rows. If the ASBVd status of an orchard is known, it will be easier to use equipment without the risk of spreading ASBVd. Trees showing fruit symptoms should be marked and the fruit should not be used as seed source. A tree with only a few symptomatic fruit was probably infected through pollen and the

rest of the tree will remain healthy. These trees will test negative with molecular detection methods. When trees with fruit symptoms are detected, the probability of finding symptomless carrier trees in close proximity are high. Systematic testing of orchards is recommended.

3.6 References

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CHAPTER 4

Genetic differences between Avocado sunblotch viroid (ASBVd) variants detected in a clonal population of young, symptomless 'Fuerte' trees.

Abstract

Avocado sunblotch viroid (ASBVd) is an infective single-stranded, covalently closed, circular RNA molecule inducing avocado sunblotch disease (ASBD) on avocado (*Persia americana*. Mill). Symptomless carrier trees play an important role in the epidemiology of ASBVd and have been described as the primary sources of infection by spreading the disease through budding and grafting practices. Here we report on the molecular differences between ASBVd variants detected in a clonal population of symptomless 'Fuerte' avocado plants. There were a total of 103 symptomless carrier trees in which ASBVd was detected by real-time qRT-PCR using a 99 bp primer pair from a population of 453 young 'Fuerte' trees. Thus, 22% of this population was infected but still growing and looking like normal healthy trees. Whole genomes of ASBVd variants were obtained by using a conventional PCR with primer sequences that yielded a 250 bp product. The genomes obtained had sequence lengths that varied between 248 and 253 nucleotides (nt). From the 76 ASBVd positive sequences, only 42 variants were obtained and variants were repeatedly detected from different trees in the population. The variants' sequences were deposited in the NCBI GenBank® database and were assigned accession numbers ON135462 to ON135503. The phylogenetic analysis of the variants obtained from this study showed a high sequence identity of 97% with the reference ASBVd variants obtained from GenBank® database. The current study is crucial for the development of accurate detection techniques of ASBVd and contributes to the ASBVd action plan goals that promote the removal of all infected material from avocado orchards to prevent the further spread of the viroid.

Keywords: *qRT-PCR, RT-PCR, sequencing, sequence lengths, ASBVd variants, symptomless carrier trees, 'Fuerte' clonal trees*

4.1 Introduction

Avocado sunblotch viroid (ASBVd) is classified under the smallest viroid family *Avsunviroidae*, the family is only represented by four species classified under three genera (Di Serio *et al*., 2017). The genus *Pelamoviroid* has two members namely peach latent mosaic viroid (PLMVd) and chrysanthemum chlorotic mottle viroid (CChMVd). The genus *Elaviroid* has a single member namely eggplant latent viroid (ELVd) (Di Serio *et al*., 2017). ASBVd is the single member of genus *Avsunvoroid* (Di Serio *et al*., 2017). This viroid family does not consist of a central conserved region and the members of this family have the self-cleavage ability (Delan-Forino, 2014). ASBVd 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) and is an infective single-stranded, covalently closed, circular RNA molecule (Palukaitis *et al*., 1979; Symons, 1981; Saucedo-Carabez *et al*., 2015). It forms a small rod-like secondary structure between 246 and 251 nt in length (Saucedo-Carabez *et al*., 2019).

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

There are 108 known ASBVd variants deposited in the GenBank® database. The variants are associated with the different symptoms they induce in the avocado host. The symptom-associated variants include a symptomless carrier (ASBVd-Sc), a bleached symptom associated (ASBVd-B) and a variegated symptom associated (ASBVd-V) variant (Semancik and Szychowski, 1994). Different ASBVd variants arise

from slight sequence variations on the ASBVd sequence (Running *et al*., 1996). Most of the changes occur between U-A nucleotide bases leading to the sequence variations (Shnell *et al*., 1997). However, because the variants share similar biological properties, they are therefore not identified as different strains (Semancik and Szychowski, 1994). The nucleotide differences found between ASBVd variants is a crucial factor to consider when developing molecular detection techniques for ASBVd. Symptomless carrier trees play an important role in the epidemiology of ASBVd, as they have been described as the primary sources of infection by spreading the disease through budding and grafting practices (Saucedo Carabez *et al*., 2019). There are no studies that focus specifically on the differences between the variants involved in the symptomless carrier infected trees. It is, therefore, crucial to study all the nucleotide bases associated with symptomless carrier trees. Against this background, the main objective of the current study was to investigate the genetic differences between ASBVd variants within a clonal symptomless carrier 'Fuerte' tree population.

4.2 Material and methods

4.2.1 Plant material

Plant material was kindly donated by a nursery based in Mbombela, Mpumalanga (longitude: 30.773893; latitude: -25.402504) from a batch that was previously diagnosed with ASBVd, all trees were asymptomatic. The batch consisted of 453 young 'Fuerte' trees grafted on the 'Bounty' rootstock. The trees were kept and monitored at ARC-TSC under the shade net, each tree was tested individually for ASBVd. After extracting all required RNA material, the infected trees were destroyed.

4.2.2 ASBVd RNA extraction

The old and the new leaves were selected from each young tree separately and the samples were taken to the plant pathology laboratory at the ARC-TSC, Mbombela for processing. RNA extraction from the samples was done using the cellulose column chromatography method as described in chapter 2, section 2.2.2.2.

4.2.3 Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

The presence of ASBVd was detected with an ASBVd-specific primer set (5'-AGAGAAGGAGGAGTCGTGGTGAAC -3'; 5'- TTCCCATCTTTCCCTGAAGAGAC -3') amplifying a 99 bp product in a one-step reverse transcription-quantitative

polymerase chain reaction (qRT-PCR) (PCRBIOSYSTEMS) using the QIAGEN Rotor-Gene Q instrument. The cycling conditions were as follows: 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. Samples were analysed using cycling threshold values (Ct) and endpoint analysis. The trees sampled were scored into three infection levels, *viz.* high, medium and low infection based on Ct values. All trees with Ct values equal to or less than 10 were rated as highly infected, Ct values from 10.1 to 18 were rated as medium and Ct values from 18.1 to 25.4 were rated as low/slightly infected.

4.2.4 Reverse transcription-polymerase chain reaction (RT-PCR)

Subsequently, the primer pair 5'-ATCACTTCGTCTCTTCAGGGAAAGA-3' and 3'-CAAGAGATTGAAGACGAGTGAACTA-5', amplifying a 250bp product (Lutting and Manicom a, 1999), was used to detect ASBVd in a conventional RT-PCR in a ProFlex PCR system by Life technologies using qPCRBIO SyGreen 1-Step Go Lo-ROX kit (PCRBIOSYSTEMS). 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. PCR products were visualised on a 1% agarose gel using 1x TAE buffer and stained with ethidium bromide.

4.2.5 Sequencing of ASBVd variants

Full-length amplicons were sequenced at Inqaba Biotechnical Industries (Pty) Ltd. (Pretoria, South Africa). 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. Sequences were analysed by BLASTn and aligned on MAFFT version 6 (Katoh *et al*., 2019). The analysis of the sequences and the evolutionary analyses were conducted using known ASBVd GenBank® sequences (Table 4.1). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 11 (Tamura *et al*., 2021). The sequences were deposited to the NCBI GenBank® database using BankIt. The sequences were submitted using an aligned version, the order of the sequences appeared according to the order of the alignment with accessions from ON135462 to ON135503 (Zwane *et al*., 2014; Table 4.1).

2620 **Table 4.1** Accession numbers for sequences obtained in the current study with ASBVd
2621 variants and outgroups obtained from NCBI GenBank® used in phylogenetic studies

Sequence	Reference	Accession
Avocado sunblotch variant ScFuerte04	Zwane *et al*. (2022)	ON135462
Avocado sunblotch variant ScFuerte44	Zwane *et al*. (2022)	ON135463
Avocado sunblotch variant ScFuerte27	Zwane *et al*. (2022)	ON135464
Avocado sunblotch variant ScFuerte8	Zwane *et al*. (2022)	ON135465
Avocado sunblotch variant ScFuerte80	Zwane *et al*. (2022)	ON135466
Avocado sunblotch variant ScFuerte70	Zwane *et al*. (2022)	ON135467
Avocado sunblotch variant ScFuerte94	Zwane *et al*. (2022)	ON135468
Avocado sunblotch variant ScFuerte03	Zwane *et al*. (2022)	ON135469
Avocado sunblotch variant ScFuerte54	Zwane *et al*. (2022)	ON135470
Avocado sunblotch variant ScFuerte65	Zwane *et al*. (2022)	ON135471
Avocado sunblotch variant ScFuerte45	Zwane *et al*. (2022)	ON135472
Avocado sunblotch variant ScFuerte76	Zwane *et al*. (2022)	ON135473
Avocado sunblotch variant ScFuerte15	Zwane *et al*. (2022)	ON135474
Avocado sunblotch variant ScFuerte71	Zwane *et al*. (2022)	ON135475
Avocado sunblotch variant ScFuerte96	Zwane *et al*. (2022)	ON135476
Avocado sunblotch variant ScFuerte88	Zwane *et al*. (2022)	ON135477
Avocado sunblotch variant ScFuerte95	Zwane *et al*. (2022)	ON135478
Avocado sunblotch variant ScFuerte07	Zwane *et al*. (2022)	ON135479
Avocado sunblotch variant ScFuerte20	Zwane *et al*. (2022)	ON135480
Avocado sunblotch variant ScFuerte67	Zwane *et al*. (2022)	ON135481
Avocado sunblotch variant ScFuerte64	Zwane *et al*. (2022)	ON135482
Avocado sunblotch variant ScFuerte101	Zwane *et al*. (2022)	ON135483
Avocado sunblotch variant ScFuerte05	Zwane *et al*. (2022)	ON135484
Avocado sunblotch variant ScFuerte86	Zwane *et al*. (2022)	ON135485
Avocado sunblotch variant ScFuerte72	Zwane *et al*. (2022)	ON135486
Avocado sunblotch variant ScFuerte29	Zwane *et al*. (2022)	ON135487
Avocado sunblotch variant ScFuerte10	Zwane *et al*. (2022)	ON135488
Avocado sunblotch variant ScFuerte16	Zwane *et al*. (2022)	ON135489
Avocado sunblotch variant ScFuerte33	Zwane *et al*. (2022)	ON135490
Avocado sunblotch variant ScFuerte55	Zwane *et al*. (2022)	ON135491

Avocado sunblotch variant ScFuerte06	Zwane *et al*. (2022)	ON135492
Avocado sunblotch variant ScFuerte25	Zwane *et al*. (2022)	ON135493
Avocado sunblotch variant ScFuerte98	Zwane *et al*. (2022)	ON135494
Avocado sunblotch variant ScFuerte100	Zwane *et al*. (2022)	ON135495
Avocado sunblotch variant ScFuerte48	Zwane *et al*. (2022)	ON135496
Avocado sunblotch variant ScFuerte50	Zwane *et al*. (2022)	ON135497
Avocado sunblotch variant ScFuerte11	Zwane *et al*. (2022)	ON135498
Avocado sunblotch variant ScFuerte01	Zwane *et al*. (2022)	ON135499
Avocado sunblotch variant ScFuerte17	Zwane *et al*. (2022)	ON135500
Avocado sunblotch variant ScFuerte09	Zwane *et al*. (2022)	ON135501
Avocado sunblotch variant ScFuerte23	Zwane *et al*. (2022)	ON135502
Avocado sunblotch variant ScFuerte46	Zwane *et al*. (2022)	ON135503
Avocado sunblotch viroid Sc isolate CF4, complete sequence	Schnell *et al*. (2001a)	AF229828.1
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, variant ls11-6.	Ambros *et al*. (1999)	AJ241849
Eggplant latent viroid complete genome, isolate 2	Fadda *et al* (2003)	NC_039241

4.3 Results

4.3.1 Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) has been defined as a more sensitive, faster and reliable hybridization method allowing the detection of variations between samples (Bar-Joseph *et al*., 1986). Using this technique, in this study, 103 trees were diagnosed as positive from a population of 453 young ‘Fuerte’ trees. Therefore, 22.7% of the population was ASBVd positive

symptomless carrier trees that were growing normally and looking healthy. However, concentration levels varied between low, medium and high on different trees.

4.3.3 Reverse transcription-polymerase chain reaction (RT-PCR)

Complete ASBVd genomes cannot be amplified using an qRT-PCR, therefore, the 103 samples that tested ASBVd positive in qRT-PCR assay were amplified using a primer that yielded a 250 bp amplification product (Luttig and Manicom, 1999) in a conventional RT-PCR. The products were run through a 1% agarose gel with ethidium stain. There were only 76 samples that produced bands on the gel, even though not all of the bands were clear. The remaining 27 samples were not detected on the conventional RT-PCR, therefore, yielding false-negative results (Figure 4.1).

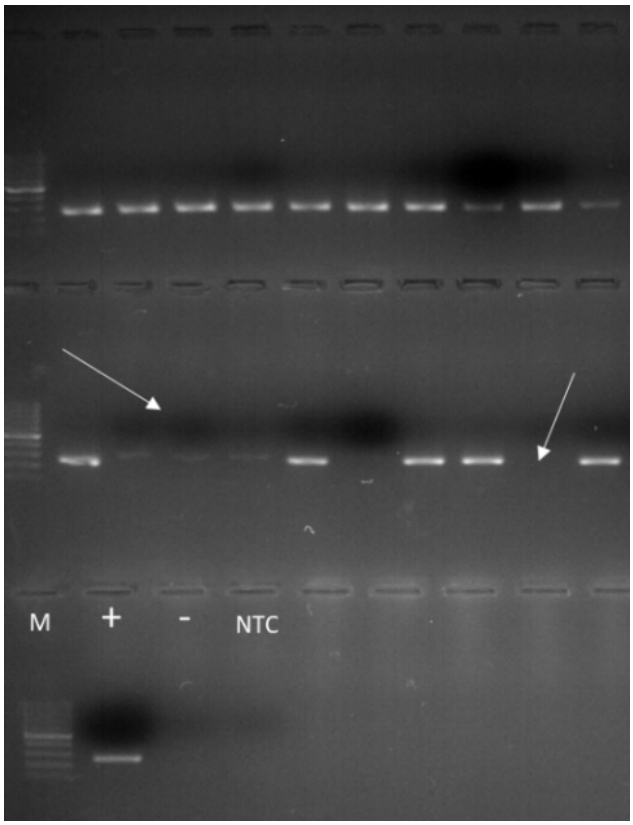

Figure 4.1 A 1% agarose gel showing different bands obtained from amplifying the positive ASBVd samples using Luttig and Manicom (1999) primer pair amplifying a 250 bp product. **M** refers to a 100bp molecular marker, **+** refers to ASBVd positive control, **-** refers to negative control and **NTC** refers to a non-template control deionized H_2O. The arrow on the left is showing products with faint bands and the right arrow points to products with the band. Other samples produced clear bands with sizes of approximately 250 bp.

4.3.4 Sequencing of ASBVd variants

The 76 products of the samples with visible bands were sent for sequencing at Inqaba Biotechnical Industries (Pty) Ltd. (Pretoria, South Africa). The primers used for sequencing amplified a 250 bp product (Luttig and Manicom 1999). Analysis of the sequences showed that sequence lengths varied between 248 and 253 nucleotides (Table 4.2). The blast results of the sequences matched the ASBVd sequences in the GenBank® database. There were 13 sequences with a length of 252 nt and 6 sequences with a 253 nucleotide lenth implying that 19 sequences had nucleotide lenths that were out of the previously defined sequence length range. The majority of the sequence lengths, however, fell well into the defined range; there were three sequences with 248 nt lengths, and seven sequences with 249 nt lengths. Most of the sequences were found between the 250 and 251 nt lengths with 24 and 23 sequences respectively (Table 4.2).

Table 4.2 Different avocado sunblotch viroid (ASBVd) variant lengths and the frequency of their occurrence detected in the population of young symptomless carrier 'Fuerte' trees.

Sequence length (nt)	Total number of sequences
248	3
249	7
250	24
251	23
252	13
253	6
TOTAL	**76**

Sequences were aligned and analysed using BioEdit. The results showed that 42 different variants were detected from 76 sequences and some of the sequences were either duplicated or repeated frequently in a population. The nucleotide sequences were then deposited into the NCBI GenBank® database and the accessions for 42 variants were obtained (Table 4.1). The variants that were duplicated included accessions ON135464, ON135462, ON135481, ON135489, ON135487 and ON135470. Sequence ON135499 was repeated three times and sequence

accessions ON135465 and ON135474 were the most occurring variants with each repeating itself 14 times in the population (Table 4.3).

Table 4.3 Frequently occurred ASBVd variants in a 'Fuerte' symptomless carrier population

Variant	Accession	Sequence length (nt)	Times repeated
Avocado sunblotch variant ScFuerte27	ON135464	248	2
Avocado sunblotch variant ScFuerte04	ON135462	249	2
Avocado sunblotch variant ScFuerte67	ON135481	251	2
Avocado sunblotch variant ScFuerte16	ON135489	252	2
Avocado sunblotch variant ScFuerte29	ON135487	253	2
Avocado sunblotch variant ScFuerte54	ON135470	250	2
Avocado sunblotch variant ScFuerte01	ON135499	249	3
Avocado sunblotch variant ScFuerte8	ON135465	250	14
Avocado sunblotch variant ScFuerte15	ON135474	251	14

A comparison of the nucleotide changes showed that the different lengths arose from the addition of the nucleotides between the residue positions 155-159, the more nucleotides added in these positions the longer the sequence lengths (Table 4.4). There were other nucleotide exchanges in other positions as shown in table 4.4 below. Most common exchanges came from A → G, C→ T and T→ C leading to different variants of the symptomless carrier associated infections (Table 4.4).

2684 **Table 4.4** Sequence differences among ASBVd sequences isolated from the young
2685 'Fuerte' trees symptomless carriers aligned with GenBank® ® sequences

Nucleotide change	Residue position	Variant number
T → A	3, 123,	S74687.1
	124	AF229828.1
A → T	6	AF229828.1
	161	ON135473
	193	ON135472
A → G	28, 149	S74687.1
	180	ON135472, ON135470, ON135471, ON135467
	186	ON135503
	191	ON135502
	192	ON135503
C → T	105, 216	S74687.1
	160	ON135473
	193	ON135503
+TAA	119-121	S74687.1
T → C	138	ON135469, ON135492, ON135490, ON135491, ON135475, ON135476, ON135482
	143	ON135476
	150	ON135479, ON135475
	152	ON135469, ON135479, ON135466
G → A	132	ON135492
	182	ON135479
G → T	154	ON135482
G → C	197	ON135478
T → G	194	ON135503
+AA	155-156	ON135463
+AAA	155-157	ON135472, ON135470, ON135471
+AT	155-156	ON135480, ON135503
+AAT	155-157	ON135481, ON135468

+ATT	155-157	ON135499, ON135465, ON135498, ON135473, ON135466
+AATT	155-158	ON135474, ON135489, ON135493, ON135490, ON135497, ON135491, ON135475, ON135478, ON135476, ON135494, ON135495
+AATA	155-158	ON135501, ON135477
+AAATT	155-159	ON135485, ON135483
+AATTT	155-159	ON135502, ON135486
+AATTA	155-159	ON135487
+C	196	ON135484, ON135492, ON135501, ON135488, ON135489, ON135480, ON135487, ON135490, ON135491, ON135482, ON135481, ON135494, ON135495
+G	198	ON135497
	199	ON135485
	200	ON135503
+GA	197-198	ON135498
+GAT	155-157	ON135469
+GATA	155-158	ON135496
+GATT	155-158	ON135492, ON135488
+GAATT	155-159	ON135484
+GTT	155-157	ON135467
+T	155	ON135464
+TT	155-156	ON135462, ON135500
+TTTT	155-158	ON135479

The sequences were aligned to four known ASBVd GenBank®® sequences used as references, AF229828.1 (Schnell *et al*., 2001b) S73860.1, S73861.1, S74687.1 (Semancik and Szychowski, 1994, Table 4.1). The phylogenetic analysis of the variants obtained from this study showed a high nucleotide identity of 97% with the reference ASBVd variants obtained from GenBank®. However, some variants shared a low identity. Sequence accession ON135493 and ON135498 shared an nucleotide identity of 50% indicating a high diversity between the variants (Figure 4.2). These variants had the same nucleotide lengths, the differences occurred between positions

156 and 196 where there were TC and GA exchanges. Sequence accessions ON135472, ON135470 and ON135471 shared a nucleotide identity of 67%, also indicating a high sequence diversity between the variants (Figure 4.2). The latter variants all had the same length of 250 nt and there were only TA exchanges in positions 153 and 193 positions between the variants.

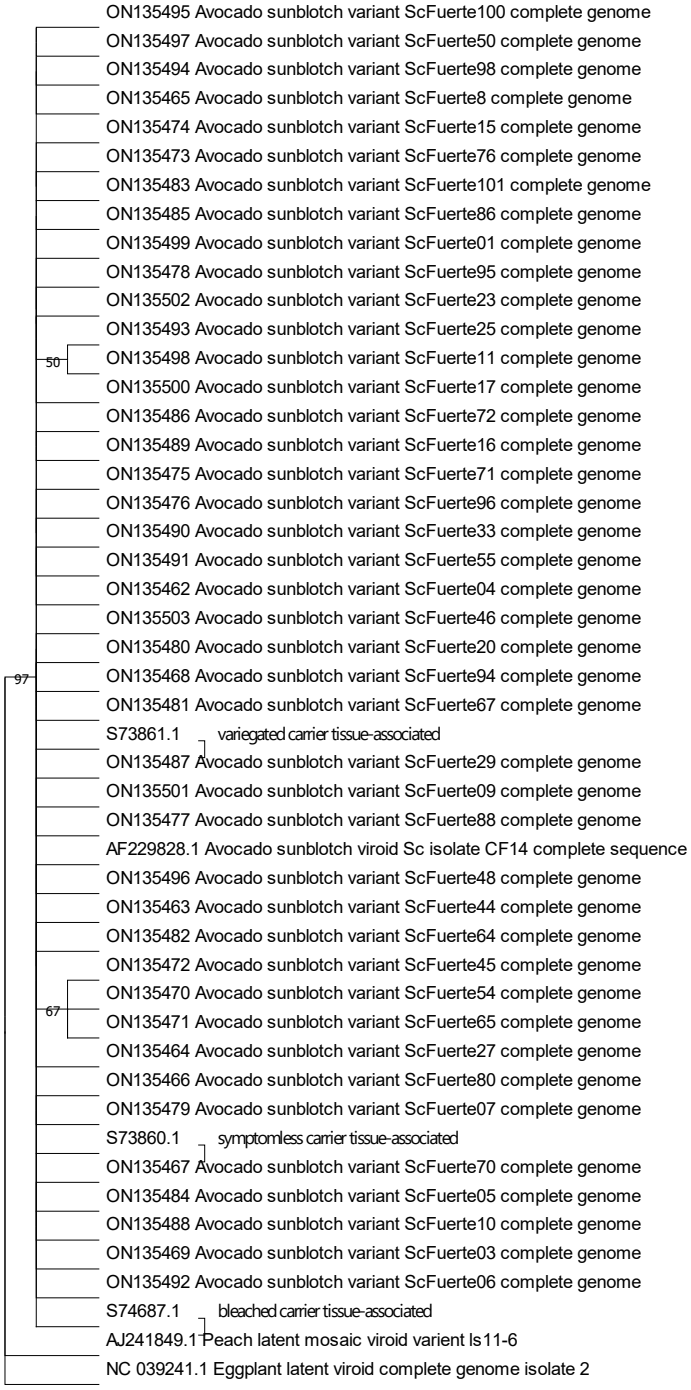

Figure 4.2 Evolutionary relationships of ASBVd variants of 'Fuerte' symptomless carrier trees. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 1.73398079

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 (AJ241849) and Egg latent mosaic viroid (NC_039241) from the family Avsunviroidae were used as an out-group.

4.4 Discussion

qRT-PCR has been defined as a more sensitive, faster and reliable detection method (Bar-Joseph *et al*., 1986). The sensitivity of the qRT-PCR has allowed the detection of low concentrations of ASBVd thus making it possible to detect ASBVd in the early stages of infection (Morey-Leon *et al*., 2018). The conventional PCR method can miss some positives when ASBVd concentrations are too low and the results can be false-negative with the formation of primer-dimers (Morey-Leon *et al*., 2018). In this study, the qRT-PCR assay detected 103 ASBVd-positive trees compared to the conventional method which only detected 76 samples Therefore, qRT-PCR was more sensitive compared to the conventional RT-PCR, however, the latter is still preferable when studying the whole genomes since the qRT-PCR only amplifies partial genomes. Moreover, the fact that 22.7% of the young 'Fuerte' trees were symptomless carrier trees growing normally and looking healthy emphasises the importance of the symptomless carrier trees in the epidemiology of ASBVd, as they have been described as the primary sources of infection in spreading the disease through budding and grafting practices (Saucedo Carabez *et al*., 2019).

According to literature, ASBVd forms a small rod-like secondary structure between position 246 and 251 nt (Saucedo-Carabez *et al*., 2019). In this study, sequences detected had lengths that varied between 248 and 253 nt. The most commonly occurring variants were between the lengths of 250 and 251 nt implying that the frequently replicated variants (Schnell *et al*., 2001a) are between these two lengths. Therefore, in a population of 'Fuerte trees, it is most likely to find variants between 250 and 251 nt in size. Moreover, the variants occuring repeatedly were also between the 250 and 251 nt in length . Two variants occured repeatedly 14 times each and both had lengths of 250 and 251 nt, respectively. From a total of 76 sequences, 13 accessions had sequence lengths of 252 nt and only six accessions had sequence lengths of 253 nt. These two seqeunce lengths have not been described before and in this study we confirmed the presence of these sequences in the 'Fuerte' population.

Based on our results, it is more likely to find ASBVd variants with a length of 252 nt compared to 253 nt in the population of ‘Fuerte’ avocado trees.

The ASBVd variants are associated with different symptoms they induce in the avocado host. The symptom-associated variants include a symptomless carrier (ASBVd-Sc), a bleached symptom associated (ASBVd-B) and variegated symptom associated (ASBVd-V) variants (Semancik and Szychowski, 1994). Different ASBVd variants arise from slight sequence variations on the ASBVd sequence (Running *et al*., 1996). Most of the changes occur between U-A bases leading to sequence variations (Shnell *et al*., 1997). Schnell *et al*. (2001b) conducted a similar study; however, they focused on the sequence diversity among avocado sunblotch viroids isolated from single avocado trees. These researchers found that a single infected tree could have more than one ASBVd variant; it can contain a set of unique variants made of unique assemblages of the most common sites for mutation. In this study, we also report similar results; where there were six different sequence lengths (nt) detected in the young population of ‘Fuerte’ cultivar trees. The variants also had minor changes between their nucleotides and most changes occurred in similar positions. The majority of the detected sequences were clustered with known ASBVd variants. The low identities were found between sequences that had the T-A exchanges implying that these changes are the most important in the ASBVd variants.

A similar study was conducted by Semancik and Szychowski (1994), where they discovered that the most common initial symptoms to appear in the foliar had a bleached appearance. This was supported in the current study where we found most of the highest identity was between the bleached carrier tissue associated variants and most of the mutations were found within the variegated and symptomless carrier tissue associated variants. Semancik and Szychowski (1994) also described that a single leaf could develop a mix of bleached, variegated and symptomless symptoms0 and that the symptomless part of the tissue that developed variegated symptoms was found to have lower ASBVd concentrations which were insufficient for the identification of ASBVd. Because all the trees were symptomless in this study, symptomless carrier variants were able to replicate and dominate the population and still produce sufficient concentrations to be detected.

The current findings are crucial for the selection of primers in the future; the current primers would be aligned to the different variants of symptomless carrier variants detected in the current experiment. Changes in the nucleotides of the ASBVd viroid also play a crucial role in the expression of the viroid in different plants. Some have lower, and medium and others have higher viroid concentrations. This also affects the yield and the quality of trees with different infection levels.

4.5 Conclusions

There is a wide range of variants associated with the symptomless carriers, however, they remain detectable with the current detection techniques. Symptomless carrier trees are the most prevalent in South African orchards, and therefore indexing all propagation material is important for the prevention and effective management of ASBVd in the orchards. Knowledge of ASBVd variation, especially as illustrated in this study from clonal 'Fuerte' plants, is crucial for the development of accurate detection techniques of ASBVd and contributes to the ASBVd action plan goals that promote the removal of all infected material from avocado orchards to prevent the further spread of the viroid. The variants deposited to the NCBI GenBank® will contribute to adding new information regarding ASBVd variation.

4.6 References

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CHAPTER 5

General overview

5.1 Major findings

Differences were observed between ASBVd infected and healthy trees; medium and highly infected trees excessively produced flowers, lost leaves during flowering and ultimately bore few to no fruit at the end of the season. The dry matter content results showed that avocado sunblotch viroid (ASBVd) did not affect the time to maturity of the fruit; fruit from infected and healthy trees matured at the same time. Yield measurements indicated that medium and highly infected trees produced between 83% and 96% lower yields compared to healthy trees. Postharvest studies showed that medium and highly infected fruit significantly lost firmness and developed colour more rapidly than healthy fruit. Infected fruit also developed external rots and shrivels for non-stored fruit, however, these injuries were reduced for fruit stored at 5^0 C for 28 days.

Graft transmission was demonstrated by grafting 30 healthy and 30 infected 'Hass' scions onto a healthy 'Bounty' rootstock. There was a 53% grafting success rate for the infected symptomless carrier trees compared to the 43.3% for the healthy trees. This implies a 53% transmission success rate of ASBVd when grafting. Binding of ASBVd to different surfaces was demonstrated for all treatments. The treatment of plant sap macerated in water will be the closest to simulating the use of metal pruning equipment in the field. After 24 hours, the ASBVd particles remained intact on the metal surface. The finding demonstrate the importance of sanitation practices in field and in nurseries. ASBVd was successfully detected on the pollen and bees from all three sampled sites. The phylogenetic analysis of detected viroid genomes gave a 93% nt identity between them and the existing ASBVd variants retrieved from the GenBank® database.

There were a total of 103 positive symptomless carrier trees detected using a real-time qRT-PCR , from a population of 453 young 'Fuerte' trees which translated to 22% of this population being infected but still growing and looking like normal healthy trees. Seventy six samples were sequenced and analysed. The samples had sequence lengths ranging between 248 and 253 nucleotides (nt). Blast analysis showed that all the sequences were ASBVd variants. From the 76 sequences, only 42 distinct variants

were identified with the remaining sequences being repeated several times in the population. The variants were deposited into the NCBI GenBank® database and were given accession numbers ON135462 to ON135503. Phylogenetic analysis of the variants obtained from this study showed a high nt identity of 97% with the reference ASBVd variants obtained from GenBank® .

5.2 Implications of findings

The qRT-PCR was more sensitive in detecting ASBVd compared to the conventional RT-PCR, however, the latter is still preferable when studying the whole genomes since the qRT-PCR only amplifies the partial genome. Moreover, the fact that 22.7% of the young 'Fuerte' trees were symptomless carrier trees growing normally and looking healthy emphasises the importance of the symptomless carrier trees in the epidemiology of ASBVd as these trees have been described as the primary sources of infection in spreading the disease through budding and grafting practices (Saucedo Carabez *et al*., 2019). The infected symptomless carrier trees produce significantly lower yields and should be removed from the orchards because they contribute to the transmisiion of ASBVD to the healthy trees.

Graft transmission (top-work) was demonstrated to be the most important transmission method for the ASBVd in South African orchards. ASBVd was found to thrive up to 24 hours on metal surfaces, this emphisises the importance of sanitation whenpruning trees because there is a possibility to spread ASBVd to healthy trees. The honey bees are carriers of ASBVd and play an important role in pollen transmission.

ASBVd forms a small rod-like secondary structure between 246 and 251 nt in length (Saucedo-Carabez *et al*., 2019). In the current study, ASBVd sequences with nt lenghts ranging between 248 and 251 were described with the most sequences ranging between 250 and 251 nt. The two sequence lengths, 252 and 253 nt, have not been described previously. However, it is more likely to find variants with a size of 252 nt compared to 253 nt in the population of 'Fuerte' avocado trees.

5.3 Way forward

Prevention is better than cure; however, this does not apply to ASBVd because there is no cure. The indexing of all propagation material should be emphasised especially to all nurserymen for all avocado trees that are used for propagation regardless of the physical symptoms. Molecular indexing should be a standard practise because

symptomless carrier trees behave exactly like healthy trees in their early development stages. The symptomless carrier ‘Hass’ trees are overbearing; they produce a lot of flowers only to lose them at the end of the season, ending up producing lower yields. These observations can be incorporated as part of ASBD management strategies in 'Hass' orchards. Further, the information can be used to conduct similar research on other avocado cultivars. The removal of the symptomless carrier trees is crucial. In the current study 42 new variants were identified in a single population of trees that would have been used to establish a new orchard if they were not identified through indexing. This alone demonstrates that the source plants for propagation was the symptomless carrier trees that went unnoticed and undetected in the field and were mistaken for healthy trees. It is, therefore, crucial to remove all infected trees, even if they have low infection levels. These trees are, however, very complicated because it was demonstrated in the current study that they produce a significant yield. Therefore, it is highly recommended to establish programs for regular scouting for symptoms and to mark and remove ASBVd-infected trees from orchards.

An extensive study on testing pollen transmission is required to verify that pollen transmission is only confined in the fruit. There were no measures taken to prevent the leaves from brushing each other. This is an important point that was overlooked and will be considered in the future when conducting similar research on root grafting. The viroid stability experiment will be extended longer in future (Month). Illumina sequencing or TA cloning of the PCR amplicons would give more insight to the sequence population. Moreover. Modelling the secondary structures of some of the ASBVd sequence variants would show the effect of mutations at certain positions in the secondary structure

5.4 References

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Conferences to date

Zwane, Z. R, Gubba, A., and Jooste, A.E.C. The effect of avocado sunblotch disease (ASBD) on tree morphology, fruit maturity, yield and quality of 'Hass' avocado in South Africa. ARC-TSC postgraduate Open Day 2021

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Research outputs

An unpublished manuscript (Chapter 2) was submitted to the European Journal of Plant Pathology, titled "The effect of Avocado sunblotch disease (ASBD) on tree morphology, fruit maturity, yield and quality of 'Hass' avocado in South Africa" EJPP-D-22-00234

The sequencing results of the study (Chapter 4) on Genetic differences between Avocado sunblotch viroid (ASBVd) variants detected in a clonal population of young, symptomless 'Fuerte' trees detected 42 new variants that were deposited in the NCBI GenBank® database (Table 5.1)

Table 5.1 Accession numbers for variants obtained in the current study with NCBI GenBank® used in phylogenetic studies

Sequence	Reference	Accession
Avocado sunblotch variant ScFuerte04	Zwane *et al*. (2022)	ON135462
Avocado sunblotch variant ScFuerte44	Zwane *et al*. (2022)	ON135463
Avocado sunblotch variant ScFuerte27	Zwane *et al*. (2022)	ON135464
Avocado sunblotch variant ScFuerte8	Zwane *et al*. (2022)	ON135465
Avocado sunblotch variant ScFuerte80	Zwane *et al*. (2022)	ON135466
Avocado sunblotch variant ScFuerte70	Zwane *et al*. (2022)	ON135467
Avocado sunblotch variant ScFuerte94	Zwane *et al*. (2022)	ON135468
Avocado sunblotch variant ScFuerte03	Zwane *et al*. (2022)	ON135469

Avocado sunblotch variant ScFuerte54	Zwane *et al*. (2022)	ON135470
Avocado sunblotch variant ScFuerte65	Zwane *et al*. (2022)	ON135471
Avocado sunblotch variant ScFuerte45	Zwane *et al*. (2022)	ON135472
Avocado sunblotch variant ScFuerte76	Zwane *et al*. (2022)	ON135473
Avocado sunblotch variant ScFuerte15	Zwane *et al*. (2022)	ON135474
Avocado sunblotch variant ScFuerte71	Zwane *et al*. (2022)	ON135475
Avocado sunblotch variant ScFuerte96	Zwane *et al*. (2022)	ON135476
Avocado sunblotch variant ScFuerte88	Zwane *et al*. (2022)	ON135477
Avocado sunblotch variant ScFuerte95	Zwane *et al*. (2022)	ON135478
Avocado sunblotch variant ScFuerte07	Zwane *et al*. (2022)	ON135479
Avocado sunblotch variant ScFuerte20	Zwane *et al*. (2022)	ON135480
Avocado sunblotch variant ScFuerte67	Zwane *et al*. (2022)	ON135481
Avocado sunblotch variant ScFuerte64	Zwane *et al*. (2022)	ON135482
Avocado sunblotch variant ScFuerte101	Zwane *et al*. (2022)	ON135483
Avocado sunblotch variant ScFuerte05	Zwane *et al*. (2022)	ON135484
Avocado sunblotch variant ScFuerte86	Zwane *et al*. (2022)	ON135485
Avocado sunblotch variant ScFuerte72	Zwane *et al*. (2022)	ON135486
Avocado sunblotch variant ScFuerte29	Zwane *et al*. (2022)	ON135487
Avocado sunblotch variant ScFuerte10	Zwane *et al*. (2022)	ON135488
Avocado sunblotch variant ScFuerte16	Zwane *et al*. (2022)	ON135489
Avocado sunblotch variant ScFuerte33	Zwane *et al*. (2022)	ON135490
Avocado sunblotch variant ScFuerte55	Zwane *et al*. (2022)	ON135491
Avocado sunblotch variant ScFuerte06	Zwane *et al*. (2022)	ON135492
Avocado sunblotch variant ScFuerte25	Zwane *et al*. (2022)	ON135493
Avocado sunblotch variant ScFuerte98	Zwane *et al*. (2022)	ON135494
Avocado sunblotch variant ScFuerte100	Zwane *et al*. (2022)	ON135495
Avocado sunblotch variant ScFuerte48	Zwane *et al*. (2022)	ON135496
Avocado sunblotch variant ScFuerte50	Zwane *et al*. (2022)	ON135497
Avocado sunblotch variant ScFuerte11	Zwane *et al*. (2022)	ON135498
Avocado sunblotch variant ScFuerte01	Zwane *et al*. (2022)	ON135499
Avocado sunblotch variant ScFuerte17	Zwane *et al*. (2022)	ON135500
Avocado sunblotch variant ScFuerte09	Zwane *et al*. (2022)	ON135501
Avocado sunblotch variant ScFuerte23	Zwane *et al*. (2022)	ON135502

Avocado sunblotch variant ScFuerte46	Zwane *et al*. (2022)	ON135503

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