Genetic structure analysis and assessment of susceptibility to *Fusarium euwallaceae* of popular avocado cultivars

by

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Declaration

I, Raven Wienk, hereby declare that this thesis, which I hereby submit for the degree Master of Science in Microbiology at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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Ethics statement

The author, whose name appears on the title page of this dissertation/thesis, has obtained, for the research described in this work, the applicable research ethics approval.

The author declares that she has observed the ethical standards required in terms of the University of Pretoria's Code of Ethics for Researchers and the Policy guidelines for responsible research.

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Preface

Avocado is an important economical crop, with breeding cycles of more than 20 years to produce improved individuals. The utilisation of molecular marker technologies can aid in speeding up breeding cycles by providing insights into the mechanisms underlying improved performance against biotic and abiotic stressors. An emerging biotic threat to the avocado industry is the Polyphagous Shot Hole Borer (PSHB) and its fungal symbiont *Fusarium euwallaceae*. Currently there is scarce information available regarding the resistance capacity of local cultivars to this pest-pathogen complex. Additionally, no genetic marker information or genetic population studies have been performed on germplasms available in South Africa, thus, hindering the selection of individuals with valuable characteristics.

Chapter 1 of this thesis presents a review of the literature on the study of avocado populations using molecular markers and the emergence of new threats to the industry in South Africa. In this review, the basics of avocado are discussed including the origin of available varieties, their propagation and the current breeding methods used in the selection of improved genotypes. This is followed by a discussion of using molecular markers in avocado population management. Lastly, the common pathogens and pests affecting avocado and the emerging threat of the PSHB and its fungal symbiont *F. euwallaceae* to the South African avocado industry are discussed. .

Chapter 2 evaluates an avocado fruiting cultivar breeding population in South Africa using single nucleotide polymorphism (SNPs) markers. This evaluation includes the identification of mislabelled samples, as well as, the validation of clonal material. The population was also assessed to verify horticultural variety and determine genetic diversity and population structure.

Chapter 3 involves the assessment of popular avocado cultivars' susceptibility towards *F. euwallaceae*, firstly, using a detached branch pathogenicity trial involving lesion measurements, and secondly, using a plantlet pathogenicity trial involving a real-time Polymerase Chain Reaction (PCR) approach to assign susceptibility or tolerance status.

Chapter 4 reflects on the outcomes of this project and includes recommendations that may be of interest for future research.

List of abbreviations

APA	Affinity propagation analysis
AFLP	Amplified fragment length polymorphism
DAPC	Discriminant analysis of principal components
DFPs	DNA fingerprints
DNA	Deoxyribonucleic acid
MAS	Marker-assisted selection
PCA	Principal component analysis
PCR	Polymerase chain reaction
SNPs	Single nucleotide polymorphisms
SSRs	Simple sequence repeats
RAPDs	Randomly amplified polymorphic DNAs
RFLP	Restriction fragment length polymorphism
RFU	Relative fluorescence unit
VNTR	Variable number of tandem repeats

Standard units of measure and symbols

°C	Degrees Celsius
h	Hours
L	Litre
µl	Microliter
µM	Micromolar
mg	Milligram
ml	Millilitre
mM	Millimolar
mm	Millimetre
min	Minutes
ng/µl	Nanograms per microliter
%	Percentage
sec	Seconds

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CHAPTER 1 Molecular markers and breeding practices used against threats in the avocado industry

1.1 **Introduction**

Persea americana Mill. (avocado) is an important economical crop and, like most members of the Lauraceae family, grows in tropical or subtropical climates (Bergh & Ellstrand, 1986). Avocado consists of three main botanical varieties, *P. americana* var. *guatemalensis* Williams. ('Guatemalan'), *P. americana* var. *drymifolia* Blake. ('Mexican') and *P. americana* var. *americana* Mill. ('West Indian') (Lahav & Lavi, 2002, Scora *et al.*, 2002). These varieties are vegetatively propagated through grafting, thereby allowing growers to select valuable traits such as improved fruit yield and/or quality and resistance to pathogens and pests (Lewis & Alexander, 2008) in chimeric trees. The prolonged juvenile period of avocado trees makes for a long breeding cycle of new cultivars and rootstocks, commonly extending over 20 years. During breeding cycles phenotypic evaluations can take several years, including fruit formation and other fruit quality traits, such as flowering density and timing, fruit shape, and fruit maturation (Schaffer *et al.*, 2013). After traits have been evaluated, avocado breeders can make informed selections to yield improved cultivars for cultivation. Unfortunately these breeding practices make avocado breeding programmes costly and time-consuming (Köhne, 2005). Ongoing climate change and an increase in emerging pests and pathogens are expected to continue, therefore, making the improvement of avocado cultivars an invaluable resource to the industry (Hardham & Blackman, 2018).

To speed up the time consuming practices of traditional breeding programmes, plant breeders have begun to utilise molecular marker technology to perform large-scale analyses, in determining genetic diversity, identifying botanical varieties and selecting

resistant individuals (Batley, 2015). Microsatellite markers have been used to clarify genetic relationships among individuals in avocado germplasms (Lavi *et al.*, 1993, Mhameed *et al.*, 1997, Schnell *et al.*, 2003). More recently, single nucleotide polymorphisms (SNPs) were identified in avocado genomes and were used to further clarify genetic relationships and determine genetic diversity among individuals (Ge *et al.*, 2019a). Kuhn *et al.* (2019c) designed a 6000 SNP Infinium II chip to potentially aid in genetic mapping, this was further reduced to 384 SNPs to evaluate avocado populations (Kuhn *et al.*, 2019b). The utilization of molecular marker technologies, specifically SNPs, are proposed to be of great value to the avocado industry, since they are currently the best markers for breeding, due to their abundance in the genome and their potential to genetically differentiate cultivars that are resistant/tolerant to abiotic and biotic stresses (Batley, 2015).

The emergence of new biotic stresses, which pose a threat to avocado include the root pathogen *Rosellinia necatrix* Berl. ex Prill (van den Berg *et al.*, 2018, Zumaquero *et al.*, 2019) as well as the Polyphagous Shot Hole Borer (PSHB) and its fungal symbiont *Fusarium euwallaceae* Freeman (Eskalen *et al.*, 2013, Freeman *et al.*, 2013, van den Berg *et al.*, 2018). The PSHB has a wide host range, affecting over 335 tree species in 58 plant families (Eskalen *et al.*, 2013). The beetle and its symbiont have been found in Israel (Mendel *et al.*, 2012) and the United States of America (California) as an invasive pest (Eskalen *et al.*, 2013), where it has caused significant damage in avocado orchards, resulting in severe economic losses within the avocado industry (Eskalen *et al.*, 2013). *F. euwallaceae* is introduced into the host by the beetle, after which the fungus invades the vascular tissue of the tree, preventing the transport of water and nutrients, thus causing Fusarium dieback and death of infected juvenile and mature trees (Mendel *et al.*, 2012,

Eskalen *et al.*, 2013). Recently, this pest-pathogen complex was detected on London plane trees in South Africa (Paap *et al.*, 2018), on a backyard avocado tree (van den Berg *et al.*, 2018), and it has been observed in a commercial orchard in 2021 (Unpublished data). This presents an alarming threat to the avocado industry in this country.

The South African avocado industry relies heavily on resistant/tolerant rootstocks and cultivars to manage potential diseases, but phenotypic information on local cultivars' resistance to PSHB and its fungal symbiont is currently lacking. Additionally, very few genetic population studies have been performed on germplasm in local breeding programmes, hampering the selection and breeding of individuals with valuable characteristics, specifically resistance to biotic stresses. In this review, avocado varieties and their propagation will be discussed, together with genetic population studies using molecular markers towards the selection of improved genotypes. In addition, the most devastating pathogens and pests affecting the avocado industry, particularly in South Africa, and the increasing threat of PSHB and its fungal symbiont *F. euwallaceae* will be reviewed.

1.2 **Avocado varieties & hybrids**

Avocado species are highly polymorphic with several different growing forms occurring within the same species, that arose from the *Persea* genus that originated in Central America to the central and eastern highlands of Mexico, including Guatemala (Popenoe, 1920), about 35 to 56 million years ago during the Tertiary (Eocene period) (Schaffer *et al.*, 2013). The *Persea* genus contains about 150 species, including avocado "*americana*", *P. borbonia*, *P. schiedeana*, and *P. indica* (Bergh & Ellstrand, 1986). *Persea* can be divided into two main subgenera, *P. americana* and *Eriodaphne* (Bergh & Ellstrand, 1986). These two subgenera are distinct from each other, which has limited hybridization attempts, *Eriodaphne* contains a few species that are completely resistant to Phytophthora Root Rot (PRR) – a devastating avocado disease caused by *Phytophthora cinnamomi* Rands - but grafting of these species with *P. americana* has failed (Bergh & Ellstrand, 1986).

1.2.1 The three varieties & hybrid cultivars

Avocado comes mainly from three landraces or 'varieties' (Lahav & Lavi, 2002, Scora *et al.*, 2002), *P. americana* var. *americana* ('West Indian'), *P. americana* var. *guatemalensis* ('Guatemalan') and *P. americana* var. *drymifolia* ('Mexican') (Wolstenholme, 2003). These landraces were possibly domesticated at least 5000 BC by Mesoamerican groups (Smith, 1966). Each variety has its own preferred growing conditions and possesses unique characteristics, like taste and shape of the fruit, and resistance against biotic and tolerances of abiotic stresses (Williams, 1977, Bergh & Ellstrand, 1986, Knight & Robert, 1999). The lack of reproductive barriers between varieties has led to extensive hybridization with

varying agronomical traits (Davis *et al.*, 1998, Ashworth & Clegg, 2003). Varieties with desirable traits are the result of the exploration, collection, evaluation and conservation of germplasms (Karp *et al.*, 1997). Germplasms are a valuable genetic resource for breeding and selection programmes for the development of new rootstocks and scion cultivars (Karp *et al.*, 1997). Most commercial avocado rootstocks and cultivars are hybrids (Popenoe & Williams, 1947). Hybrids can have phenotypic similarities, thus, making it difficult to trace the variety background (Popenoe & Williams, 1947), and as genetically related hybrids are easily crossed causing hybrid breakdown and inbreeding depression, hybrid uncertainty undermines breeding programmes' ability to develop improved and unique individuals.

***P. americana* var. *guatemalensis* - Guatemalan**

The Guatemalan variety of avocado has the highest fruit averages and horticultural quality in comparison to the other varieties, with a thick rough skin, small seed and round shape (Bergh & Ellstrand, 1986). This variety has the advantage of maturing later which provides a later harvesting season and therefore, bridges the maturity gap between the earlier hybrid varieties, and allows a year-round harvest (Bergh & Ellstrand, 1986). Guatemalan varieties are more sensitive to time-induced chlorosis, salt, heat, Dothiorella canker and, Verticillium wilt (Ben-Ya'acov & Michelson, 1995).

***P. americana* var. *drymifolia* - Mexican**

The Mexican variety has a thin waxy skin and large seed, with fruit size being elongated and generally smaller than commercially desirable (Bergh & Ellstrand, 1986). This variety is mainly used for rootstocks and hybridization, where it is used to produce hybrids which increase season harvest periods (Bergh & Ellstrand, 1986). The Mexican variety has reduced tolerance with regards to salinity, though it is more tolerant of poorly aerated soil,

cold, Dothiorella canker, and Verticillium wilt (Ben-Ya'acov & Michelson, 1995). This variety has shown some tolerance towards cold and resistance to *P. cinnamomi* (Sánchez-González *et al.*, 2019). It is difficult to morphologically differentiate between the Mexican and Guatemalan varieties in less tropical areas (Williams, 1977).

***P. americana* var. *americana* - West-Indian**

The West Indian variety has a thin, shiny skin and elongated shape with an early harvest period and hybrids with the Guatemalan variety bridges the harvesting gap in tropical regions (Bergh & Ellstrand, 1986). Additionally, this variety is the most tolerant to salinity, calcareous soil and heat. It does, however, have a low tolerance for poorly aerated soil and cold (Ben-Ya'acov & Michelson, 1995). The West-Indian variety and West Indian x Guatemalan hybrids are highly susceptible to Laurel wilt caused by *Raffaelea lauricola* T.C. Harr. Fraedrich and Aghayeva (Ploetz *et al.*, 2012). The West-Indian varieties share similar fruit characteristics with the Mexican variety, but are dissimilar in climatic adaptation (Williams, 1977).

1.3 **Avocado propagation and selection**

Throughout history farmers have tried to improve their orchards and crops by choosing and keeping the most productive or desirable individuals (Lewis & Alexander, 2008). There are two major strategies for plant breeding; classical and molecular methods (He *et al.*, 2014). Classical breeding entails the intentional breeding of two members of a species, each of which possesses one or more different and desirable traits and recombining these traits into a single offspring. This type of breeding includes methods such as crossing and backcrossing (He *et al.*, 2014), followed by asexual propagation of desirable genotypes by grafting (Lewis & Alexander, 2008). Molecular breeding, in contrast, exploits biotechnology and biological tools that target biomarkers such as marker-assisted selection (MAS) where individuals are selected based on their genetic makeup rather than on phenotype alone (Moose & Mumm, 2008).

Grafting involves the cutting of a shoot from the original plant (called the scion – fruit bearing) and attaching it to another plant (rootstock – root system), thus, uniting the plants and allowing them to grow as one (Lewis & Alexander, 2008). The new plant grows from a fragment of the parent plant and is genetically identical, which maintains all of the characteristics for which the variety was selected (Lewis & Alexander, 2008). Grafting connects genetically distinct individuals to form genetic chimeras that can improve crops, by conferring unique traits to both below and above ground components of the plant (Mudge, 2009). Grafted plants are selected for valuable characteristics, such as improved fruit yield, better fruit quality, desirable form and resistance/tolerance to biotic factors, such as soil-borne pathogens (oomycetes, fungi and nematodes), as well as abiotic factors

(soil type, drought, salinity and waterlogging) (Wolstenholme, 2003, Lewis & Alexander, 2008).

Avocados are clonally propagated through grafting (Frolich & Platt, 1972) using any of the splice, wedge, bark graft, or side graft techniques (Lewis & Alexander, 2008). The avocado industry uses clonally propagated rootstocks and scions (Roe *et al.*, 1999, Wolstenholme, 2003, Köhne, 2005, Vorster, 2005, Kremer-Köhne & Köhne, 2007), to achieve a combination of desired traits, which are usually in opposite selective directions, such as root rot tolerance (rootstocks) and high productivity (scions) (Roe *et al.*, 1995). Vegetatively propagated avocado rootstocks may impact the physical characteristics of the grafted cultivar by overcoming problems with dwarfing, precocity, productivity (flowering, fruit set, fruit weight, overall yield), shoot pest/pathogen resistance and fruit quality (texture, sugar and nutrient content, weight, flavour and colour) (Köhne, 2005, Warschefsky *et al.*, 2016). The main selection criteria for rootstocks in the South African avocado industry are based primarily on resistance or tolerance to PRR, salinity and calcareous soils (Ben-Ya'acov & Michelson, 1995, Köhne, 2005, Kremer-Köhne & Köhne, 2007, Warschefsky *et al.*, 2016). Currently, the avocado rootstocks of choice are ‘Dusa®’, ‘Duke 7’, and ‘Latas’ (Schaffer *et al.*, 2013). The main criteria for avocado cultivar selection in South Africa is based on the production of high yields, the size, shape and flavour of the fruits, as well as genetic resistance to fruit diseases (Arpaia & Smith, 2004). The most popular cultivars produced in South Africa are ‘Hass’, ‘Carmen-Hass’, ‘Fuerte’, ‘Pinkerton’, and ‘Gwen’ (Schaffer *et al.*, 2013). Another cultivar gaining popularity is ‘Maluma®’, which outperforms ‘Hass’ on production, fruit size, precocity and distribution (Ernst, 2007).

1.4 **Avocado breeding programmes**

Avocado has a concurrent dichogamous system which enforces outcrossing as the pistils and stamens of the plant mature at different times (Davenport, 1986). Avocado has high levels of heterozygosity which produces unpredictable hybrids, where evaluations require several years and utilizes large areas (Lahav & Lavi, 2002). Breeding programmes rely heavily on identifying self-pollinated and/or mislabelled seedlings (Kuhn *et al.*, 2019c), and screen for PRR resistance (Köhne, 2005), before further resources are expended on undesirable, insignificant or susceptible individuals. Avocado breeding and the development of new cultivars and rootstocks can extend over 20 years due to the protracted juvenile phase (Köhne, 2005), which involves the flowering and fruiting age ranging from 3 to 11 years (up to 14 years) (Lavi *et al.*, 1992), and fruit maturity taking approximately 18 months (Schaffer *et al.*, 2013). The juvenile period in avocado can be significantly shortened by choice of parents (Lavi *et al.*, 1992). Consequently, any innovations that reduce cost and time of breeding, or accelerates and improves breeding efficiency, are therefore especially valuable to the industry (Clegg, 2004).

Breeding programme - South Africa

The majority of the South African avocados are produced in the warm subtropical areas of the Limpopo and Mpumalanga provinces (Donkin, 2007). Around the mid-1920s, Dr Hans Merensky imported superior avocado grafted trees from the California, USA; they were established at Westfalia Estate in South Africa and were comprised of several Mexican, Guatemalan and hybrid cultivars (Kremer-Köhne *et al.*, 2001). In the 1960s and 1970s, South African growers favoured and selected the Guatemalan variety, although this variety was sensitive to *P. cinnamomi* (Wolstenholme, 2003). However, avocado production

continued to develop from the early 1970s, with approximately 2000 ha being expanded to over 12 000 ha by the year 2003 (Donkin, 2007).

Initially, the Institute for Tropical and Subtropical Crops rootstock selection programme screened over 40 000 seedlings for root rot tolerance during 1992 to 2000 (Bijzet, 1999). Merensky Technology Services (today known as Westfalia Technological Services – WTS™) began a rootstock selection programme in 1993, choosing mostly Mexican varieties for root rot tolerance (Wolstenholme, 2003). In 2001, the programme released the clonal rootstock 'Dusa®', which outperformed the Californian 'Duke 7' with regards to tree health, yield and PRR tolerance (Kremer-Köhne *et al.*, 2001, Wolstenholme, 2003). 'Dusa®' is of Guatemalan/Mexican origin and was selected specifically for South Africa's climate and conditions (Kremer-Köhne *et al.*, 2001) and for its tolerance to PRR, poor soil aeration in high clay areas, and high soil acidity due to high rainfall in granite and dolerite soil types (Wolstenholme, 2003). Recently, the new rootstock Leola™ was released, which exhibited healthier trees and higher-yielding orchards in difficult growth conditions while demonstrating tolerance to *P. cinnamomi* root-rot under heavy infection pressure (Bekker, 2020).

Breeding programme - USA

The breeding programmes of California in the USA selected varieties with cold tolerance, however, they showed *P. cinnamomi* sensitivity (Wolstenholme, 2003). In the 1950s, California began breeding for rootstock tolerance to *P. cinnamomi*, using mostly Mexican seedlings (Wolstenholme, 2003). The University of California at Riverside in the USA has an avocado breeding programme which focuses on breeding of root rot resistance in rootstocks and the development of molecular markers to improve germplasm identification

and preservation (Arpaia & Smith, 2004, Dixon, 2012). Some of the most popular clonal rootstocks from California with PRR tolerance have been ‘Duke 7’, ‘Thomas’, ‘Barr Duke’, and ‘D9’ (Ben-Ya'acov & Michelson, 1995, Whiley, 2002).

Traditional breeding methods have yielded significant contributions to the avocado industry, while technological advances in molecular biology and genomics have allowed for new and improved strategies for avocado breeding, as most breeding programmes have underutilized the accessible genetic variation available (Schaffer *et al.*, 2013). Molecular phylogenetics could resolve avocado taxonomy, which may complement the morphological techniques in resolving taxonomic problems and improve breeding in the future (Schaffer *et al.*, 2013).

1.5 **Genetics and breeding**

Genetic diversity is the extent and abundance of genetic variations within and between populations of organisms, as they are produced and accumulated over evolutionary time (Magurran & McGill, 2011), and sometimes these changes allow members of a species to adapt to various environmental conditions and stressors (Schleif, 1993, Ellegren & Galtier, 2016). This diversity was previously assessed on the phenotypic/morphological level, which are readily available and require minimal equipment, however, these evaluations are time-consuming to perform and require an expert in the species, unfortunately, they are expensive, their numbers are limited, and their evaluations are subjective (Karp *et al.*, 1997).

However, with advances in technology it can now be assessed on the DNA sequence level (Karp *et al.*, 1997). Modern breeding programmes and plant genetics rely heavily on the analysis of genomic variation (Deschamps *et al.*, 2012). These genetic variations cause changes in the DNA sequence, affecting the synthesis of proteins and the interactions between proteins, which in turn affect the development of observable traits, or the phenotype. Molecular markers are fragments of DNA that are associated with a certain location within the genome, and they are used to identify and record variations in individuals at the DNA level (Botstein *et al.*, 1980, He *et al.*, 2014). As all molecular markers are derived from polymorphisms in the DNA sequence, they have been successfully applied in plant science, including all large-scale genomic analyses (genome assembly, trait mapping, genetic diversity and evolutionary studies), as well as, in marker-assisted breeding for plant and crop improvement (Batley, 2015). Molecular markers can provide an alternative approach to breeding which negates the costs and constraints of phenotypic evaluations (Karp *et al.*, 1997).

1.5.1 Avocado genetic research

Utilising molecular markers can benefit the avocado industry by accelerating the genetic improvement of rootstocks and cultivars and improving the understanding of the genetic determination of complex traits (Clegg, 2004). Here, the history of avocado genetic research using molecular markers over the last few decades will be discussed.

1.5.1.1 Isozymes, RFLPs, RAPDs and Minisatellites

Torres & Bergh (1978) used three isozyme systems to analyse two groups of 'Pinkerton' seedlings to determined their rate of outcrossing. Later, Torres & Bergh (1980) analysed over 100 avocado cultivars and eight other *Perse*a species using six enzyme systems to determine isozyme variation. Three isozyme systems were used to show that the 'Hass' cultivar resulted from cross-pollination (Goldring *et al.*, 1987). Furnier *et al.* (1990) used restriction fragment length polymorphism (RFLP) markers to study the relationships between *Persea* species and avocado cultivars, which indicated that *P. americana* is not a monophyletic group. As well as, suggested that the Guatemalan variety originated as a hybrid between *P. steyermarkii* and *P. nubigena*. This study supported the classification of the Guatemalan, Mexican and West-Indian varieties as a single species of *P. americana* (Furnier *et al.*, 1990). A quantitative genetic analysis was performed on several cultivar crosses and selfed individuals using isozyme markers, which determined parentage, and the seedling progenies were assessed on eight avocado traits (Lavi *et al.*, 1991a). Lavi *et al.* (1991b) used four DNA fingerprint probes for the genetic analysis of 14 offspring from a cross between 'Pinkerton' and 'Ettinger'. The DNA fingerprint probes could distinguish between each race and help characterize relationships between races and cultivars. Bufler and Ben-Yaacov (1992) studied variation in the nuclear ribosomal DNA which demonstrated a close phylogenetic relationship between the Guatemalan and West-Indian variety and that the Mexican variety is the most distant (Ben-Ya'acov *et al.*, 1992). Additionally, a quantitative analysis of several traits was conducted to estimate the variance components and their heritability between and within avocado crosses (Lavi *et al.*, 1993). Nine quantitative traits were evaluated and showed that the additive variance was non-significant for all traits, whereas the value of the non-additive genetic variance was significantly higher for all traits (Lavi *et al.*, 1993). The significant non-additive variance

and low narrow and broad-sense heritability values indicate that the traits may be controlled more by environment than by genetic factors (Lavi *et al.*, 1993). They concluded that morphological traits in avocado are probably coded by several loci with several alleles in each locus (Lavi *et al.*, 1993). Another study by Mhameed *et al.* (1997) used variable number of tandem repeats (VNTR) markers to analyse 24 genotypes assessing the genetic relationships between *Persea* species and avocado cultivars. The phylogenetic analysis supported the morphological and racial classification of the cultivars, which allocated to three varieties, and supported the hypothesis that the Guatemalan and West-Indian varieties are closer to each other than to the Mexican variety (Mhameed *et al.*, 1997). Davis *et al.* (1998) evaluated a set of 36 avocado cultivars using RFLPs and reached a similar conclusion. This study also revealed three main clusters representing the major varieties as well as smaller clusters that represent interracial hybrids (Davis *et al.*, 1998). In another study, Fiedler *et al.* (1998) used 22 Randomly Amplified Polymorphic DNA (RAPD) markers to discriminate 16 avocado accessions to identify their horticultural variety, additionally, variety-specific markers were identified and the similarities indicated three groups of equal rank (Fiedler *et al.*, 1998).

1.5.1.2 Microsatellites

Microsatellites are very short DNA sequences repeated in tandem, which can be used in genotyping as a molecular marker (Pierce, 2012). Genetic relatedness among avocado varieties has previously been assessed using 30 microsatellite markers (Lavi *et al.*, 1994). The Mexican and the West Indian varieties seemed to be less related compared with the more closely related Mexican and Guatemalan varieties and the Guatemalan and West Indian (Lavi *et al.*, 1994). Two VNTR molecular markers, DNA fingerprints (DFPs), and

simple sequence repeats (SSRs) were used to study the mode of inheritance and estimate heterozygosity levels in avocado cultivars (Mhameed *et al.*, 1996). The average heterozygosity varied from 0.50 to 0.66 and the gene diversity varied from 0.42 to 0.66 in this study (Mhameed *et al.*, 1996). These results indicate that avocado accessions of Mexican and Guatemalan varieties are highly heterozygous and heterogenous, whereas those of the West Indian variety are more homozygous and homogenous (Mhameed *et al.*, 1996). Sharon *et al*. (1997) used 34 markers, including 25 SSRs, three RAPD markers and six DFP bands to create a crude linkage map, which is randomly dispersed in the avocado genome with 12 linkage groups covering 352.6 cM (Sharon *et al.*, 1997, Viruel *et al.*, 2007).

Ashworth & Clegg (2003) used 25 microsatellite markers to infer genealogical alignments among 35 cultivars, rootstocks, and two wild relatives of *P. americana*. This study suggests that the hybrid genotypes represent an ancient hybridization or a recent origin of the avocado varieties, due to low bootstrap support during analysis. The average heterozygosity was high (60.7%), this could differentiate between the varieties and suggested that the Guatemalan and West-Indian varieties are more closely related than to the Mexican variety (Ashworth & Clegg, 2003). Schnell *et al.* (2003) used 14 microsatellite markers to estimate the genetic diversity among 254 avocado accessions and clarified the genetic relationships between individuals within a large germplasm collection. This study also clarified the anecdotal information on parentage of mixed variety hybrids and seedling selections from commercial cultivars. Additionally, the phylogenetic analysis indicated three separate populations, Guatemalan, Mexican and West Indian varieties, with each variety being distinct and significantly different from each other. The 14 microsatellite loci had an average unbiased genetic diversity of 0.83 and the total

propagation error in the collection, was estimated to be 7.0% and the average observed heterozygosity was 0.64 (Schnell *et al.*, 2003). Alcaraz & Hormaza (2007) analysed 75 avocado accessions from different locations using 16 microsatellite loci, which classified most of them into three variety groups, however, the presence of several interracial hybrids caused low bootstrap support for some accessions. Acheampong *et al.* (2008) used 12 SSR loci to investigate 172 Ghanaian accessions, which indicated that the Ghanaian accessions are genetically more closely related to the West-Indian variety.

Douhan *et al.* (2011) used 61 amplified fragment length polymorphism (AFLP) markers to genetically characterize 83 selected cultivars and rootstocks. In this study, no obvious trends were observed based on the cluster analysis with regards to known PRR-tolerant and susceptible rootstocks. The AFLP analysis did, however, determine that there is substantial variation among the rootstocks; these results are not surprising given the phenotypic variability within avocado due to hybridization (Chen *et al.*, 2009). Gross-German & Viruel (2013) developed 47 SSR and expressed sequence tag (EST-SSR) markers to analyse 42 avocado accessions. These accessions clustered into three groups based on their varieties, and also found that the Guatemalan and Mexican varieties were more closely related to each other than either were to the West Indian variety (Gross-German & Viruel, 2013). Ferrer Pereira *et al.* (2017) investigated 49 accessions from a Venezuelan germplasm using 13 SSR loci, which could classify the accessions according to their variety composition. Guzmán *et al.* (2017) used 28 SSR loci to analyse 318 accessions from a Mexican germplasm, which could not differentiate among the three varieties, however, could classify accessions into two groups according to ecological regions. Boza *et al.* (2018) analysed 354 accessions of different *Persea* species from different locations using 55 SSR loci, which classified the *P. americana* accessions into three varieties.

Recently, 226 avocado trees sampled from eight districts in Tanzania were investigated using 10 microsatellite markers, with an average expected and observed heterozygosity of 0.84 ± 0.02 and 0.65 ± 0.04 reported, respectively (Juma *et al.*, 2020). This study revealed a lack of genetic differentiation for seven of the 28 population pairs tested with mixing of avocado trees from different districts (Juma *et al.*, 2020).

1.5.1.3 Single nucleotide polymorphisms (SNPs)

Another method which has been used to analyse genetic diversity within avocado is SNPs, which are the differences in individual nucleotides at a particular locus, which occur in at least 1% of the population (Ganal *et al.*, 2009). They are more powerful than microsatellite markers due to their widespread prevalence in the genome, they are stably inherited, bi-allelic, highly reproducible and allow for high-throughput automation (Reale *et al.*, 2006, Seeb *et al.*, 2011, Batley, 2015). SNPs are currently the best molecular markers to be applied in agriculture to perform genetic diagnostics of individuals and germplasm identification for breeding programmes (Batley, 2015). Chen *et al.* (2008) identified 176 SNPs using targeted resequencing from 21 wild avocado accessions from Mexico, Costa Rica, Ecuador and the Dominican Republic. The results showed a relatively low genetic variation in the species, with the observed heterozygosity to be 40–70.6% (Chen *et al.*, 2008). The diversity was determined to be 0.0071, which is low for an outcrossing species, and lastly, the interlocus linkage disequilibrium indicated substantial population structure, potentially induced by the admixture of the three varieties (Chen *et al.*, 2008). The follow-up study involved the same wild genotypes and markers, with an additional 33 cultivars, this revealed substantial genetic differentiation among three geographic groups of the wild germplasm that corresponded to the three varieties of avocado (Chen *et al.*, 2009).

Another study identified >250 000 polymorphic SNPs from 21 avocado individuals which clarified genetic relationships and determined genetic diversity among individuals in two avocado germplasms in China (Ge *et al.*, 2019a). These results indicated that Guatemalan-Mexican accessions and hybrids, possessed the highest genetic diversity, whereas the West Indian accessions and Guatemalan-West Indian hybrids had the lowest genetic diversity (Ge *et al.*, 2019a). Rubinstein *et al.* (2019) studied 100 accessions from the three varieties in the Israeli avocado germplasm using 192 randomly selected SNPs, these SNPs were identified by genotyping the accessions within each variety. The genetic relationships and the genetic diversity of the germplasm was evaluated using a subset of 109 SNPs, which indicated three distinct varieties (Rubinstein *et al.*, 2019).

Most recently, a study described SNPs from leaf- and flower-expressed genes using RNA sequencing from two large mapping populations and a reference transcriptome from 'Hass' involving 1339 cultivars, which identified over 660 000 SNPs (Kuhn *et al.*, 2019b). After filtering, 5050 SNPs were selected to design a Illumina SNP genotyping platform to create a high density linkage map (Kuhn *et al.*, 2019b). These researchers further reduced this to 384 SNPs and created a database that estimated genetic diversity in four germplasm collections, identified mislabelled and self-pollinated individuals and identified potential paternal parents of progeny from known maternal parents. Additionally, a minimum of 13% mislabelling in the germplasm collection was determined (Kuhn *et al.*, 2019c).

A variety of valuable crops have exploited the advances in technology to identify molecular markers that are linked to abiotic and biotic stresses (Kage *et al.*, 2016, Younis *et al.*, 2020). Numerous breeding programmes utilize these markers to develop new

varieties of crops with improved resistance to these stresses (Jain *et al.*, 2010). This technology may be used to screen seedlings for the presence of molecular markers correlated with desirable traits, thus saving time, land, labour and money (Clegg, 2004). With new threats to avocado production emerging, the use of molecular markers to correlate genotypes with resistant phenotypes is more important than ever.

1.6 **Avocado threats**

There are several significant pathogens and pests impacting avocado production throughout the world (Schaffer *et al.*, 2013). The most economically important avocado threats include Phytophthora root rot, White root rot, Laurel Wilt and most recently, Fusarium Dieback, caused by the PSHB and its fungal symbiont *F. euwallaceae*. Understanding host and pathogen interactions, specifically for avocado, is vital to the development and improvement of disease management strategies (Schaffer *et al.*, 2013).

1.6.1 Phytophthora and Rosellinia root rot

Phytophthora root rot, caused by the soil-borne pathogen *P. cinnamomi* affects more than 5000 plant species (Hardham & Blackman, 2018) and is the most serious and important threat in global avocado production. White root rot is caused by *R. necatrix,* an ascomycete pathogen that affects a wide variety of plant species (Kulshrestha *et al.*, 2014) and is a serious disease of avocado in Israel, Spain (López-Herrera *et al.*, 1998) and South Africa (van den Berg *et al.*, 2018). Both pathogens infect the fine feeder roots, disrupting the vascular cambium, which leads to wilting, chlorosis of the foliage and fruit, and eventually

the death of the host plant (ten Hoopen & Krauss, 2006, Hardham & Blackman, 2018). Currently, there is no effective control strategy for *P. cinnamomi* or *R. necatrix*, as the pathogens have a wide host range, while being able to survive in the soil and in asymptomatic or tolerant host plants (Jang & Tainter, 1991). Thus, an integrated approach is essential to enable economic production of avocado in the presence of these pathogens (Guest & Grant, 1991), which includes prevention (quarantine, nursery hygiene), cultural practices (drainage, irrigation), biological control (use of suppressive soils, bio-enhanced mulches) (Yang *et al.*, 2001), chemical control (phosphonate injections (Guest & Grant, 1991, Hardy *et al.*, 2001) and fluazinam (Ruano-Rosa *et al.*, 2018), respectively) and breeding for rootstock tolerance/resistance (Garbaye, 1991).

1.6.2 Redbay Ambrosia Beetle (RAB) causing Laurel wilt

A devastating pest of avocado is an ambrosia beetle (*Xyleborus glabratus* Einhoff), also known as the Redbay Ambrosia Beetle (RAB), which is a vector of *R. lauricola*, an ascomycete systemic virulent pathogen, which causes Laurel wilt (LW) (Fraedrich *et al.*, 2008). As the beetle penetrates the tree, its fungal symbiont is deposited and develops in the brood galleries, which acts as a food source for the offspring and adult beetles (Ploetz *et al.*, 2017). The fungal pathogen invades the xylem and disrupts the transport of water, causing the development of initial symptoms such as leaf discoloration, wilting, defoliation, and finally the death of the host tree (Ploetz *et al.*, 2017). This pest pathogen complex originated in Asia (Fraedrich *et al.*, 2008) and has become a significant problem for the avocado industry in the USA (Mayfield *et al.*, 2008). Limitations to the pest-pathogen management in avocado includes the current lack of information about the biology of *X. glabratus*, and the lack of fungicides and pesticides. However, cultural

practices such as the restricted movement of firewood into avocado production areas in Florida, as well as the chipping of infected wood has shown promise in reducing the spread of this pest-pathogen complex (Ploetz *et al.*, 2017). Currently, the presence of the pest-pathogen complex has not been detected in South Africa.

1.6.3 Polyphagous Shot Hole Borer (PSHB) and its fungal symbiont *Fusarium euwallaceae*

An ambrosia beetle (Coleoptera: Curculionidae: Scolytinae), commonly known as PSHB and its fungal symbiont *F. euwallaceae*, has emerged in Israel (Mendel *et al.*, 2012) and the California, USA, as an invasive pest (Eskalen *et al.*, 2013). This complex has been shown to cause Fusarium dieback on a variety of over 350 plant species, from over 50 families (Eskalen *et al.*, 2013). High levels of mortality, significant damage and economic losses have occurred in these countries due to the invasion, affecting urban landscapes, forests, agricultural and ornamental trees with severe damage to the avocado industry (Eskalen *et al.*, 2013).

Xyleborini are a tribe of ambrosia beetles of the subfamily Scolytinae, which cultivate ambrosial fungal symbionts that concentrate nutrients from their woody hosts. *Euwallacea* is the only genus known to cultivate fusaria fungi to date (Beaver, 1989). *Euwallacea* is a genus of over 40 species of Xyleborini (Kasson *et al.*, 2013). Scolytine ambrosia beetles have a mutualistic relationship with a specific fungus, *F. euwallaceae* (Kasson *et al.*, 2013). However, Lynch *et al.* (2016) recorded *F. euwallaceae*, and two additional fungal symbionts, *Graphium euwallaceae*, and *Paracremonium pembeum*, associated with the PSHB. *Euwallacea* has spread from its native areas of Southeast Asia – where they

develop in dead or weak/dying trees - into Israel and the California, USA, where they develop in live healthy trees (Eskalen *et al.*, 2013, Mendel *et al.*, 2017). Stressed trees may be susceptible to ambrosia beetle attacks if predisposed to damaging biotic and abiotic factors (Ranger *et al.*, 2010).

Female beetles transport their fungal symbiont from one tree to another, in specialised mandibular cavities situated within their mandibles called mycangia (Beaver, 1989). They bore into trees creating breeding/brood galleries in the xylem, this allows for the establishment and development of the fungal symbiont, which acts as a food source for the offspring and adult beetles (Freeman *et al.*, 2013). This fungal symbiont colonises the gallery walls which invades the vascular tissue (xylem) of the tree, causing cambial discolouration and necrosis, branch dieback, loss of yield and the subsequent death of both juvenile and mature host trees (Mendel *et al.*, 2012, Eskalen *et al.*, 2013).

The most prominent invasion of this pest-pathogen complex is on avocado. Fusarium dieback was first observed on avocado in Israel in 2005, causing little concern due to trees showing few external symptoms. Presence of PSHB was subsequently recorded in 2009, however, this complex has since caused severe damage to agricultural crops in the country (Mendel *et al.*, 2012). By 2016, the beetle had spread to nearly all commercial avocado orchards in Israel (Mendel *et al.*, 2017). In California, USA, the ambrosia beetles was first detected in 2003, though dieback was not observed until nine years later in 2012 (Eskalen *et al.*, 2013), however, there has yet to be a report of PSHB infestation in other parts of the USA or Mexico. Recently, this pest-pathogen complex was detected in KwaZulu Natal, South Africa, on London plane trees showing signs of ambrosia beetle attacks and Fusarium dieback (Paap *et al.*, 2018). Following the first report of the complex in the

country, tree health surveys were conducted and a diseased backyard avocado tree was observed in another province Gauteng, with the tree exhibiting white powdery exudates on the external, as well as, internal necrotic lesions and galleries (van den Berg *et al.*, 2018). However, in 2021, the PSHB was detected in a commercial orchard in the Western Cape (Unpublished data).

Avocado has been identified as a reproductive host for the PSHB, and galleries appear at the base of branches and near branching points in this host (Mendel *et al.*, 2017). Beetle entry and the ensuing spread of the pathogen in avocado trunks and branches later causes the production of white eruptions or 'sugar volcanoes' of 7-carbon sugars mannoheptulose and perseitol (Liu *et al.*, 1999, Mendel *et al.*, 2012, Freeman *et al.*, 2013). With the severe disease progression caused by this complex, the susceptibility or resistance status of popular cultivars is vital to the avocado industry. Mendel *et al.* (2017) studied the interaction between popular avocado cultivars and the PSHB, which showed that 'Ettinger' displayed partial resistance, whereas 'Hass' displayed susceptibility towards the beetle, additionally, 'Reed', 'Nabal', and 'Pinkerton' were noticeably affected (Mendel *et al.*, 2017).

The presence of PSHB and *F. euwallaceae* in South Africa is worrying, since 70% of avocados produced by South African nurseries are 'Hass' (Donkin, 2007), which has been shown to be highly susceptible to the beetle and its fungal symbiont (Mendel *et al.*, 2017). The emergence of the complex is also of concern due to the limitations of control management strategies for the pest-pathogen complex. Management of the insect pest is not currently possible, due to inefficient trapping mechanisms and the lack of biocontrol measures such as predators or parasitoids to control the PSHB. Control strategies for the

fungus are also inadequate at this time, due to the limited movement of systemic fungicides within the tree (Mendel *et al.*, 2017).

1.7 **Conclusion**

Avocado breeding previously involved the selection of valuable material based on their phenotypes. Many molecular markers have been used on avocado in the past, however, as technology advances, better markers become available to perform large-scale genomic analyses. Future work on this topic should look at the population of avocados in South Africa and the diversity it holds, specifically, with the use of molecular markers. As well as look at the susceptibility of popular avocado cultivars grown in South Africa against the PSHB and its fungal symbiont. No genetic population studies have been performed on avocado cultivar germplasms in South Africa. Likewise, very little phenotypic information is available, on South African cultivar resistance towards the PSHB and *F. euwallaceae*. Maintaining a high level of genetic variation in rootstock and cultivar selections is vital to the avocado industry, to ensure that the species can adapt to different environmental conditions and stressors. This applies especially in the case of emerging threats to avocado. Without sufficient genetic diversity in avocado germplasm collections, the avocado industry will not have the resources to breed cultivars and rootstocks that are resistant to these new threats. This dissertation will address the lack of population studies performed on avocado cultivar germplasms in South Africa by implementing molecular marker technologies for the validation of clonal material, verification of horticultural varieties and determining the genetic diversity and population structure of an avocado cultivar germplasm in South Africa. Additionally, this dissertation also addressess the lack of information on cultivar resistance towards the PSHB and its fungal symbiont *F. euwallaceae,* by conducting pathogenicity trials on a selection of popular avocado cultivars using qualitative and quantitative assessments to assign susceptibility or tolerance status towards *F. euwallaceae*.

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CHAPTER 2 Genetic diversity, population structure and clonal verification in South African avocado cultivar germplasm using single nucleotide polymorphism (SNP) markers

2.1 **Abstract**

Since the start of avocado cultivation in South Africa, superior rootstocks and fruit cultivars have been selected and bred based on morphologically important traits, which is time-consuming and expensive to evaluate. Recent technological advances in genomics, including the development of a single nucleotide polymorphism (SNP) genotyping platform for avocado, can uncover cryptic relatedness, verify clonal identity, and detect genotypes with improved disease tolerance faster, thereby improving the breeding efficiency of avocado. However, the use of molecular markers to verify clonal material and assess genetic diversity and population structure has yet to be explored in the South African avocado industry. This underutilized resource presents an opportunity for increased turnaround of genetic improvement through molecular breeding. The aim of this study was to implement molecular marker technologies for the validation of clonal material, verification of horticultural varieties and determining the genetic diversity and population structure of avocado cultivar germplasm in South Africa. An avocado cultivar breeding population, containing 375 individuals, was genotyped using 384 SNP markers. Our analysis indicated a 10.74% identity mismatch ratio in the cultivar germplasm. Following this result, Principal Component Analysis (PCA) and Discriminate Analysis of Principal Components (DAPC) suggested that the South African cultivar germplasm was admixed in relation to the three known avocado varieties, Guatemalan, Mexican, and West-Indian. Additionally, the ancestral origins were determined for 27 individuals with unknown ancestry. Furthermore, the population diversity was assessed and revealed moderate levels of differentiation in the germplasm, suggesting a high level of gene flow between the different populations. This research highlights the value of clonal verification and horticultural variety identification in the reliable propagation of material with desired

traits that are specific for South African conditions. The accurate propagation of material and clonal identity could aid avocado growers to link morphological characters and stress tolerance to accurate genetic backgrounds, which could improve the selection of avocados for current and future environmental stressors, especially as Africa is set to be significantly impacted by climate change.

Keywords: Avocado, SNPs, Population structure, Genetic admixture, Breeding

2.2 **Introduction**

Avocados is an economically important crop that are clonally propagated via grafting, including both rootstocks and scions (Roe *et al.*, 1999, Wolstenholme, 2003, Köhne, 2005, Vorster, 2005, Kremer-Köhne & Köhne, 2007). This maintains all of the valuable morphological characteristics for which a variety was selected (Wolstenholme, 2003, Lewis & Alexander, 2008), forming genetic chimeras that can confer unique traits to both below and above ground components of the plant (Mudge, 2009). However, these traits are usually in opposite selective directions, such as high productivity (scions) and root rot tolerance (rootstocks) (Roe *et al.*, 1995). Avocado breeding and propagation require morphological assessments, identification of undesirable self-pollinated seedlings (Kuhn *et al.*, 2019c), and screening for pathogen resistance, which are laborious and resource intensive (Köhne, 2005). Consequently, morphological assessments, selection and development of new cultivars and rootstocks can extend over 20 years, due to the protracted juvenile phase (Köhne, 2005), which involves flowering, fruiting, and fruit maturation (Schaffer *et al.*, 2013) and this is a costly process to growers (Köhne, 2005, Schaffer *et al.*, 2013). Advances in technology can now assess an individual on the genotypic level using molecular markers (Karp *et al.*, 1997). Molecular markers are excellent for genomic and evolutionary studies, clonal verification, identifying cryptic relatedness among individuals, and identifying favourable genotypes linked to phenotypic performances in certain environmental conditions (Batley, 2015). Consequently, these technologies have the potential to advance and improve genomic selection, by reducing the time and costs involved in phenotyping large numbers of individuals, which is vital to the avocado industry (Clegg, 2004).

Avocado comes mainly from three ‘varieties’, *P. americana* var. *americana* Mill. (‘West Indian’), *P. americana* var. *guatemalensis* Williams. (‘Guatemalan’) and *P. americana* var. *drymifolia* Blake. (‘Mexican’) (Lahav & Lavi, 2002, Wolstenholme, 2003). Intraspecies reproduction between varieties has led to extensive hybridization, resulting in hybrids with varying agronomical traits (Davis *et al.*, 1998, Ashworth & Clegg, 2003). The South African avocado industry relies heavily on superior rootstocks and cultivars, with important morphological traits such as improved fruit yield, better fruit quality, and resistance/tolerance to abiotic and biotic factors, which are usually hybrids (Popenoe & Williams, 1947). These hybrids usually show remarkable morphological similarities, making selection and verification of propagated material difficult (Popenoe & Williams, 1947). However, with technological advances, DNA markers may be used to genetically classify and verify horticultural varieties in new and existing avocado germplasms during propagation (Schnell *et al.*, 2003).

The most current and popular molecular markers that have been used to study avocado are microsatellites and single nucleotide polymorphisms (SNPs). These markers have been used to investigate sequence nucleotide diversity (Chen *et al.*, 2008), study population structure (Chen *et al.*, 2009, Ge *et al.*, 2019a, Ge *et al.*, 2019b, Juma *et al.*, 2020), assign horticultural varieties (Chen *et al.*, 2009), determine genetic diversity (Rubinstein *et al.*, 2019, Ge *et al.*, 2019b, Juma *et al.*, 2020), clarify phylogenetic relationships (Ge *et al.*, 2019a), provide clonal and cultivar verification (Kuhn *et al.*, 2019c) and create linkage maps (Kuhn *et al.*, 2019b).

No research has been conducted on the genetic diversity and population structure of avocados in South Africa. The aim of the present study was to address the lack of genetic

diversity and population structure knowledge of avocado by sampling and SNP genotyping individuals from an avocado fruit cultivar population by using the 384 SNP platform developed by Kuhn *et al.* (2019c). An affinity propagation analysis (APA) (Frey & Dueck, 2007) was used for clonal verification and identification of mislabelled individuals. In addition, a phylogenetic analysis was performed to study individuals in the population with similar genetic relationships, as well as, compare the APA and industry breeding/ selection records to aid in the identification of mislabelled accessions. Furthermore, the population structure and genetic diversity was investigated using principal component analysis (PCA) and discriminant analysis of principal components (DAPC) (Jombart *et al.*, 2010). These results will be valuable in the establishment of molecular tools that can be used for the effective execution of conservation and breeding practices in the avocado industry.

2.3 **Materials and methods**

2.3.1 Biological material - Germplasm accessions

This study used an avocado breeding population from Tzaneen, Limpopo (South Africa) selected and provided by Allesbeste™, consisting of 375 fruiting cultivar individuals, of which 108 individuals were genetically unique. As sample collection could be error prone, some trees were sampled in duplicate. Each accession had a unique “accession ID”, thus, individuals with identical “accession IDs” that were sampled from different trees were classified as “clonal/clones”. These were sampled to confirm clonal identity and determine the technical error rate. Whereas individuals with identical “accession IDs” that were sampled from the same tree were classified as “duplicates”, these were sampled to determine the machine error rate. Allesbeste™ provided proprietary material for this study

and as such, all accessions have been anonymised. All individuals that were genotyped are summarized in **Supplementary File 1 (**10.25403/UPresearchdata.19145087**)**.

2.3.2 SNP genotyping

Ten green, fleshy leaves at intermediate expansion with minimal damage were sampled from each tree. DNA was isolated from the leaf material by the United States Department of Agriculture (USDA) collaborator using the method described by Kuhn *et al.* (2017). Briefly, 3 mm leaf disks, totalling approximately 50 mg per sample, were punched from leaves using a PlantTrak Lx - Benchtop Leaf Punching System (Brooks Life Sciences, Massachusetts, USA). Leaf material was disrupted by shaking with 1/52″ metallic beads, and extracted using the Mag-Bind Plant DNA DS 96 Kit (Omega BioTek (M1130-01), Norcross, Georgia, USA) with automated steps run on a Hamilton Starlet liquid handling robot (Nevada, USA). DNA was quantified by fluorometry and all DNA samples were adjusted to a concentration of 10–20 ng/µl using a Hamilton liquid handling robot. Each avocado individual was genotyped with 384 bi-allelic SNP markers designed as assays to be run on the Fluidigm EP1™ system with the 96.96 IFC (Fluidigm, San Francisco, CA, USA; Table S1); with 91 DNA samples and five controls, as previously described by Kuhn *et al.* (2019c). Samples were SNP genotyped by the USDA, Florida, U.S.A. Additional published SNP genotypic data (Kuhn *et al.*, 2019c) was incorporated for population structure analyses and horticultural variety verification, these individuals were from three horticultural varieties, believed to be ancestral to the South African germplasm. These individuals were labelled as “UCR”, that included two Guatemalan (G), six Mexican (M), and four West-Indian (WI) individuals, kindly provided by the University of California, Riverside, USA (Kuhn *et al.*, 2019c).

2.3.3 Affinity propagation analysis and visualisation of genotypic data

The data was reformatted to proceed with downstream processes using a custom Perl (Version 5.28.1) script to extract and reformat the genotype information into four categories as homozygous allele 1 (1), homozygous allele 2 (2), heterozygous (3), or missing data (0) (Kuhn *et al.*, 2019c). Markers and individuals with greater than 5% missing data were removed in a recursive fashion. Consequently, 61 individuals and eight markers were removed and excluded from further analysis. Therefore, 326 individuals (including the 12 references & 107 unique "accession IDs") and 376 markers remained from the cultivar data. This dataset was named "APA Dataset", as seen in **Table 2-1**.

Custom Python distance and similarity scripts were used to generate pairwise distances (Python - Version 3.8.6), as described by Kuhn *et al.* (2019c). The similarity matrix was used to perform an Affinity Propagation Analysis (APA) that generated clusters and aided in the identification of mislabelled individuals and confirmation of clonal material (Frey & Dueck, 2007, Bodenhofer *et al.*, 2011, Pedregosa *et al.*, 2011, Kuhn *et al.*, 2019a). Additionally, individuals were assigned silhouette scores (Rousseeuw, 1987), as described by Kuhn *et al.* (2019a). In an affinity group, the silhouette scores determine the quality of the membership of a particular accession in a group. If two or more individuals share the same or similar silhouette score, they were considered to be genetically identical or "clonal" within the limits of machine genotype error. Low or negative scores indicated weak membership in the group and that the group was broadly dispersed in the dataspace (Rousseeuw, 1987).

Genotype statistics were obtained by the visualisation and sorting of the genotypic data, as described by Kuhn *et al.* (2019c). The reformatted genotypic data encoded into the four categories (0, 1, 2, and 3), grouping information from the APA and silhouette scores were collated in Microsoft Excel (2019). Numbers of 0, 1, 2, and 3 were calculated for each row (individual) and column (marker) using the COUNTIF function. The number of missing data (0) and heterozygous allele calls (3) were calculated using the MIN and MAX functions, as well as averaged using the AVERAGE function.

The data were sorted by "accession IDs", affinity groups, silhouette scores, and genotypic profiles, as described by Kuhn *et al.* (2019c). The number of SNP differences, machine genotyping error, and technical error were calculated for each "clonal" and "duplicate" set of individuals. The machine genotyping error was calculated using the "duplicate" individuals – individuals sampled from the same tree multiple times. The technical error was calculated using "clonal" individuals – identical "accession ID" individuals sampled from different trees. Mislabelled individuals were identified in two ways; firstly, individuals with identical "accession IDs", but had different genotypic SNP profiles beyond machine genotyping error, were classified as mislabelled type 1. Secondly, individuals with different "accession IDs", but had similar genotypic SNP profiles within machine genotyping error rate, were classified as mislabelled type 2. Mislabelled individuals were highlighted in red in **Supplementary File 1 (**10.25403/UPresearchdata.19145087**).**

2.3.4 Phylogenetic analysis

The APA Dataset was used to perform a hierarchical cluster analysis to study the individuals with similar genetic characteristics and aid in the identification of mislabelled accessions. The dataset was aligned using MUSCLE and subsequently used to construct a condensed unweighted pair group method with arithmetic mean (UPGMA) tree using the maximum composite likelihood method, with the confidence examined using bootstrap values calculated for 1000 replicates in MEGA X (Kumar *et al.*, 2018). The dendrogram was exported in the Newick format to be visualized and customized in Interactive tree of life (iTOL) v6 (Letunic & Bork, 2019).

Table 2-1: The number of individuals genotyped and the germplasm sources used in this study, including the published dataset – 12 references. [* individuals with greater than 5% missing data were removed.]

Population	**Source**	**Location**	**Number of individuals sampled (Original Dataset)**	**Number of individuals retained * for APA analysis (APA Dataset)**	**Number of individuals retained for Population Analysis (Population Analysis Dataset)**
Cultivar Germplasm	Allesbeste™	Tzaneen, Limpopo, South Africa	375	326 (Including the 12 references)	159 (Including the 12 references)
Published Horticultural References	University of California, Riverside	Various Locations	12	12	12

(Kuhn *et al.*, 2019c)					

2.3.5 Population structure analysis

After the identification of the mislabelled individuals through the APA and phylogenetic analysis, individuals with the least missing data for each "clone" and "duplicate" within machine genotyping error rate were retained. Consequently, 167 individuals were removed and excluded from further analysis, these were highlighted in yellow in **Supplementary File 1 (**10.25403/UPresearchdata.19145087**).** Thus, 159 cultivar individuals, of which 12 were published horticultural references, were retained and this second dataset was named "Population Analysis Dataset", as seen in **Table 2-1**, and was used to perform the principal components analysis (PCA), discriminant analysis of principal components (DAPC), structure and diversity analysis. Additionally, one non-polymorphic marker was detected during this analysis and removed – marker SHRSPaS006061, thus, 375 markers remained for the structure analysis. The reduced dataset was reformatted in Microsoft Excel (2019) into a four-bit binary code with A as (1), C as (2), G as (3), and T as (4).

The PCA (Patterson *et al.*, 2006, Reich *et al.*, 2008), DAPC, and allele composition analysis was performed using the Adegenet package (Jombart, 2008, Jombart *et al.*, 2010), whereas the genetic diversity was determined using the mmod 1.3.3 package (Winter, 2012). These analyses were all performed in RStudio, version 1.3.1093 (RStudio Team, 2016) using R version 4.0.3 (R Development Core Team, 2020).

The PCA was performed to display the genetic relationships among individuals, genetically classify and verify the horticultural variety of individuals and detect structure within the germplasm. The germplasm was analysed in relation to published SNP genotypic data (Kuhn *et al.*, 2019c), which represented the three horticultural varieties (Guatemalan - G, Mexican - M, West-Indian - WI). The number of principal components (PCs) retained was based on preserving majority of the variance while retaining the fewest PCs. The variance explained by each PC was calculated as the ratio of each eigenvalue to the sum of all calculated eigenvalues.

DAPC was performed to determine the genetic differentiation between different clusters of individuals using the find.clusters() function to determine the number of groups (K) *de novo*, with the optimal K selected using the diffNgroup method (Jombart, 2008). The number of PCs to retain was determined using the optim.a.score() function (Jombart, 2008). The clusters are considered as populations, as it may indicate the individual's horticultural variety. The allele composition analysis/membership probabilities were displayed using the compoplot() function (Jombart, 2008). PCA and DAPC data were imported and visualised using the Plotly R package (Plotly Technologies Inc, 2015).

Measures of genetic diversity were evaluated with several "F_{ST} analogues", specifically, Nei's G_{ST} (Nei, 1973, Nei & Chesser, 1983), Hedrick's G_{ST} (Hedrick, 2005), and Jost's D (Jost, 2008) estimators for Hs and Ht using the diff_stats function (Meirmans & Hedrick, 2011, Winter, 2012). Hs and Ht are estimates of the heterozygosity expected for this population with and without sub-populations, respectively. Population divergence was estimated between all combinations of population clusters nested within varieties, using the pairwise_G_{ST}_Nei, pairwise_G_{ST}_Hendrick and pairwise_D functions, furthermore, the

chao_bootstrap function was applied to the populations to determine the robustness of the analysis (Winter, 2012). The 12 reference individuals were removed from the “Population Analysis Dataset”, to prevent the reference samples from skewing the analysis.

2.4 **Results**

2.4.1 SNP genotyping statistics and affinity propagation analysis

After removing individuals and markers with more than 5% missing data, the cultivar population contained 326 individuals (including 12 reference individuals and 107 unique "accession IDs") and 376 markers. Missing data per individual varied from 0 to 17 markers of the 376 markers, thus, the average missing data from individuals was 3.0 or 0.8%. Missing data for markers varied from 0 to 15 for the 326 individuals, thus the average of missing data of all markers was 2.6 or 0.8%. The heterozygous allele calls for individuals ranged from 2.4% (9/370, six missing data) for accession "UCR524 (WI)" to 75% (282/376) for accession "AB042", and the heterozygous allele calls for markers ranged from 0% (0/324, two missing data) for SNP marker "SHRSPaS006061" to 79.3% (253/319, seven missing data) for SNP marker "SHRSPaS002697".

The APA generated 64 cultivar groups for 326 individuals and groups varied from one to 43 individuals. The machine genotyping error ranged from 0% to 2.02% for accession "AB006" with 38 SNP differences. The cultivar germplasm technical genotyping error ranged from 0% to 1.46% for accessions "AB035 & AB266" with 11 SNP differences. The cultivar germplasm contained 35 individuals which were mislabelled (31 individuals were mislabelled type 1 and 14 individuals were mislabelled type 2), thus, indicating that approximately 10.74% mislabelling is present in the cultivar germplasm. Eleven mislabelled type 2 individuals overlapped with the mislabelled type 1 individuals; therefore, they contributed once to the mislabelled total. Formatted data with affinity

propagation groups, silhouette scores, and genotype data are recorded in **Supplementary File 1 (**10.25403/UPresearchdata.19145087**)**.

2.4.2 Phylogenetic analysis

The genetic distance matrix of the 326 avocado individuals were used to study the genetic relationships in the population through hierarchical clustering. The phylogenetic analysis indicated that the germplasm was divided into three main populations, based on the reference individuals which are highlighted in the darker shade of the respective colours, as seen in **Figure 2-1**. Based on breeding records and suspected horticultural varieties provided by industry, the individuals were coloured accordingly. The UPGMA-based dendrogram produced three major groups, some containing individuals from different horticultural varieties, pointing at genetic admixture between varieties, as seen in **Figure 2-2**. The majority of the individuals from the phylogenetic analysis corresponded with the APA, with the exception of some of the mislabelled individuals.

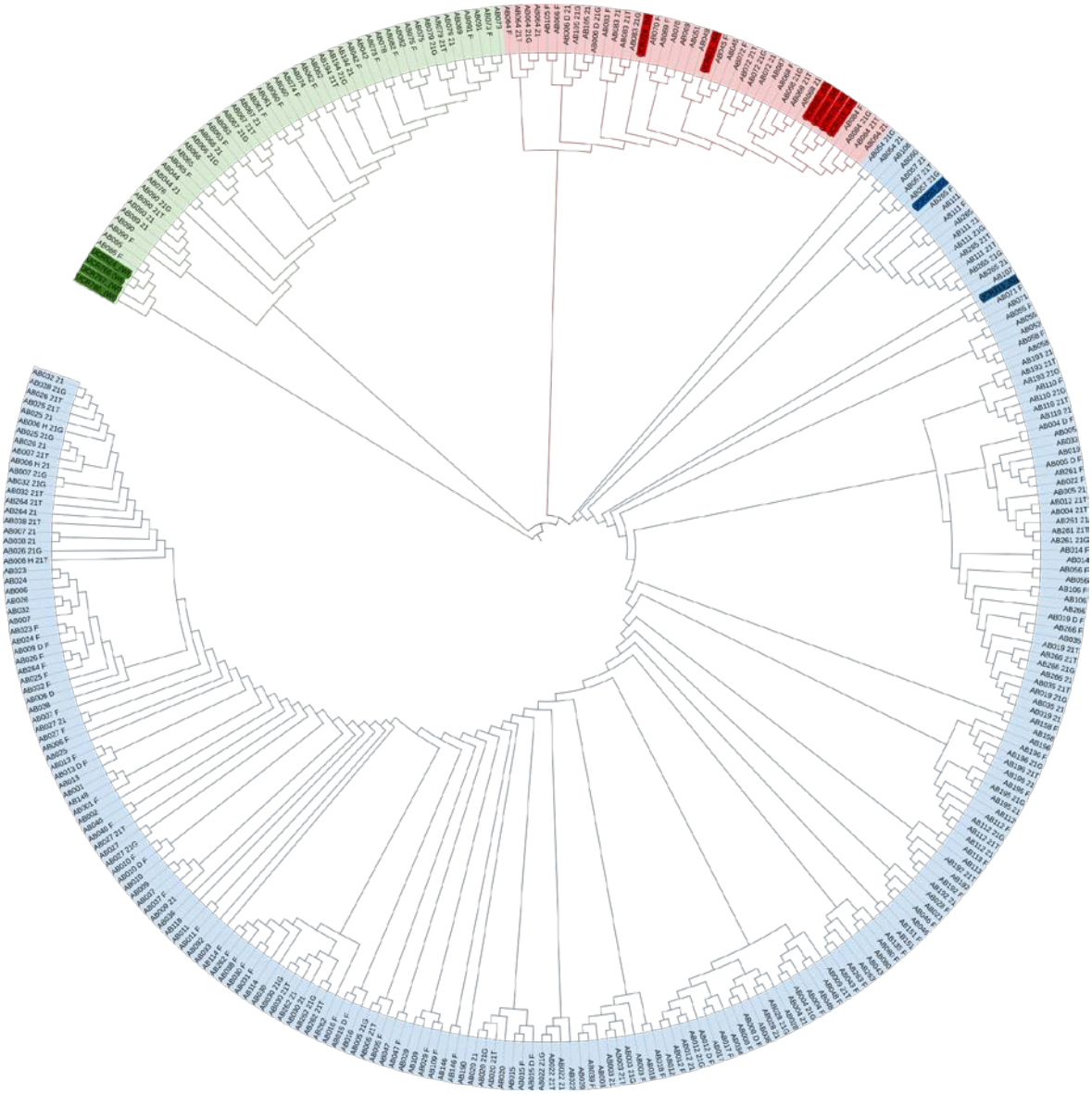

Figure 2-1: Dendrogram of the 326 avocado trees constructed with UPGMA showing genetic relationships between the analysed samples. Leaves and branches were coloured according to the horticultural variety based on the reference individuals. G: Guatemalan (blue), M: Mexican (red), WI: West Indian (green).

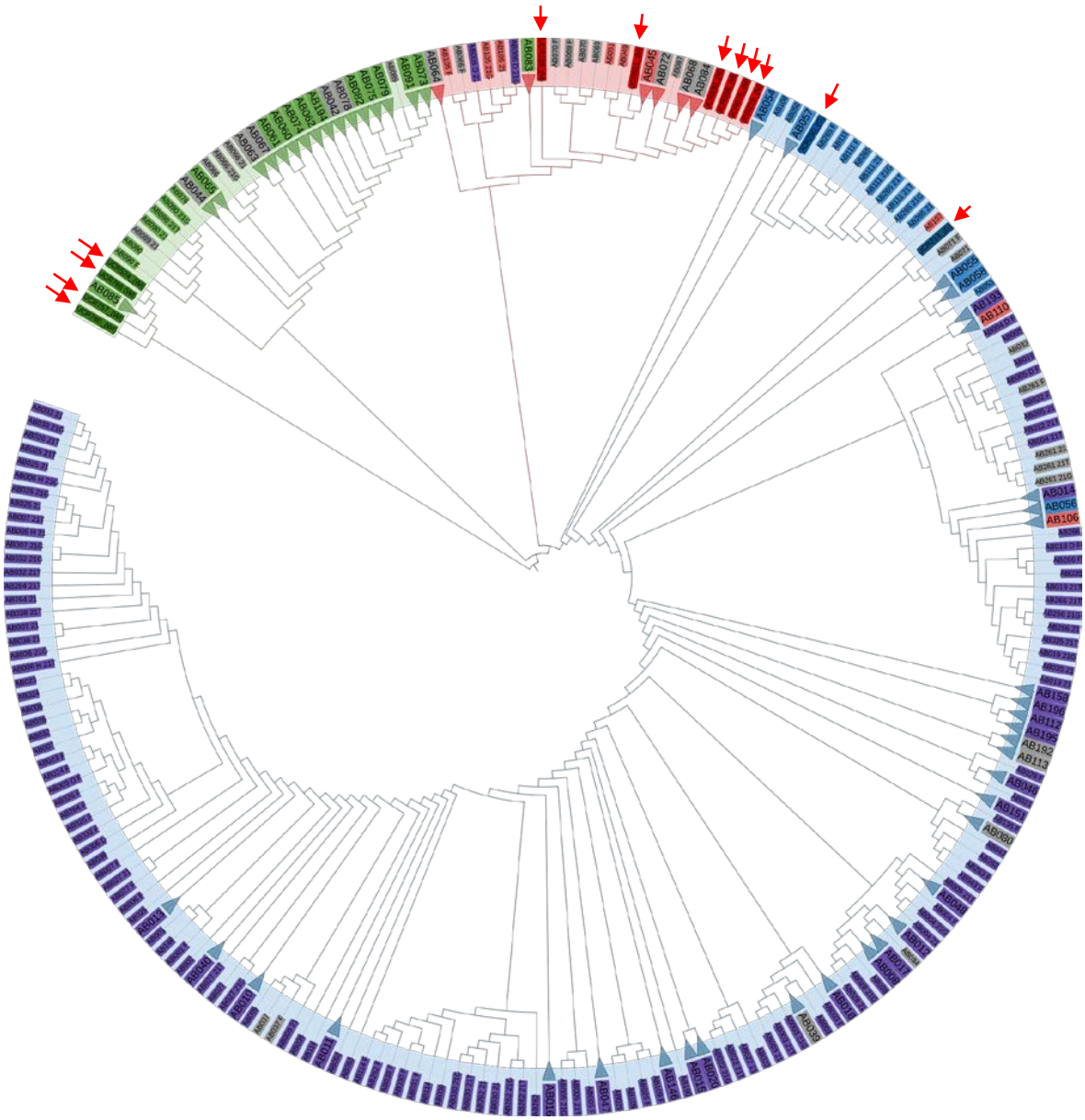

Figure 2-2: Dendrogram of the 326 avocado trees constructed with UPGMA showing genetic relationships between the analysed samples. Leaves with identical accessions were collapsed into nodes. The branches and reference individuals were coloured according to the horticultural variety based on the reference individuals, G: Guatemalan (blue), M: Mexican (red), WI: West Indian (green) as indicated by the red arrows. The leaves were coloured according to horticultural variety information provided by Allesbeste™. G: Guatemalan (blue), M: Mexican (red), WI: West Indian (green), Hybrids (purple), and Unknown (grey).

2.4.3 Principal component analysis & population structure analysis

The PCA was used to study the genetic relationships in the cultivar germplasm. The first three eigenvalues were 114.15, 51.22, and 14.73, respectively. The variance explained by the first three PCs were 30.6%, 13.7%, and 4.0%, respectively, hence, the overall variation was 48.3%. The eigenvalues of the analysis showed that majority of the genetic variance was captured by the first three PCs, as seen from the PCA eigenvalue bar graph in **Figure 2-3 (A)** and the a-score optimisation in **Figure 2-3 (B)**. The scatterplot of the first three PCs for the cultivar germplasm indicated that the reference individuals for Guatemalan (G - blue), Mexican (M - red), and West-Indian (WI - green) had well-defined clusters. The Allesbeste™ cultivar germplasm (orange) appeared to show a cline between the reference individuals, indicating possible genetic admixture, as seen in **Figure 2-3 (C)**. The WI cluster separated from the G and M clusters along the first PC, whereas the M cluster separates from the G cluster along the second PC. The Allesbeste™ cultivar germplasm clustered mainly between the G and M clusters, with most individuals grouping closer to the G cluster, as seen in **Figure 2-3 (C)**.

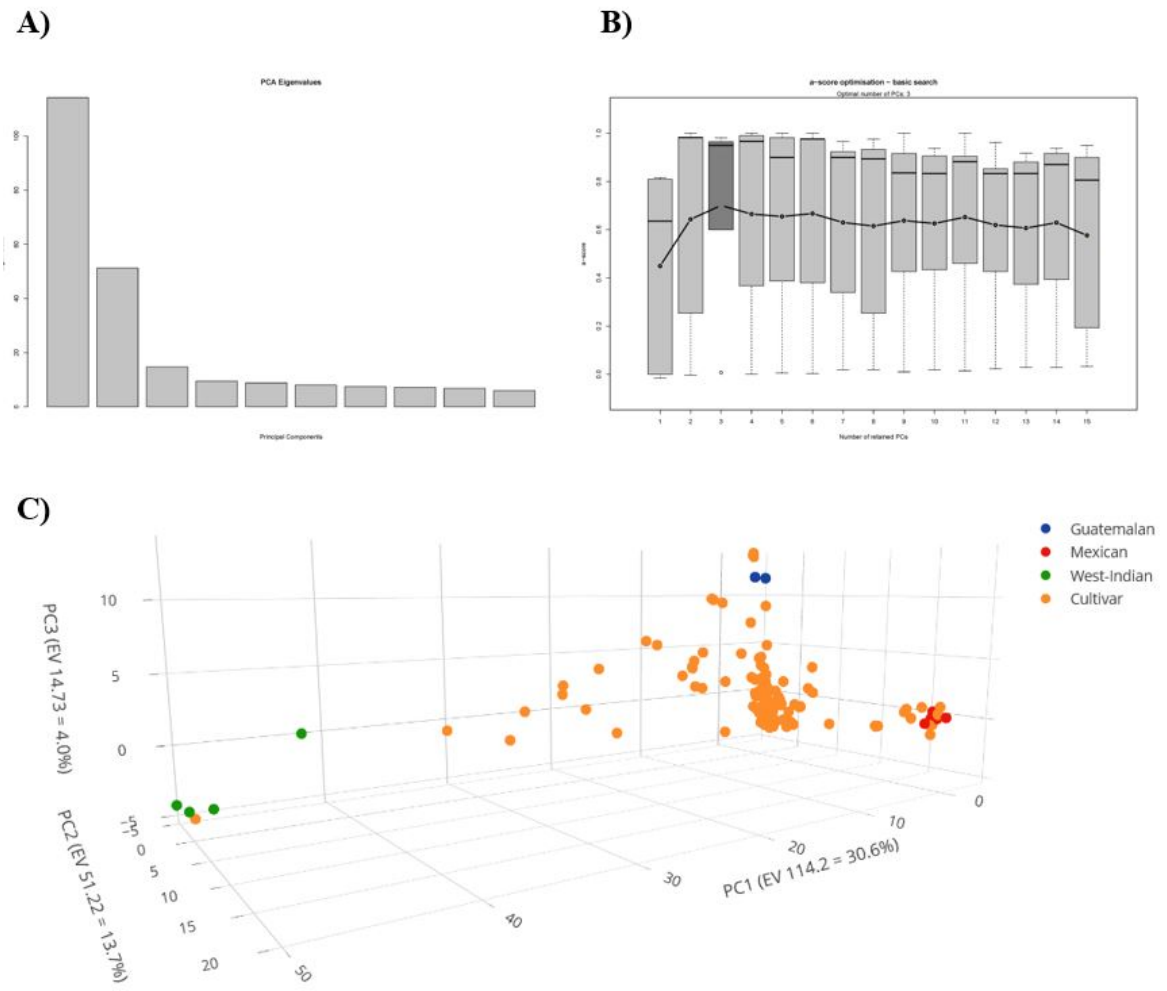

Figure 2-3: Principal Component Analysis (PCA) of the South African cultivar germplasm using 375 single nucleotide polymorphisms (SNPs). (A) PCA eigenvalues of the analysis (B) The optimal a-score indicating the number of principal components (PCs) to retain for analysis, indicating three PCs. (C) The eigenvalues and variance of each PC are found within parentheses on each axis. Individuals are represented as dots and the reference varieties are represented by G: Guatemalan (blue), M: Mexican (red), WI: West Indian (green) and the Allesbeste™ cultivar germplasm is represented in orange. https://plotly.com/~RavenWienk/165/

Further analysis of the cultivar germplasm based on the above PCA, with the individuals now coloured according to the suspected horticultural variety provided by Allesbeste™ based on breeding records, revealed that the majority of the individuals were G and or GxM hybrids. The cultivar germplasm had 27 individuals with unknown horticultural variety, as seen in **Figure 2-4**, which was resolved with 16 individuals assigned as G, eight as M, and three as WI. Additionally, there were 17 misclassified individuals, which were reassigned.

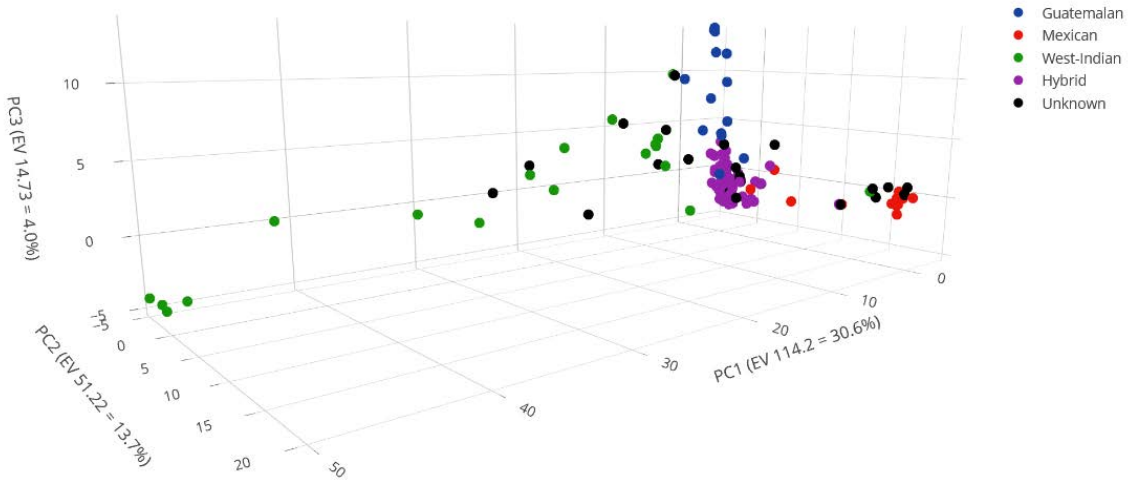

Figure 2-4: Principal Component Analysis (PCA) of the South African cultivar germplasm and the suspected horticultural varieties and hybrids based on Allesbeste™ breeding records. The eigenvalues of each principal component are found within parentheses on each axis. Individuals are represented as dots and the horticultural varieties are G: Guatemalan (blue), M: Mexican (red), WI: West Indian (green), GxM: Guatemalan x Mexican hybrid (purple) and Unknown variety (black). https://plotly.com/~RavenWienk/167/

The DAPC was used to investigate the population differentiation between groups of individuals and identified clusters of genetically related individuals. Based on information from literature and industry, hybrids are common between the horticultural varieties, therefore, a DAPC analysis was performed from K = 2 until K = 7 to identify potential hybrids in the germplasm. The eigenvalues of the analysis showed that majority of the genetic variance was captured by the first three PCs, as seen from the PCA eigenvalue bar graph in **Figure 2-5 (A)** and the a-score optimisation in **Figure 2-5 (B)**. According to the diffNgroup method, the optimum number of genetic clusters were K = 5, which was best supported and appeared to be the most biologically relevant scatterplot. This scatterplot shows the first two PCs of the DAPC for K = 5, as seen in **Figure 2-5 (C)**. Clusters are shown by different colours and inertia ellipses, while dots represent individuals, indicating the Guatemalan (blue), Mexican (red), and West-Indian (green), Cluster 1 (cyan - possible GxWI hybrids), and Cluster 2 (magenta - possible GxM hybrids). Three groups of genetically closer clusters can be identified, Guatemalan, Cluster 1 and Cluster 2, as seen in **Figure 2-5 (C)**. This scatterplot also indicates that majority of the West-Indian accessions are hybrids. Additionally, the scatterplot was shown using the first three PCs of the DAPC of the cultivar germplasm for K = 5, indicating the Guatemalan (blue), Mexican (red), and West-Indian (green), Cluster 1 (cyan), and Cluster 2 (magenta), as seen in **Figure 2-5 (D)**. The scatterplot showed a cline, indicating genetic admixture between the genetic clusters. The cultivar germplasm consisted of 9.4% G, 13.2% M, 3.8% WI, 10.7% Cluster 1 (possible GxWI hybrids), and 62.9% Cluster 2 (possible GxM hybrids). Majority of the results from the DAPC matched the suspected horticultural variety provided by Allesbeste™.

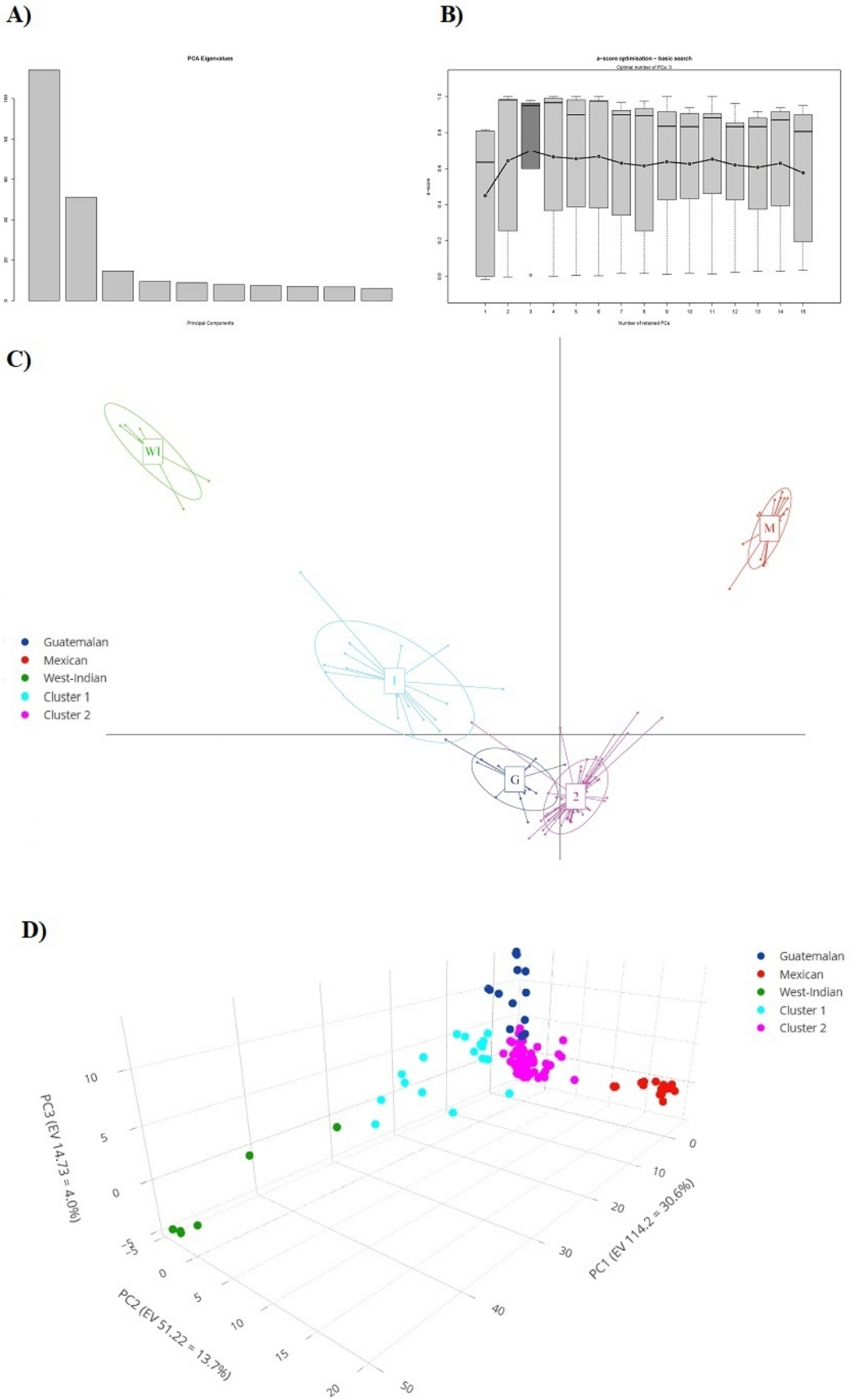
A)
PCA Eigenvalues
Principal Components
B)
a-score optimisation - basic search
Optimal number of PCs: 3
Number of retained PCs
C)
WI
M
I
G
2
Guatemalan
Mexican
West-Indian
Cluster 1
Cluster 2
D)
PC3 (EV 14.73 = 4.0%)
PC2 (EV 51.22 = 13.7%)
PC1 (EV 114.2 = 30.6%)
Guatemalan
Mexican
West-Indian
Cluster 1
Cluster 2

Figure 2-5: Discriminant Analysis of Principal Components (DAPC) of the South African cultivar germplasm using 375 single nucleotide polymorphisms (SNPs). (A) PCA eigenvalues of the analysis (B) The optimal a-score indicating the number of PCs to retain for analysis, indicating three PCs. (C) Scatterplot shows the first two PCs of the DAPC for K = 5, with clusters shown by different colours and inertia ellipses, while dots represent individuals (D) The eigenvalues and variance of each principal component are found within parentheses on each axis. Individuals are represented as dots and the reference varieties are represented by G: Guatemalan (blue), M: Mexican (red), WI: West Indian (green), Cluster 1 (cyan) and, Cluster 2 (magenta). https://chart-studio.plotly.com/~RavenWienk/175

The allele composition analysis of the cultivar germplasm indicated the inferred structure and membership probabilities, where each individual is represented by a coloured bar with length proportional to the estimated membership to each cluster, as seen in **Figure 2-6**. Majority of the germplasm individuals were composed of the G cluster and Cluster 2 (GxM hybrid), which corresponds to the DAPC results. The reference individuals are located in the enclosed area, from individual 147 to 159 in the genomic composition plot, as seen in **Figure 2-6**. All genomic composition plots from K=2 until K=7 is recorded in **Supplementary Figure 2-1**.

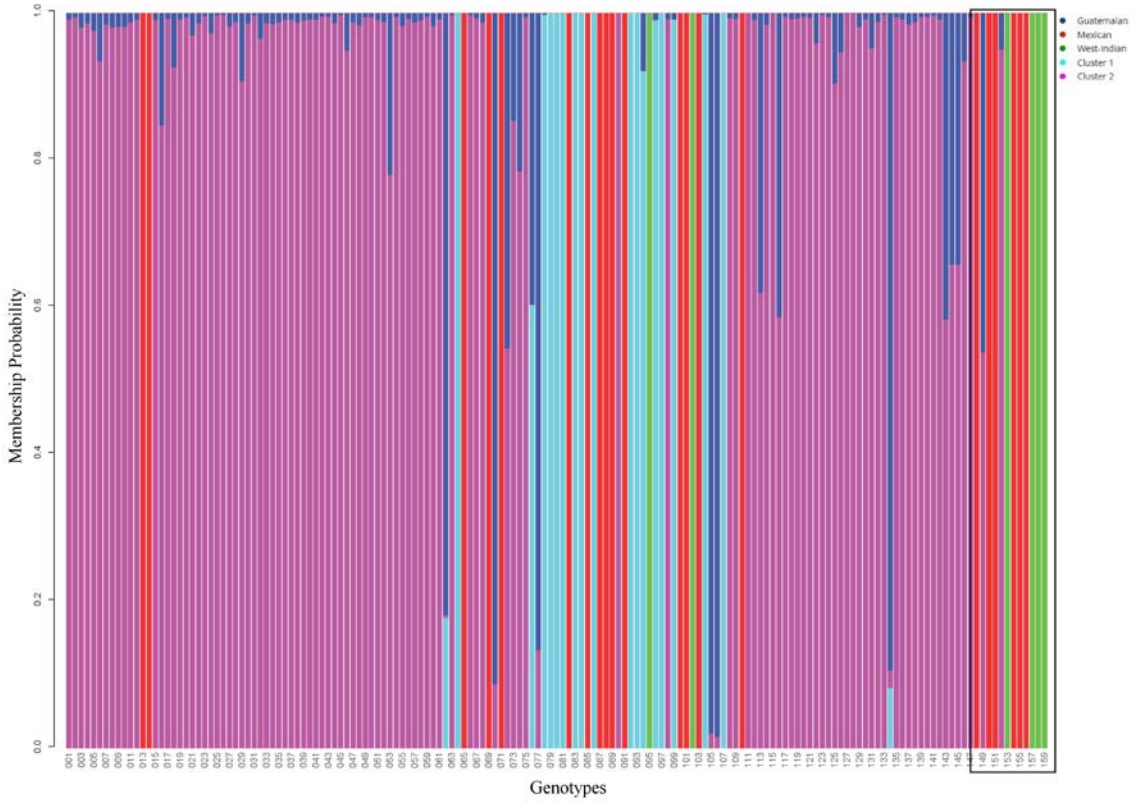

Figure 2-6: Genomic composition plot of the discriminate analysis of principal components (DAPC) indicating the cluster's composition for the cultivar germplasm for 159 genotypes. Each thin vertical line in the bar plot represents one individual and each colour represents one inferred ancestral population. The length of each colour in a vertical bar represents the proportion of that individual's ancestry that is derived from the inferred ancestral population corresponding to that colour. The same colour in different individuals indicates that they belong to the same cluster, indicating they share the same ancestral population. Clusters: Guatemalan (blue), Mexican (red), West-Indian (green), Cluster 1 (cyan), and Cluster 2 (magenta). Reference individuals are located in the enclosed area.

2.4.4 Diversity analysis

The genetic diversity present in the germplasm population was evaluated with three "F_{ST} analogues", Nei's G_{ST}, Hedrick's G_{ST}, and Jost's D, additionally Hs and Ht are estimates of the heterozygosity expected for this population with and without the sub-populations defined in the data, respectively. This analysis indicated that the WI vs. Cluster 1 (Nei's G_{ST} = 0.050, Hedrick's G_{ST} = 0.148, Jost's D = 0.058) had the least differentiation, whereas M vs. WI (Nei's G_{ST} = 0.525, Hedrick's G_{ST} = 0.847, Jost's D = 0.509) had the highest differentiation, as seen in **Table 2-2**. The genetic differentiation among the five clusters identified by DAPC was investigated further by computing population pairwise F_{ST} estimates. The genetic differentiation between the G cluster and Cluster 2 (possible GxM hybrids) was the second lowest (Pairwise Nei's G_{ST} = 0.069, Pairwise Hedrick's G_{ST} = 0.181, Pairwise Jost's D = 0.059) comparable to the lowest genetic differentiation seen between the WI cluster and Cluster 1 (possible GxWI hybrids; Pairwise Nei's G_{ST} = 0.050, Pairwise Hedrick's G_{ST} = 0.148, Pairwise Jost's D = 0.058). The greatest genetic differentiation was between the M and WI (Pairwise Nei's G_{ST} = 0.525, Pairwise Hedrick's G_{ST} = 0.847, Pairwise Jost's D = 0.509), as seen in **Table 2-3**.

Table 2-2: Global differentiation statistics among the population at K=5. Hs and Ht are estimates of the heterozygosity expected for this population with and without the sub-populations defined in the data respectively.

	Hs	**Ht**	**Nei's G_{ST}**	**Hedrick's G_{ST}**	**Jost's D**
G vs. M	0.226	0.313	0.278	0.562	0.225
G vs. WI	0.287	0.438	0.345	0.719	0.423
G vs. Cluster 1	0.375	0.429	0.126	0.359	0.173
G vs. Cluster 2	0.285	0.306	0.069	0.181	0.059
M vs. WI	0.187	0.394	0.525	0.847	0.509
M vs. Cluster 1	0.287	0.399	0.280	0.613	0.313
M vs. Cluster 2	0.199	0.266	0.252	0.502	0.167
WI vs. Cluster 1	0.356	0.374	0.050	0.148	0.058
WI vs. Cluster 2	0.254	0.440	0.422	0.796	0.499
Cluster 1 vs. Cluster 2	0.345	0.424	0.187	0.481	0.242

Clusters: Guatemalan (G), Mexican (M), West-Indian (WI), Cluster 1 (possible GxWI hybrids), Cluster 2 (possible GxM hybrids).

Table 2-3: Population Pairwise F_{ST} comparison among the five clusters (K=5) identified by the Discriminant Analysis of Principal Components.

Pairwise Nei's G_{ST}					
	G	**M**	**WI**	**Cluster 1**	**Cluster 2**
G	0	0.278	0.345	0.126	0.069
M	0.278	0	0.525	0.280	0.252
WI	0.345	0.525	0	0.050	0.422
Cluster 1	0.126	0.280	0.050	0	0.187
Cluster 2	0.069	0.252	0.422	0.187	0
Pairwise Hedrick's G_{ST}					
	G	**M**	**WI**	**Cluster 1**	**Cluster 2**
G	0	0.562	0.719	0.359	0.181
M	0.562	0	0.847	0.613	0.502
WI	0.719	0.847	0	0.148	0.796
Cluster 1	0.359	0.613	0.148	0	0.481
Cluster 2	0.181	0.502	0.796	0.481	0
Pairwise Jost's D					
	G	**M**	**WI**	**Cluster 1**	**Cluster 2**
G	0	0.225	0.423	0.173	0.059
M	0.225	0	0.509	0.313	0.167
WI	0.423	0.509	0	0.058	0.499
Cluster 1	0.173	0.313	0.058	0	0.242
Cluster 2	0.059	0.167	0.499	0.242	0

Clusters: Guatemalan (G), Mexican (M), West-Indian (WI), Cluster 1 (possible GxWI hybrids), Cluster 2 (possible GxM hybrids).

2.5 **Discussion**

The aim of this study was to use a set of previously developed SNP markers for the validation of clonal material, verification of horticultural variety and determination of the genetic diversity and population structure of an avocado cultivar breeding population in South Africa. In this study, an APA was performed that validated clonal material and identified 35 mislabelled individuals. The horticultural variety was verified using PCA which resolved 27 and 17 individuals of unknown and misclassified horticultural varieties, respectively. The population structure was investigated using DAPC which revealed that majority of the South African cultivar germplasm are Guatemalan x Mexican hybrids.

With the advances in technology, the use of molecular markers, has become increasingly popular in breeding programmes (Lahav & Lavi, 2002, Kuhn *et al.*, 2019b). SNPs are currently the best markers for selection and breeding due to their high prevalence in the genome (Batley, 2015), stable inheritance and high-throughput automation ability (Reale *et al.*, 2006). The utilization of molecular markers may improve the understanding of the genetic elements of complex traits and the development of genetically improved avocado rootstocks and cultivars (Clegg, 2004). As all molecular markers are derived from DNA sequences, they can compare genetic properties and provide information on diversity, genetic relatedness, hybridization, speciation and phylogenetic studies (Batley, 2015).

A concern involved in this study is the precise DAPC assignment of individuals, as it may be skewed by the lack of reference samples utilized during analysis (Ottewell *et al.*, 2016). Furthermore, it is important to curate more avocado germplasms in South Africa to include potential MxWI hybrids and improve our understanding of the population. An informative

addition to this study would involve linking the genotypic data with phenotypic data to provide a more accurate description of the germplasm at hand.

An APA was used to identify mislabelled individuals and confirm clonal material. An APA uses all points simultaneously with no genetic assumptions to determine which individuals would best serve as epitomes and the clustering occurs naturally, thus, decreasing erroneous results (Frey & Dueck, 2007). Previously, Kuhn *et al.* (2019c) used an APA to identify 38 mislabelled individuals in the USDA-ARS Subtropical Horticulture Research Station (SHRS) germplasm collection, thus, indicating 13% mislabelling. Similarly, in this study, the APA identified 35 mislabelled individuals, thus, indicating approximately 10.74% mislabelling in the cultivar germplasm. Mislabelling in breeding populations can occur in every phase of avocado production, including incorrect identification in the field, propagation, as well as, during procurement of samples and sampling for genotyping (Kuhn *et al.*, 2019c). It is important to identify mislabelled individuals in germplasms to prevent the propagation of incorrect material, which could be used for budwood purposes. Therefore, identifying these mislabelled individuals may improve breeding efficiency and deployment, while reducing loss of resources and time.

The PCA was used to verify the horticultural variety of individuals in the cultivar germplasm. PCA identifies genetic structures among individuals in the absence of any assumption about the underlying population genetic model (Patterson *et al.*, 2006, Reich *et al.*, 2008), as well as summarizes the overall variability in a population. Based on the PCA, the majority of the South African cultivar germplasm grouped between the Guatemalan and Mexican varieties, and the population appeared to show a cline, rather than well-defined clusters, indicating evidence of genetic admixture. Thus, the cultivar germplasm

appeared to consist mainly of GxM hybrids. Furthermore, the cultivar germplasm had 27 individuals of unknown horticultural variety, and 17 individuals with misclassified horticultural variety, which were resolved. Genetic admixture among avocado populations is attributed to the extensive hybridisation between varieties; and this is common as avocado varieties do not have sterility barriers (Davis *et al.*, 1998, Ashworth & Clegg, 2003). Hybrids allow for a desirable blend of important traits in one individual, such as disease resistance and improved yield. Unfortunately, PCA summarises the overall variability in a population and requires an aforementioned definition of clusters to study population structures, thus, these drawbacks warranted further investigation through DAPC.

The DAPC was used to determine the population structure of the cultivar germplasm, as it is a multivariate model which assesses the genetic differentiation between different clusters of individuals into groups, while maximizing between-group variability and minimizing within-group variation (Fisher, 1936, Lachenbruch & Goldstein, 1979, Jombart, 2008, Jombart & Ahmed, 2011). DAPC has a few advantages, such as the probabilistic assignment of individuals to groups (like Bayesian approaches) and the visual assessment of structures for different population genetic models (Jombart *et al.*, 2010). In this study, the DAPC allowed for the verification of the horticultural variety of individuals in the breeding population. Based on the DAPC, the cultivar germplasm consisted of 9.4% Guatemalan, 13.2% Mexican, 3.8% West-Indian, 10.7% Cluster 1 (possible GxWI hybrids), and 62.9% Cluster 2 (possible GxM hybrids). The high percentage of Guatemalan, Mexican and possible GxM hybrids in the germplasm is coherent, as the most popular cultivar grown world-wide is Hass (Crane *et al.*, 2013), which is a GxM hybrid (Rendón-Anaya *et al.*, 2019). Furthermore, the Guatemalan variety has high fruit averages

and horticultural quality, whereas the Mexican variety has a desirable fruit size (Bergh & Ellstrand, 1986) and has shown some tolerance and resistance to *Phytophthora cinnamomi* (Sánchez-González *et al.*, 2019), which are valuable traits in the industry. Furthermore, Guatemalan and Mexican varieties are typically grown in less tropical areas (Williams, 1977), such as avocado growing regions in South Africa. Interestingly, majority of the West-Indian accessions in the cultivar germplasm appeared to be GxWI hybrids, even though the industry records indicated these are West-Indian accessions. These GxWI hybrids have been known to have an early harvest period and bridges harvesting gaps (Bergh & Ellstrand, 1986), which could explain the presence of Cluster 1 (possible GxWI hybrids) in the germplasm. However, there does not appear to be any MxWI hybrids within the population. This may be due to lack of sampling or due to the lack of breeding of MxWI hybrids in South Africa. Some West-Indian individuals are more tolerant to salinity and calcareous soils (Ben-Ya'acov & Michelson, 1995), which is not favoured by most avocado cultivars grown in South Africa. Most commercial avocado rootstocks and cultivars are hybrids (Popenoe & Williams, 1947), hence, it is important to correctly identify the horticultural variety of individuals, as this affects the ability of breeding programmes to select accurate and superior individuals.

Genetic diversity was determined with "F_{ST} analogues" that assessed the within and among population variation. Mmod is a package that allows three different "F_{ST} analogues" to be evaluated, Nei G_{ST}, Hendrick's G_{ST}, and Jost's D, which is comparable between studies (Winter, 2012). These "F_{ST} analogues" and their combined use will allow more robust analyses of population structure than what is achievable with only F_{ST} (Meirmans & Hedrick, 2011). Some previously reported F_{ST} values for avocado germplasms among the three varieties were 0.19, 0.22 and 0.25, reported by Boza *et al.* (2018), Guzmán *et al.*

(2017) and Gross-German & Viruel (2013), respectively, whereas lower F_{ST} values of 0.061 and 0.05 were reported by Juma *et al.* (2020) and Cañas-Gutiérrez *et al.* (2019) respectively. These studies show the varying levels of diversity in numerous avocado germplasms worldwide. These diversity levels can be affected by the type and number of markers used, the number of individuals and populations assessed, comparable reference samples and different parameters used for the analysis. In this study, the "F_{ST} analogues" indicated that the West-Indian vs. Cluster 1 (possible GxWI hybrids; Nei G_{ST}= 0.050) had the least differentiation, whereas Mexican vs. West-Indian had the highest differentiation (Nei G_{ST} = 0.525). The genetic differentiation among the five clusters revealed that the lowest genetic differentiation was between the West-Indian and Cluster 1 (possible GxWI hybrids; Pairwise F_{ST} = 0.058). Whereas, the greatest genetic differentiation was between the Mexican and West-Indian populations (Pairwise F_{ST} = 0.509). Genetic diversity allows for a species to adapt to various environmental conditions and stressors (Schleif, 1993), such as climate change and resistance to new emerging pathogens and pests. The cultivar germplasm analysed in this study contained moderate differentiation between varieties and hybrid clusters. The "F_{ST} analogues" values in this study were similar to other studies, such as Guzmán *et al.* (2017) and Gross-German & Viruel (2013). Moderate levels of differentiation in the germplasm suggests interbreeding between the three varieties, which is seen with Cluster 1 (possible GxWI hybrids) and Cluster 2 (possible MxG hybrids) in this study. Majority of the cultivar germplasm (62.9%) grouped into Cluster 2 (possible GxM hybrids); this would correlate with industry breeding records.

2.6 **Conclusion**

To our knowledge, this study presents the first molecular genetic assessment of an avocado cultivar germplasm in South Africa. In the present study, molecular marker technology was used to identify mislabelled individuals, validate clonal material, verify horticultural varieties, determine population structure, and genetic diversity. The results from the study may prevent the future propagation of incorrect material, establish proper management and conservation strategies and lastly, improve cultivar breeding efficiency by aiding in the selection of avocado with the ability to cope with changing environments and emerging pests and pathogens. Molecular markers are a powerful and important tool for avocado breeding programmes.

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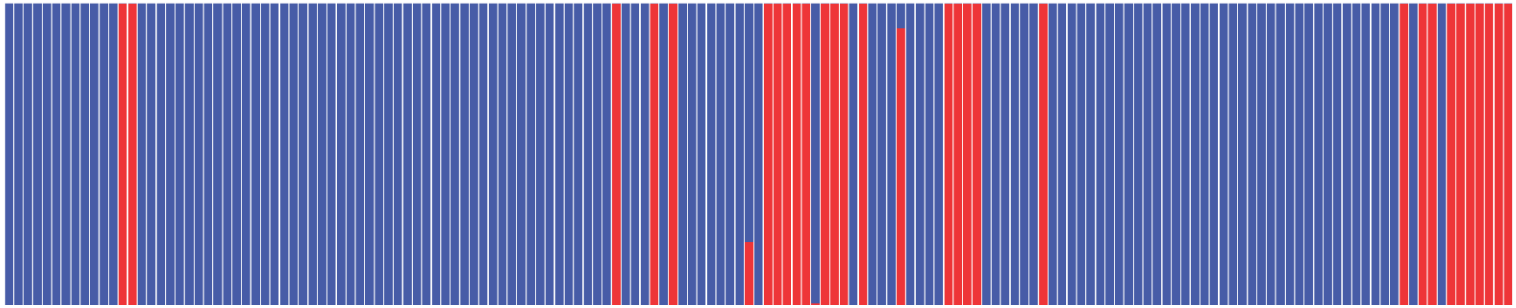
2.8 **Supplementary information**

K =2



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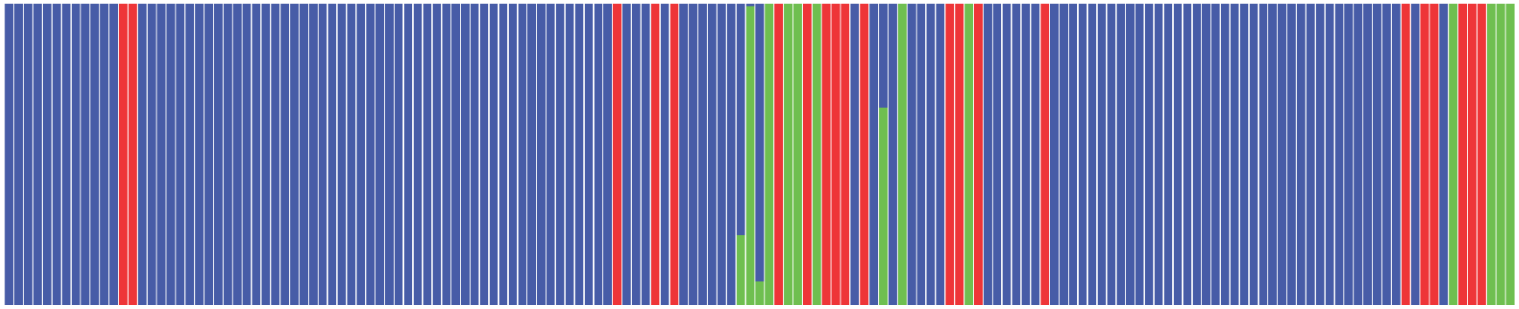

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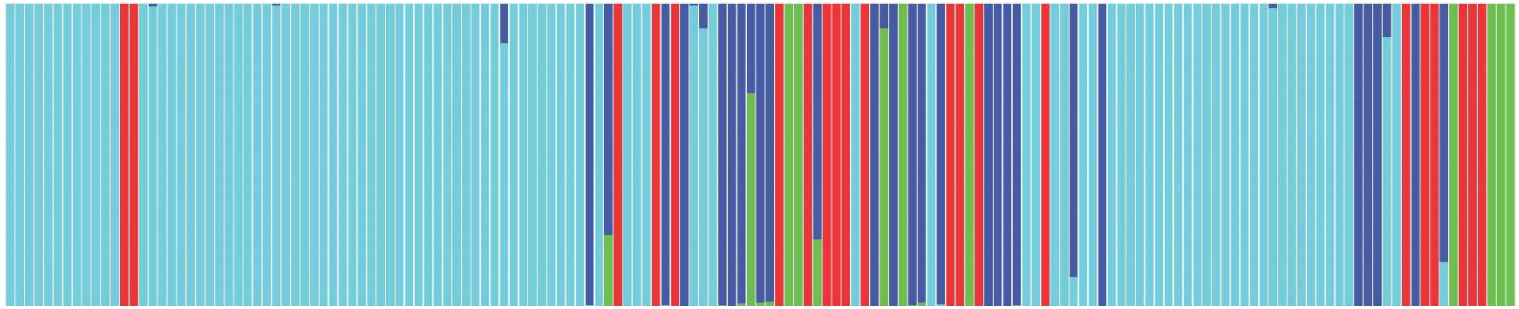

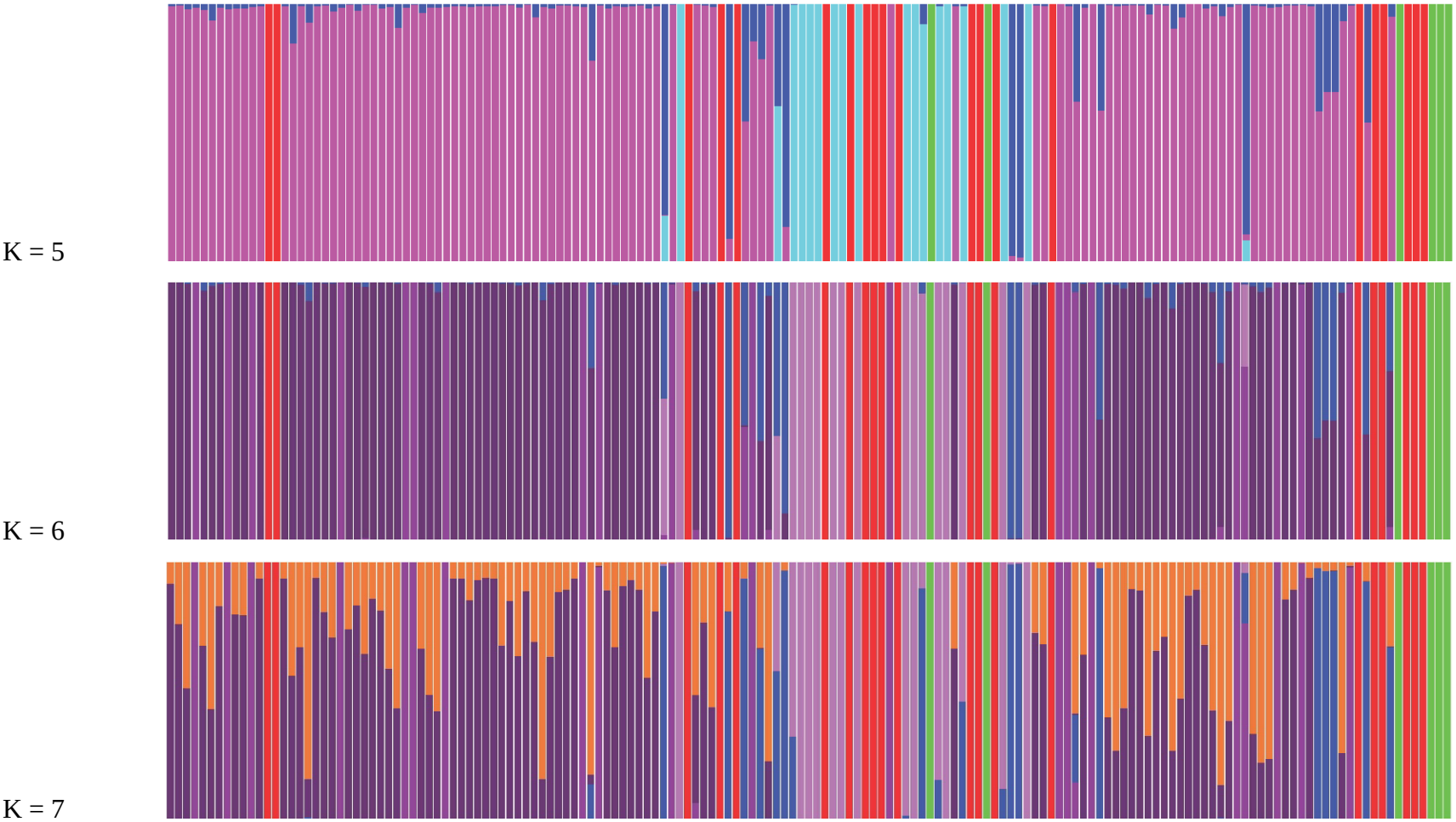

Supplementary Figure 2-1: Genomic composition plots of the discriminate analysis of principal components (DAPC) from K=2 until K=7, indicating the cluster's composition for the cultivar germplasm for 159 genotypes. Each thin vertical line in the bar plot represents one individual and each colour represents one inferred ancestral population. The length of each colour in a vertical bar represents the proportion of that individual's ancestry that is

derived from the inferred ancestral population corresponding to that colour. The same colour in different individuals indicates that they belong to the same cluster, indicating admixture.

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CHAPTER 3 Quantifying susceptibility to *Fusarium euwallaceae* in popular avocado cultivars

3.1 **Abstract**

An ambrosia beetle, commonly known as Polyphagous Shot Hole Borer (PSHB) *Euwallacea fornicatus* (Coleoptera: Curculionidae: Scolytinae), and its fungal symbiont *Fusarium euwallaceae*, has emerged as an invasive pest in Israel and the United States of America (California), causing severe damage and significant economic losses to the avocado industries. This fungal symbiont prevents the transport of water and nutrients by invading the xylem, that leads to Fusarium dieback and the eventual death of the host tree. Recently, this pest-pathogen complex was detected on avocado in South Africa, alarming the industry. Control management strategies for this pest complex are limited due to inefficient trapping mechanisms, lack of biocontrol measures and inefficient fungicides. The use of resistant/tolerant trees could serve as a potential control strategy. Recently, the interaction between popular international avocado cultivars and the PSHB or the fungal symbiont has been studied, but there is currently no information available on the susceptibility of cultivars planted in South Africa. The aim of this study was therefore to conduct, first, a detached branch pathogenicity trial using ten cultivars and second, plantlet pathogenicity trial using three cultivars towards *F. euwallaceae*, with qualitative and quantitative assessments to assign susceptibility, tolerance or resistance status. The detached branch trial indicated that the majority of the screened cultivars were susceptible towards *F. euwallaceae*, with the most severely affected cultivar being ‘Hass’, whereas, ‘Pinkerton’ and ‘Fuerte’ cultivars were the least affected indicating the potential resistance or tolerance towards *F. euwallaceae*. Whole plantlets of three cultivars (‘Hass’, ‘Maluma®’ and ‘Fuerte’) were inoculated and lesion length (upper and lower) and width (left and right) were measured. This indicated that the lower lesion was statistically

significant between infected and control for ‘Fuerte’ and ‘Hass’ cultivars. Whereas, the right lesion was statistically significant between cultivars, particularly for ‘Hass’. The amount of *F. euwallaceae* DNA present in ‘Hass’, ‘Maluma®’ and ‘Fuerte’ cultivars were determined by qPCR and indicated no statistical significance between cultivars. However, ‘Fuerte’ had the highest amount of pathogen DNA, in comparison to the other cultivars tested. As ‘Hass’ is susceptible, and the pathogen loads were similar and not statistically significant, ‘Maluma®’ and ‘Fuerte’ are classified as susceptible to *F. euwallaceae*.

Keywords: *Fusarium euwallaceae*, avocado, real-time PCR, Fusarium dieback, PSHB

3.2 **Introduction**

An emerging plant pest-pathogen complex has been detected on numerous host plants, affecting over 335 tree species, causing Fusarium dieback (Eskalen *et al.*, 2013). This complex consists of an invasive ambrosia beetle, Polyphagous Shot Hole Borer (PSHB) *Euwallacea fornicates*, and its symbiotic fungal associate, *Fusarium euwallaceae* (Mendel *et al.*, 2012, Eskalen *et al.*, 2013). Female beetles transport their fungal symbiont in specialised mandibular cavities called mycangia (Beaver, 1989). These beetles burrow into trees to establish breeding galleries and the fungal symbiont colonises the gallery walls, invading the vascular tissue of the tree, blocking the xylem, and preventing transport of water and nutrients, thus causing Fusarium dieback and the subsequent death of both juvenile and mature trees (Mendel *et al.*, 2012, Eskalen *et al.*, 2013, Freeman *et al.*, 2013). Adult female beetles establish breeding galleries in trunks and branches of trees, while the fungal symbiont, *F. euwallaceae*, develops in the xylem as a food source for larvae and adult beetles (Eskalen *et al.*, 2013, Freeman *et al.*, 2013).

Fusarium dieback was first observed on avocado in Israel in 2005, however, the PSHB was only recorded in 2009 and over time, this complex has caused severe damage to agricultural crops. By 2016, the beetle had spread to nearly all commercial avocado orchards in the country (Mendel *et al.*, 2012, Mendel *et al.*, 2017). The beetle was detected in the United States of America (California) in 2003, though dieback was not observed until 2012 (Eskalen *et al.*, 2013). Significant damage and economic losses have occurred in these countries due to the invasion, involving agricultural and ornamental trees, forest and fruit trees, with severe damage to the avocado industry (Eskalen *et al.*, 2013, Mendel *et al.*, 2017). Recently, this pest-pathogen complex was detected in a National Botanical Garden

in KwaZulu Natal, South Africa, on London plane trees showing signs of ambrosia beetle attacks and Fusarium dieback (Paap *et al.*, 2018). Following the first report of the complex in the country, tree health surveys were conducted and this pest pathogen was detected on a variety of hosts in the country, as well as on a backyard avocado tree in the Gauteng province. The diseased tree exhibited white powdery exudates on the external bark, as well as, internal necrotic lesions and breeding galleries (van den Berg *et al.*, 2018). This pest-pathogen has since been detected in a commercial avocado orchard in George, Western Cape (Unpublished data).

Control management strategies for this pest complex are limited, firstly due to inefficient trapping mechanisms, as the chemical lure Quercivorol is not strong enough to trap and kill beetles in large numbers (Eskalen *et al.*, 2020). Secondly, the lack of biocontrol measures (like predators or parasitoids) to control the PSHB, and lastly due to the limited movement of systemic fungicides in the tree to control the fungal symbiont (Mendel *et al.*, 2017). Therefore, the selection of resistant/tolerant fruit cultivars is a viable alternative to managing pests and pathogens in the avocado industry. The interaction between popular avocado cultivars and the PSHB has previously been studied in Israel, where it was shown that ‘Ettinger’ displayed partial resistance to the complex whereas ‘Hass’ exhibited susceptibility toward the beetle. Additionally, the cultivars ‘Reed’, ‘Nabal’, and ‘Pinkerton’ were noticeably susceptible (Mendel *et al.*, 2017). However, further studies are needed to assess more avocado cultivars against this pest-pathogen complex and to investigate these interactions in different avocado-growing regions of the world.

The presence of the PSHB and *F. euwallaceae* in South Africa poses a significant threat to the agriculture industry - particularly to avocado, since 70% of avocados produced by South African nurseries are 'Hass' (Donkin, 2007). To date, there is no information of the impact of this complex on South African cultivars, and investigations are needed regarding the susceptibility of local cultivars to the beetle and its fungal symbiont.

The aim of this study was to morphologically assess and molecularly quantify *F. euwallaceae* in popular avocado cultivars to determine susceptibility or resistance towards this pathogen. A detached branch pathogenicity trial towards *F. euwallaceae* was conducted using ten popular cultivars and the lesion formation was morphologically assessed. A second pathogenicity trial was conducted using three cultivars and the lesion formation was morphologically assessed. In addition, the pathogen load was quantified using real-time PCR. As the amount of pathogen DNA can be correlated with the degree of tolerance or partial resistance in the host plant (Vandemark & Barker, 2003). Identification of potentially resistant or tolerant cultivars would provide valuable information regarding the level of the threat posed by Fusarium dieback to existing avocado orchards. This information may also be used as a foundation for further investigations into breeding of specific cultivars with resistance to this pest-pathogen complex.

3.3 Materials and methods

3.3.1 Susceptibility assessment of avocado to *F. euwallaceae* using detached branches

3.3.1.1 Preparation of inoculum

The inoculum for the detached branch trial was prepared using a culture of *F. euwallaceae*, originally isolated from avocado in South Africa (CMW51808; Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa), which was cultured on ½ potato dextrose agar (PDA; 19.5 g PDA and distilled water to a volume of 1 L; Merck KGaA, Darmstadt, Germany). Wooden toothpicks were cut in half (3 cm in length and 2 mm in width) and placed in potato dextrose broth (PDB; 12 g PDB and distilled water to a volume of 500 ml; Sigma-Aldrich, Missouri, USA) and autoclaved. Toothpicks were placed on plates inoculated with *F. euwallaceae* and incubated upside down for 7 days at 25°C.

3.3.1.2 Detached branch trial

Five branches (~ 30 cm in length and 5 cm in diameter – ranged due to availability) were obtained from Allesbeste™ orchards, from 10 different avocado cultivars, as seen in **Table 3-1** (ranging in age from 5-25 years). They were cut using a Wincut 7-inch Delux Folding Saw and one end of each branch was dipped three times into paraffin candle wax. Two 3 mm holes were drilled in a spiral manner on opposing sides of each branch using a Ryobi 12V Driver drill (**Figure 3-1)**. Inoculated toothpicks were inserted into each hole in the branch, three branches from each cultivar served as infected, whereas two branches from

each cultivar served as controls - these were inoculated with toothpicks soaked in sterile PDB only. Each inoculation site was sealed with parafilm. The unsealed end of each branch was placed into moist Oasis® blocks, that were watered twice a week to prevent drying and to simulate the water flow present within live branches (**Figure 3-2)**. Incubation was allowed to occur for 6 weeks in a Phytotron at 23 - 25°C. After 6 weeks, branches were whittled until the xylem was exposed. This was done by removing thin layers of bark and stem following the length of the lesion, using a Buck folding knife. Lesion length (upper and lower) and width (left and right) were measured and recorded (**Figure 3-1)**.

Table 3-1: Popular avocado cultivars selected for the detached branch trial to assess susceptibility towards *Fusarium euwallaceae.*

Cultivar	**Variety (Industry provided)**
DA8	Hybrid (GxM)
Edranol	Unknown
Fuerte	Hybrid (GxM)
Hass	Hybrid (GxM)
Hue	Hybrid (GxM)
Marut	Hybrid (GxM)
Maluma®	Hybrid (GxM)
Pinkerton	Unknown
Q19	Hybrid (GxM)
Turner	Hybrid (GxM)

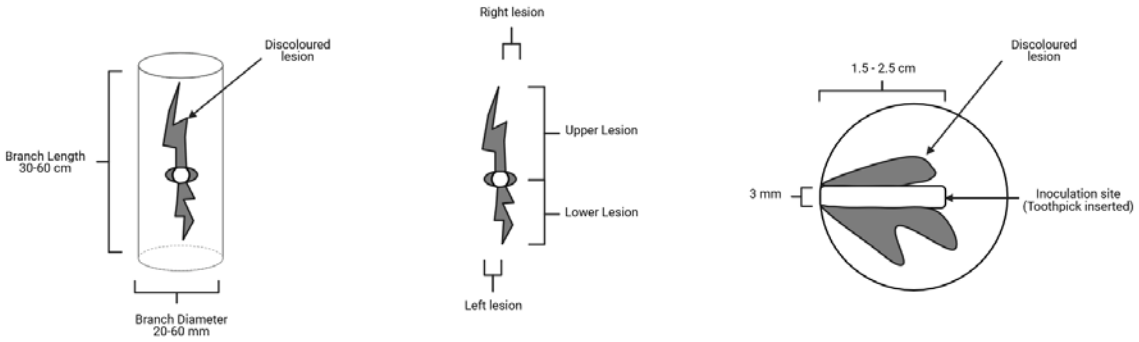

Figure 3-1: Detached avocado branch pathogenicity trial with *Fusarium euwallaceae*. Holes of 3 mm in diameters were drilled to a depth of 1.5 to 2.5 cm depending on the diameter of the avocado branch to be inoculated. Two holes were drilled in a spiral-like manner on opposing sides of the branch to avoid overlapping of lesions. The upper, lower, right and left discoloured lesions were measured.

Figure 3-2: Detached avocado branches inoculated with *Fusarium euwallaceae* using the toothpick method. Inoculated branches were sealed with wax on one side and placed in Oasis® blocks that were watered twice a week and incubated for 6 weeks.

3.3.2 Susceptibility assessment of avocado to *F. euwallaceae* in artificially inoculated avocado cultivar plantlets

3.3.2.1 Artificial inoculation and harvest of avocado cultivar plantlets

Sixty avocado plantlets from 'Hass', 'Fuerte' and 'Maluma®' cultivars (1 year-old, 20 independent biological replicates per cultivar) supplied by Allesbeste ™ were used for an *in-planta* pathogenicity trial to quantify *F. euwallaceae*. The inoculum was prepared as described previously in this study. To inoculate the avocado plantlet stems, one wound hole was drilled per plantlet and inoculated following the toothpick technique as described previously in this study. Each cultivar had fifteen plantlets inoculated with *F. euwallaceae* and five plantlets as controls. After 6 weeks, the plantlet stems were whittled until the xylem was exposed, by removing thin layers of stem following the length of the lesion,

using a Buck folding knife. Lesion length (upper and lower) and width (left and right) were measured and recorded. Then the stems were destructively sampled and cut into smaller pieces using hand shears, 10 cm stem pieces were sampled from the discoloured margins, 5 cm above and 5 cm below the inoculation site. The stem pieces were frozen in liquid nitrogen and stored at -80 °C and used to the determine the pathogen load.

3.3.2.2 DNA extraction from pure mycelia cultures and inoculated plant material

The mycelia from pure *F. euwallaceae* cultures (CMW51808) were grown in liquid yeast extract (2% malt extract, 0.5% yeast extract; Merck) in scintillation vials, shaken at 150 rpm for 4 days at 25 °C. The mycelia were collected using a dissecting needle, transferred to a 1.5 ml Eppendorf tube and freeze-dried at the FABI fungal culture collection facility (University of Pretoria, South Africa). In contrast, the avocado frozen stem material was ground to a fine powder in liquid N_2 using a IKA Mills Tube-Mill Control (IKA®-Werke GmbH & Co. KG, Staufen, Germany). DNA from freeze-dried mycelia and ground avocado powder were extracted in triplicate, for each independent biological replicate to avoid bias in pathogen quantification, using a CTAB (cetyltrimethylammonium bromide) method described by (Brunner *et al.*, 2001). Briefly, 30 mg of material was dispersed in 650 µl of extraction buffer (100 mM Tris HCl pH 8, 25 mM ethylenediaminetetraacetic acid (EDTA), 2 M NaCl, 2% (w/v) CTAB, 2% (w/v) polyvinylpyrrolidone K30 (PVP), 5% (w/v) vinyl poly pyrrolidone PVPP, supplemented with 2% (v/v) β-mercaptoethanol and 500 mg/L Spermidine) thoroughly mixed and incubated for 30 min at 65 °C, inverting the tubes every 5 min. Following incubation, the cell debris was removed through centrifugation at 12 000 rpm for 10 min and the supernatant was collected. The upper phase was extracted twice with 700 µl of chloroform, vortexed and centrifuged at 12 000

rpm for 10 min. The supernatant was precipitated with 1.5 volumes of isopropanol, incubated at -20 °C for 60 min and centrifuged at 13 000 rpm for 20 min at 4 °C. The pellet was washed with 70% cold ethanol and centrifugation at 13 000 rpm for 10 min at 4 °C. The DNA pellet was dried, resuspended in 10 mM Tris-HCl pH 8 overnight at 4°C. The concentration of the DNA samples were determined using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Massachusetts, USA) and the DNA integrity and quality was confirmed on 1% agarose gel at 110 V for 20 min. After DNA quantity and quality were confirmed for each sample, the extracted samples were pooled into their respective biological replicates (where a biological replicate consisted of the triplicate extractions). DNA samples from *F. euwallaceae* and avocado (controls) were selected to generate the serial dilutions and the concentration was determined using a Qubit® 2.0 Fluorometer (Invitrogen, Massachusetts, USA).

3.3.3 Primer testing and specificity

Fungal isolates were obtained from Mike Wingfield culture collection (CMW), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. This study included two isolates of *F. euwallaceae* and two isolates of other *Fusarium* spp., including *F. solani* and *F. oxysporum*, to confirm isolate identity and to test real-time PCR primer specificity (**Table 3-1**). All *Fusarium* spp. fungal isolates were grown on ½ PDA, as descried previously, and incubated upside-down for 7 days at 25°C. A mass of 30-50 mg of mycelia from each isolate was collected with a dissecting needle and transferred to a 1.5 ml Eppendorf tube. DNA was extracted using the PrepMan™ protocol as follows: a volume of 50 µl of PrepMan™ Ultra solution (Applied Biosystems, Massachusetts, USA)

was added to the mycelia. The mixture was vortexed and incubated for 10 min at 96 ºC on a heating block. A micropestle was used to crush and break up the mycelia in each tube. The solution was incubated for 5 min at 96 ºC on a heating block and centrifuged at 10 000 g for 5 min, after which the supernatant from each tube was transferred to a clean 1.5 ml Eppendorf tube (±20 µl). A volume of 80 µl of Tris-HCl pH 8 (10 mM) was added to the DNA solution in each tube. The DNA solutions were stored at -20 ºC.

The extracted DNA was then used in conventional PCR amplification reactions using Translation Elongation Factor (EF1α) primers (O'Donnell *et al.*, 1998) and β-Tubulin specific primers (**Table 3-2)**, FUEU-BTF and FUEU-BTR primers and FUEU-BT-probe designed by Carrillo *et al.* (2019). The Primetime FUEU-BT-probe was labelled at the 5' terminus with the fluorochrome 6-FAMTM, internally with the quencher ZENTM, and at the 3' terminus with the quencher Iowa Black® FQ (IDT, Iowa, USA). The amplification reaction was made up as follows: 20-50 ng/µl template DNA, 1 U FastStart Taq DNA Polymerase (Roche Applied Science, Penzberg, Germany), 1 µl 10x PCR reaction buffer (Roche, Basel, Switzerland), 200 µM of each dNTP (Bioline, London, UK), 0.4 µM of forward and reverse primer, respectively, and sterile water to a total volume of 10 µl. The reaction was placed in a Veriti 96 Well Thermal Cycler (Applied Biosystems), which was set for an initial denaturation at 95 °C for 3 min, 30 cycles of 35 sec denaturation at 95 °C, 35 sec annealing at 56 °C (EF1α primers) or 60 °C (β-Tubulin - FUEU primers), 1 min extension at 72 °C with a final elongation step at 72 °C for 7 min. Products were visualized using GelRed dye (Sigma-Aldrich) on a 1% agarose gel by electrophoresis at 100 V for 30 min to confirm successful amplification and expected size of the PCR products using a Gel Doc EZ Gel Documentation System (Bio-Rad, California, USA).

Table 3-2: *Fusarium* isolates tested using the EF1α primers and the FUEU-BTF and FUEU-BTR primers to assess primer specificity.

Fusarium species	Host	CMW number	GenBank accession number (EF1-α)	Resource
F. solani	*Cedrelinga cateniformis*	CMW18782	None available	(Lombard *et al.*, 2008)
F. oxysporum	*Pinus tecunumanii*	CMW25503	None available	(Herron *et al.*, 2015)
F. euwallaceae	*Platanus x acerifolia*	CMW50555	MG642810	(Paap *et al.*, 2018)
F. euwallaceae	*Persea americana*	CMW51808	MH823818	(van den Berg *et al.*, 2018)

CMW = Mike Wingfield culture collection (CMW), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa

Table 3-3: List of the oligonucleotide primers used in this study.

Name	**Sequence (5' → 3')**	**Resource**	**Product length (bp)**	**Annealing Temperature (°C)**
EF1	ATGGGTAAGGA(A/G)GACAAGAC	(O'Donnell *et al.*, 1998)	648	60
EF2	GGA(G/A)GTACCAGT(G/C)ATCATGTT	(O'Donnell *et al.*, 1998)		60
FUEU-btF	GTTACCTGACCTGCTCTGCC	(Carrillo *et al.*, 2019)	76	57.9
FUEU-btR	ACGGCTGGGAAATGTTAGC	(Carrillo *et al.*, 2019)	-	55.6
FUEU-bt-probe	56-FAM/AGTTTTGTT/ZEN/TTGACATTGGTCGAGCAA/3IABkFQ/	(Carrillo *et al.*, 2019)	-	59.9
Actin-	GTATTCATTCACCAC TACTG	(Engelbrecht	77	60

Fwd		*et al.*, 2013)		
Actin- **Rev**	AGTCAAGAGCCACATAAG	(Engelbrecht *et al.*, 2013)	-	60

3.3.4 Quantifying *F. euwallaceae* using real-time PCR

Real-time PCR was used to quantify the amount of *F. euwallaceae* DNA present within each avocado cultivar. The primer specificity was first analysed for optimal annealing temperature and cross-amplification via conventional PCR using extracted DNA from *F. euwallaceae* and avocado material using the β-Tubulin and Actin primer sets, respectively. The amplification reaction was made up as follows: 20-50 ng/µl template DNA, 1 U FastStart Taq DNA Polymerase (Roche Applied Science), 1 µl 10x PCR reaction buffer (Roche), 200 µM of each dNTP (Bioline), 0.4 µM of each forward and reverse primer and sterile water to a total volume of 10 µl. The reaction was placed in a Veriti 96 Well Thermal Cycler (Applied Biosystems), which was set for an initial denaturation at 95 °C for 3 min, 30 cycles of 35 sec denaturation at 95 °C, 35 sec annealing at 54 °C to 64 °C, 1 min extension at 72 °C with a final elongation step at 72 °C for 7 min. Products were visualized by electrophoresis as previously described.

Then the primer/probe set β-Tubulin (FUEU-BT/FR/probe) was evaluated for sensitivity in real-time PCR assays. The amount of pathogen DNA present within each sample was quantified by real-time PCR using primers for the β-Tubulin gene. For each DNA sample, the amount of pathogen DNA was calculated based on a standard curve constructed from serial dilutions of different known amounts of *F. euwallaceae* DNA (30 ng - 3 pg) against cycle threshold (Ct) values. The real-time PCR reactions were carried out in 20 µl reactions using 1× iQ Supermix (Bio-Rad), 200 nm of each primer set, 100 nm of each hydrolysis probe, and 2 µl of DNA template or sterile water for no template controls (NTCs). All PCR amplifications and biological replicates were performed in triplicate (three technical

replicates) and was carried out on a CFX Connect™ Real-Time System (Bio-Rad) using a two-step protocol: 95 °C for 3 min followed by 35 cycles of denaturation at 95 °C for 15 sec and annealing at 60 °C for 45 sec and plate readings taking place after the annealing step in real-time runs. For assessment of qPCR success and specificity, PCR products were separated by electrophoresis using 1% agarose gel at 100V for 25 min.

The amount of plant genomic DNA present within each sample was quantified by real-time PCR using primers for the avocado Actin gene (Engelbrecht *et al.*, 2013). A normal one-step real-time PCR was used for the plant Actin gene. For each DNA sample, the amount of plant DNA was calculated based on a standard curve constructed from serial dilutions of different known amounts of avocado genomic DNA (20 ng - 2 pg), against cycle threshold (Ct) values. Briefly, a 20 µl reaction for PCR amplification contained 10 µl of Sensimix SYBR No-ROX (Bioline), 0.25 µM of each forward and reverse primer, 2 µl of template, and 7 µl of sterile water. Thermal cycling conditions for Actin were as follows: pre-incubation for 10 min at 95 °C (hot start) followed by 35 cycles, each consisting of 15 s of denaturing at 95 °C, 15 s of annealing at 60 °C, and 15 s of primer extension at 72 °C. Melting curves were acquired to confirm individual real-time PCR signals corresponding to a single homogenous DNA product. All sets of reactions also included a sterile water control (negative control) to verify that no contamination was present in the reagents. All PCR reactions were performed in triplicate (three technical replicates) and analysed using a CFX Connect™ Real-Time System (Bio-Rad).

3.3.5 Statistical analyses

The statistical analysis for the detached branch and artificially inoculated avocado plantlet pathogenicity trials involved the measurement of lesion length and width. Data from each cultivar with infected and control as factors were subjected to a two-way analysis of variance (ANOVA) followed by a least-squares mean separation test ($p \leq 0.05$). Linear standard regression lines were calculated from the cycle threshold values from the serial dilutions of known *F. euwallaceae* and avocado DNA concentrations: y = mx + b, where b = y-intercept of standard curve line (crossing point) and m = slope of the standard curve line (function of PCR efficiency) (Ginzinger, 2002). Quantification data was assessed by comparing the amount of pathogen DNA with the amount of plant DNA from the mean Ct values obtained from the triple technical replicates from each biological sample, within each cultivar. Technical replicates were analysed to ensure only replicates with Ct value differences of < 0.5 were included. Pathogen load was quantified from infected avocado stem material by normalizing the β-tubulin values (*F. euwallaceae*) with the corresponding actin values (avocado) for each individual sample. This data was analysed using a Kruskal-Wallis test ($p \leq 0.05$). All data was analysed using the Statistical Analysis System (SAS; SAS Institute, Cary, NC).

3.4 Results

3.4.1 Susceptibility assessment of avocado to *F. euwallaceae* using detached branches

Ten different avocado cultivars were assessed for *F. euwallaceae* susceptibility using lesion lengths during a detached branch pathogenicity trial. The detached avocado branch inoculation trial using toothpicks was adequate to perform a cultivar susceptibility assay, with the modification of placing one end of the branch in wax to prevent drying, as well as placing the other end of the branch in moist Oasis® blocks to mimic the water flow present within live branches. The detached control branches had no/ minimal lesion formation as seen in **Figure 3-3 (A)**, whereas, lesions had formed on all inoculated branches (**Figure 3-3 (B))**. The longest upper, lower, left and right lesion measurements were recorded on infected branches at 5.5 cm (cultivar 'Turner'), 6.2 cm (cultivar 'Maluma®'), 1.1, cm (cultivar 'Hass') and 1.2 cm (cultivar 'Hass'), respectively, as recorded in **Supplementary Table 3-1**, **Supplementary Table 3-2** and **Supplementary Table 3-3.**

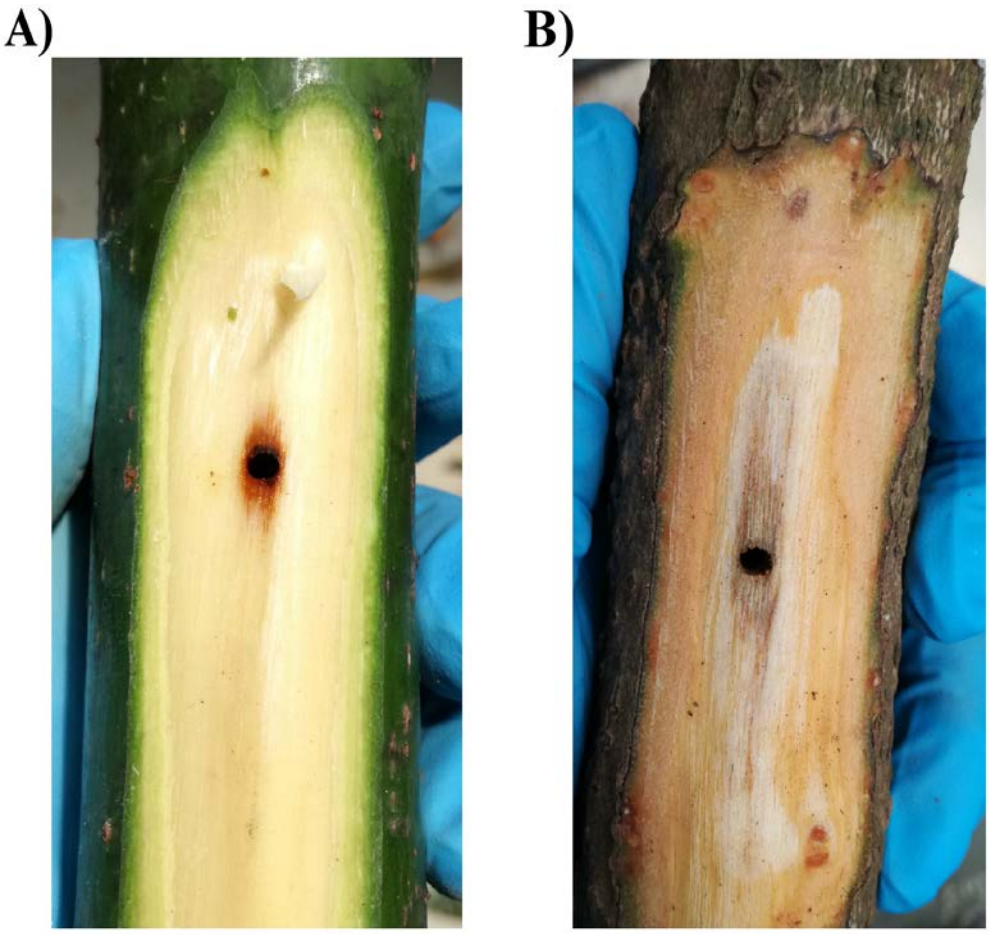

Figure 3-3: Visual results of the pathogenicity trial with *Fusarium euwallaceae* on detached avocado branches using the toothpick method. (A) Whittled control branches resulted in no/minimal lesion formation ('Hass' cultivar). (B) Whittled inoculated branches resulted in significant lesion formation ('DA8' cultivar).

The statistical analysis for the detached avocado branch pathogenicity trial compared the lesion length and width from each cultivar (infected and control as factors). The statistical analysis indicated that the mean diameter of the branches was not statistically significant in this study (**Figure 3-4)**. The upper and lower lesion comparisons were statistically significant across most cultivars at $p = 0.05$, except for cultivars 'Q19' and 'Pinkerton' for the upper lesion and cultivars 'Fuerte', 'Pinkerton', and 'Q19' for the lower lesion respectively (**Figure 3-5)**. The upper lesion comparisons across infected and control were

statistically significant at p = 0.05 for ‘DA8’, ‘Hass’, ‘Maluma®’, ‘Q19’ and ‘Turner’ (**Figure 3-5)**. The lower lesion comparisons across infected and control were statistically significant at p = 0.05 for ‘Hass’, ‘Maluma®’, ‘Q19’ and ‘Turner’ (**Figure 3-5)**.

The left and right lesion comparisons between cultivars were not statistically significant at p = 0.05, except for the cultivars ‘Hass’, ‘Marut’ and ‘Turner’ for the left lesion and cultivars ‘Hass’, ‘Hue’, and ‘Turner’ for the right lesion respectively (**Figure 3-5)**. Furthermore, the left lesion comparisons across infected and control were statistically significant at p = 0.05 for ‘Hue’ and ‘Marut’ (**Figure 3-5)**. Whereas, the right lesion comparisons across infected and control were statistically significant at p = 0.05, for ‘Edranol’, ‘Hue’ and ‘Q19’ (**Figure 3-5)**. The LSMean for ‘Hass’ was much higher than the LSMean of all the other cultivars for both the left and right lesion width. Lesion length, width and statistical outputs were recorded in **Supplementary Table 3-4, Supplementary Table 3-5, Supplementary Table 3-6, Supplementary Table 3-7** and **Supplementary Table 3-8**.

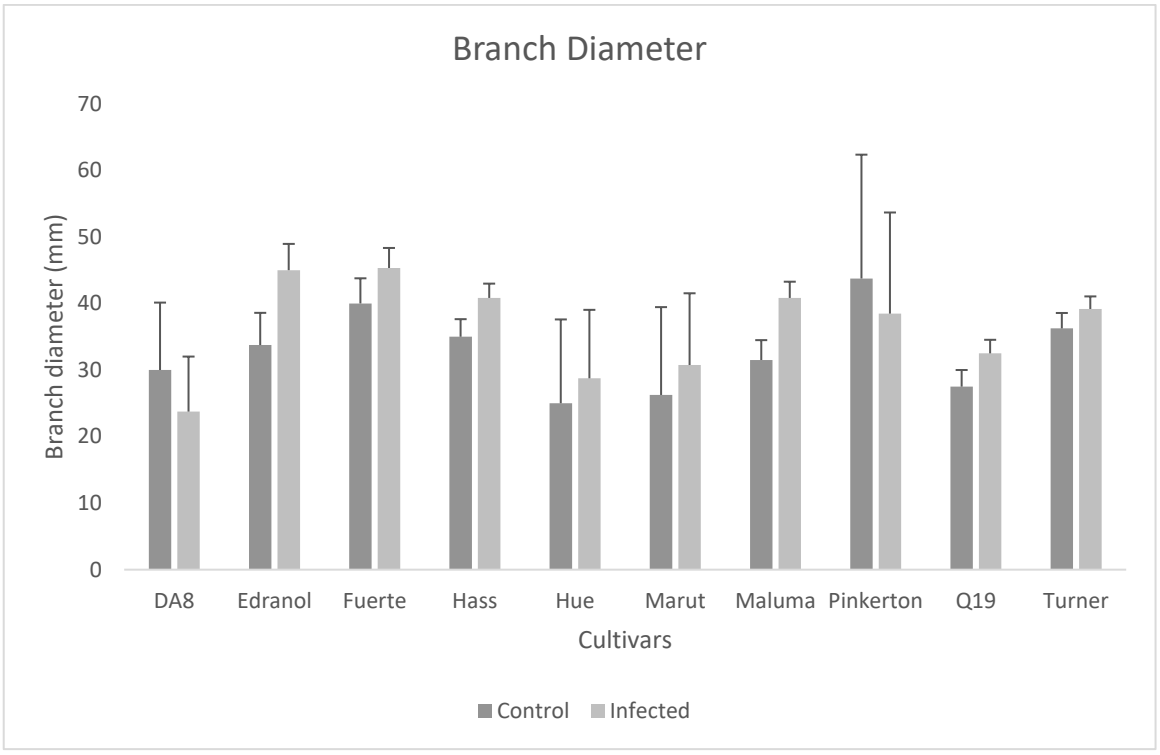

Figure 3-4: The least-squares means of the branch diameter measurements for the detached branch pathogenicity trial on various avocado cultivars. * = significant difference at p = 0.05 across control and infected and • - significant difference at p = 0.05 across cultivars.

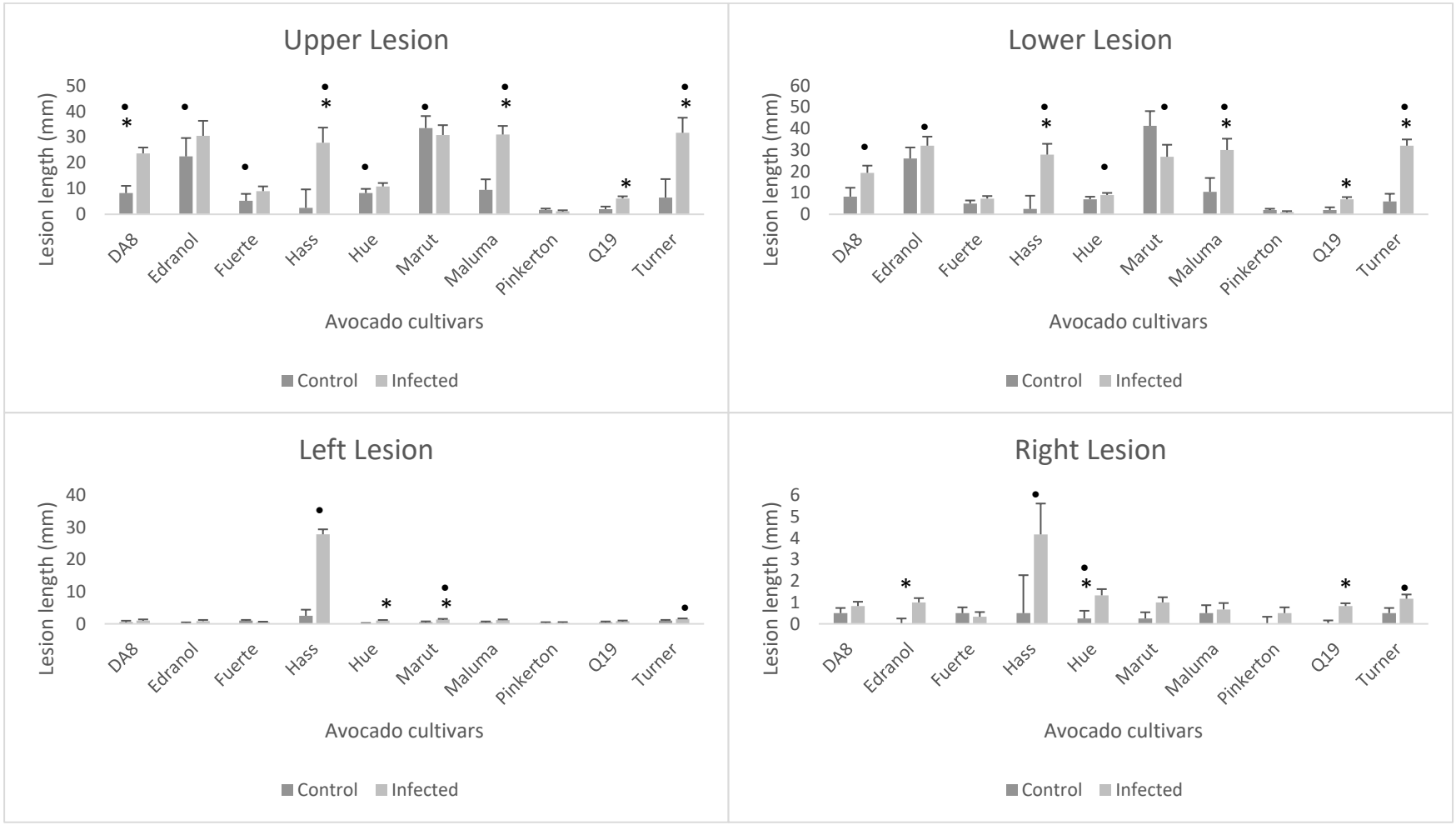

Figure 3-5: Least-squares means of lesion measurements for the detached branch pathogenicity trial on various avocado cultivars 6 weeks after inoculation with *Fusarium euwallaceae*. * = significant difference at $p = 0.05$ across control and infected and • - significant difference at $p = 0.05$ across cultivars.

3.4.2 Artificial inoculation and harvest of avocado cultivar plantlets

Avocado plantlets from ‘Hass’, ‘Fuerte’, and ‘Maluma®’ cultivars (15 infected and 5 control plantlets per cultivar) were successfully inoculated with *F. euwallaceae* using the toothpick method (**Figure 3-6)**. After 6 weeks, the plantlets were assessed for visual symptoms, however, all plantlets, including control and inoculated, appeared healthy and showed no external symptoms such as wilting. The stems were whittled and internal lesions had formed on all infected plantlets (**Figure 3-7)**. The longest upper, lower, left and right lesion measurements were recorded at 20 cm (cultivar ‘Fuerte’), 15.3 cm (cultivar ‘Hass’), 0.3 cm (cultivars ‘Hass’, ‘Fuerte’ and ‘Maluma®’), and 0.3 cm (cultivars ‘Fuerte’ and ‘Maluma®’), respectively, as recorded in **Supplementary Table 3-9** and **Supplementary Table 3-10**.

Statistical analysis for the artificially inoculated avocado pathogenicity trial compared the lesion length and width from each cultivar (infected and control as factors). The statistical analysis indicated that the stem circumference of the plantlets was not statistically significant across cultivars or between infected and control (**Figure 3-8)**. The upper and left lesion comparisons were not statistically significant across cultivars or between infected and control at $p = 0.05$, (**Figure 3-9).** Whereas, the lower lesion comparisons were statistically significant across the infected and control for ‘Fuerte’ and ‘Hass’ at $p = 0.05$, but not statistically significant across cultivars **(Figure 3-9)**. The right lesion comparison was statistically significant across cultivars at $p = 0.05$, between ‘Hass’ and ‘Fuerte’ and between ‘Hass’ and ‘Maluma®’, but not statistically significant between ‘Fuerte’ and ‘Maluma®’, additionally, the right lesion was not statistically significant across infected

and control. Lesion length, width and SAS results were recorded in **Supplementary Table 3-11, Supplementary Table 3-12, Supplementary Table 3-13, Supplementary Table 3-14** and **Supplementary Table 3-15.**

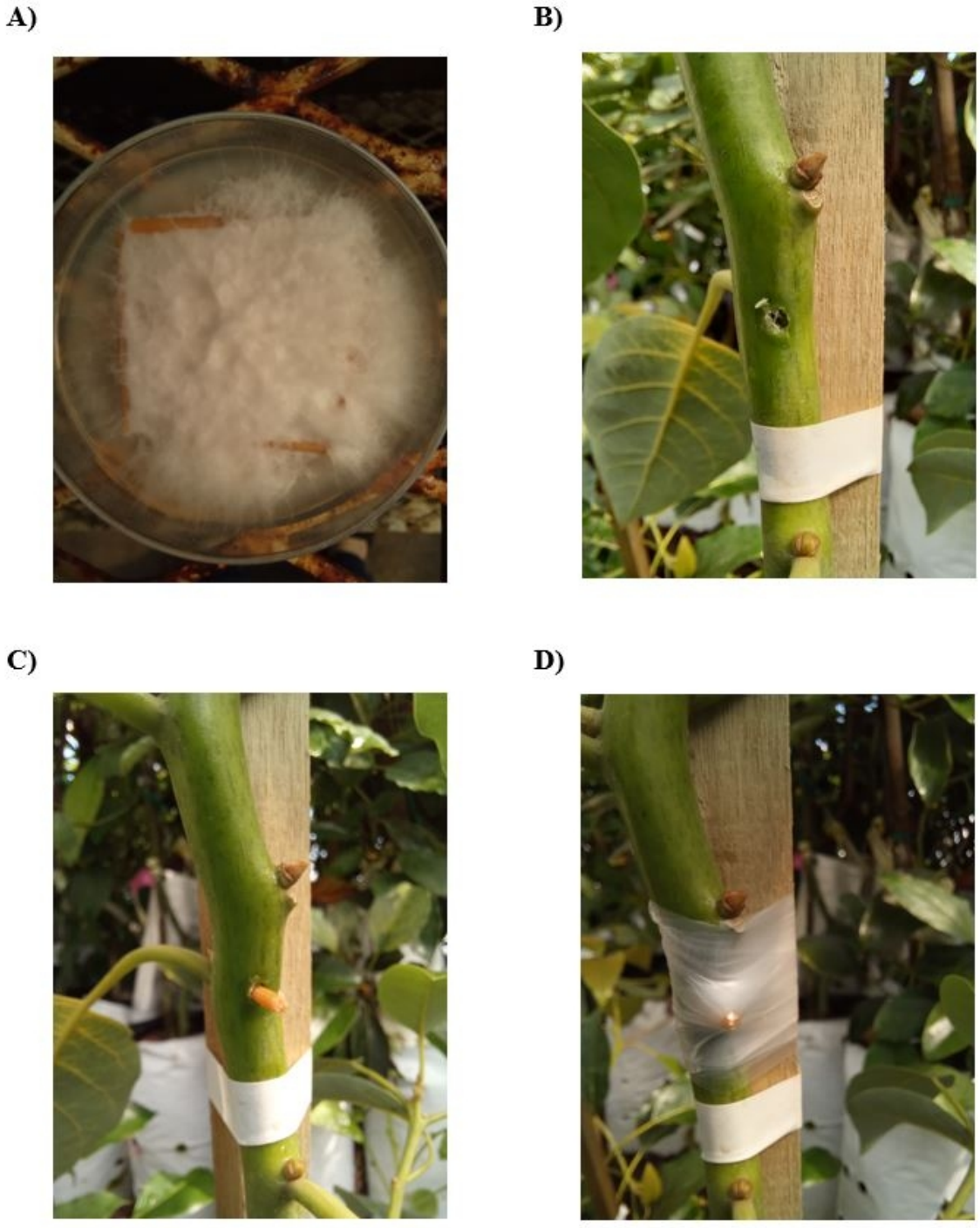

Figure 3-6: Inoculation and incubation of avocado plantlets infected with *Fusarium euwallaceae* using the toothpick method. (A) Autoclaved toothpicks overgrown with a *F. euwallaceae* isolate. (B) Inoculation site made with drill. (C) Overgrown toothpicks placed in drilled holes in avocado plantlets. (D) Inoculated sites wrapped with parafilm.

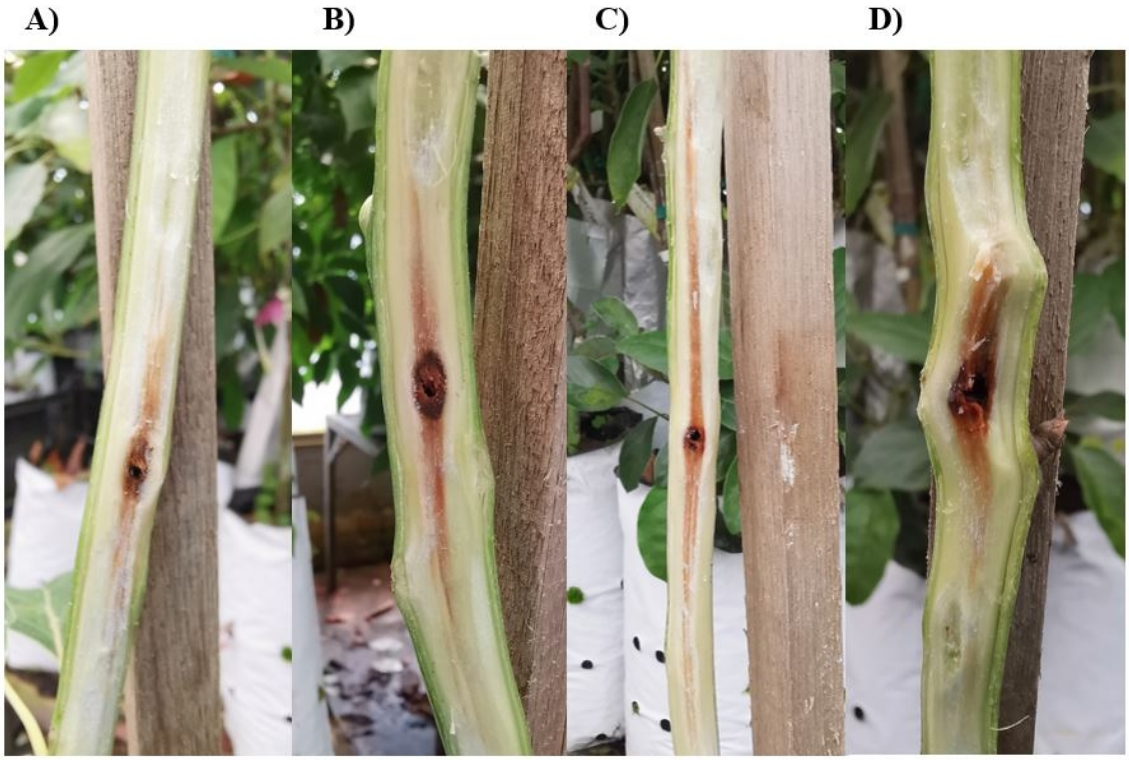

Figure 3-7: Avocado plantlet stems were whittled and lesions were exposed and measured. A) Lesion from a control plantlet. B- D) Lesions from plantlets infected with *Fusarium euwallaceae.*

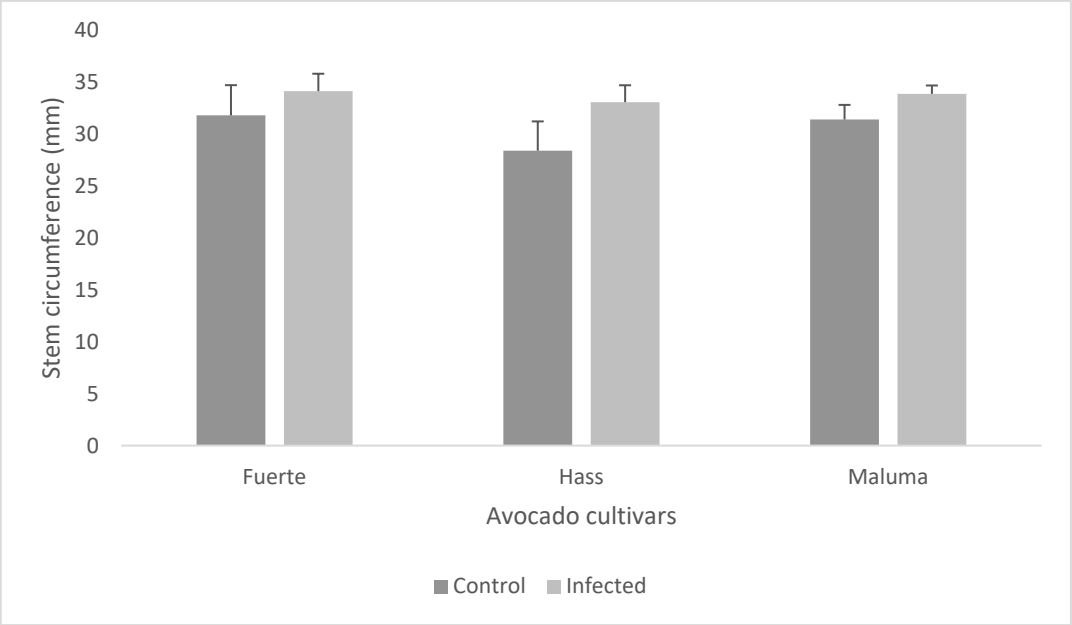

Figure 3-8: The least-squares means of the stem circumference measurements for the pathogenicity trial on various cultivars. * = significant difference at $p = 0.05$ across infected and control and • - significant difference at $p = 0.05$ across cultivars.

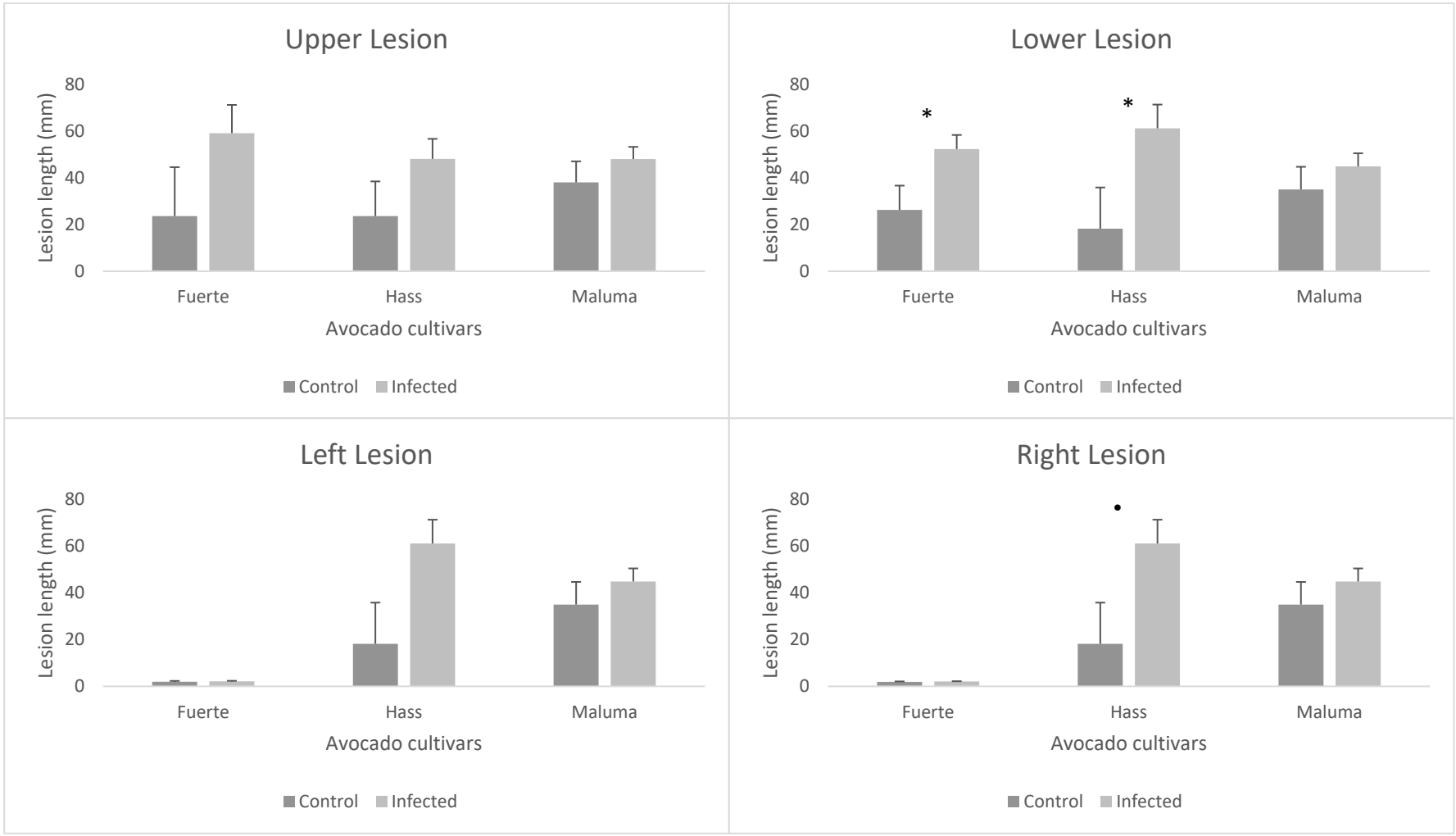

Figure 3-9: Least-squares means of the lesion measurements for the avocado pathogenicity trial on various cultivars 6 weeks after the inoculation with *Fusarium euwallaceae*. * = significant difference at $p = 0.05$ across infected and control and • - significant difference at $p = 0.05$ across cultivars.

3.4.3 DNA extraction from pure mycelia cultures and inoculated plant material

DNA was successfully extracted from pure *F. euwallaceae* mycelia (CMW51808) and avocado stem material, it was qualitatively confirmed by gel electrophoresis (**Figure 3-10** and **Figure 3-11)**, respectively. The concentrations of the DNA samples determined by Nanodrop are recorded in **Supplementary Table 3-16**. The extraction produced three bands, firstly, the well-defined intense genomic DNA and then smeared 18S and 28S ribosomal RNA bands, respectively. The well-defined intense band indicates the presence of high-quality intact DNA, and the smeared bands, indicates the presence of degraded RNA.

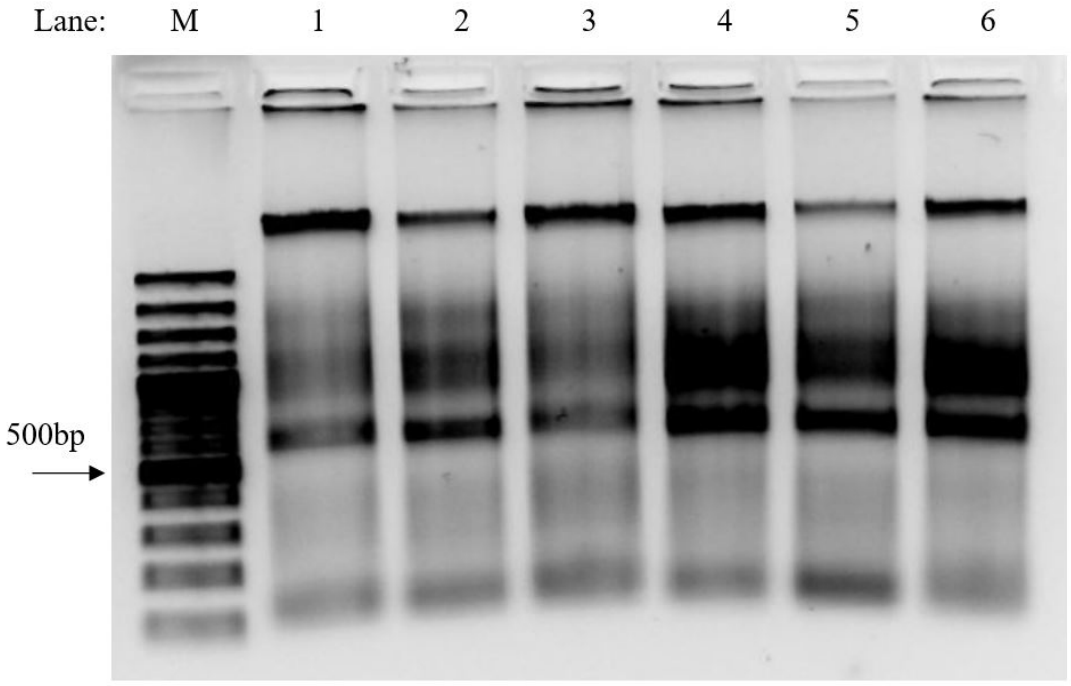

Figure 3-10: Successful *Fusarium euwallaceae* mycelia DNA extraction. Lane M contains a 100 bp molecular marker (GeneRuler 100 bp DNA Ladder, Thermo scientific), the remaining lanes represent the *F. euwallaceae* sample (CMW51808) extracted multiple times from lane 1-6. DNA samples were separated on a 1% agarose gel by electrophoresis.

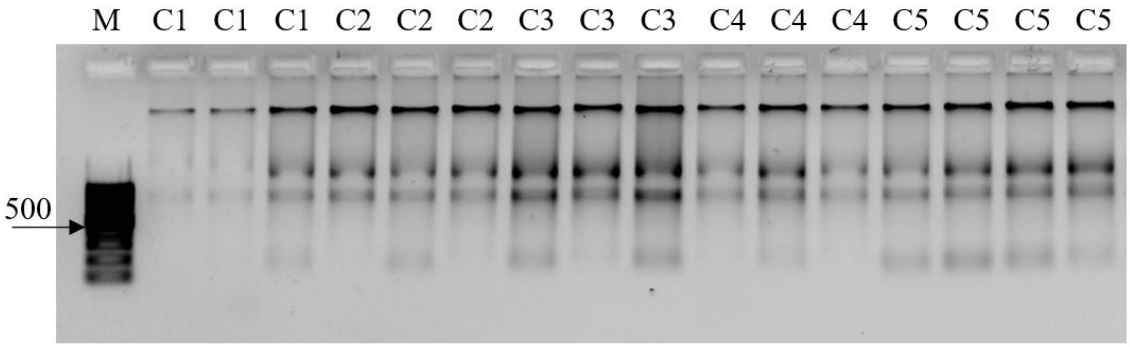

Figure 3-11: Agarose gel electrophoresis of successfully extracted DNA from inoculated avocado plantlet material. Lane M contains a 100 bp molecular marker (GeneRuler 100 bp DNA Ladder, Thermo scientific), the remaining lanes represent control plantlets in triplicate from the 'Hass' cultivar. The DNA samples were separated on a 1% agarose gel by electrophoresis.

3.4.4 Primer testing and specificity

The EF1α primers successfully amplified the DNA from all isolates tested and produced the expected sized product (**Figure 3-12)**, these were sequenced and confirmed the isolates' identity. The β-Tubulin specific primers amplified *F. euwallaceae* producing the 76 bp DNA region, and did not amplify the other *Fusarium* spp. tested (**Figure 3-13)**.

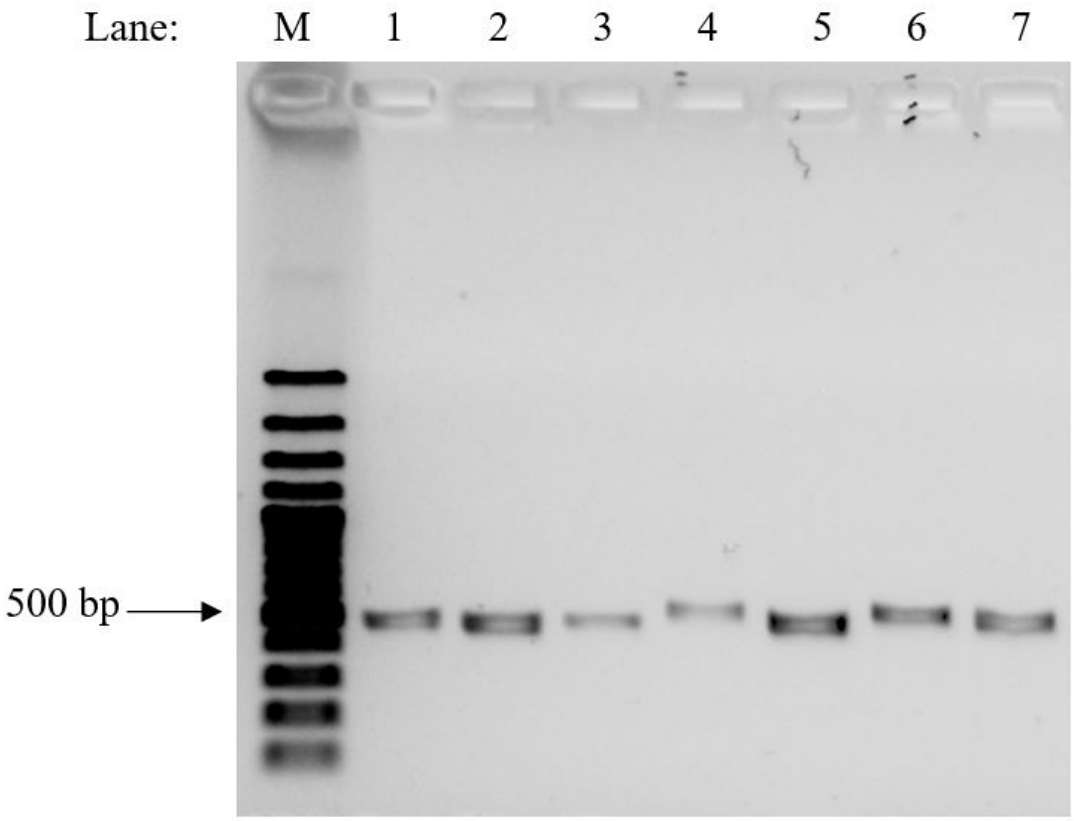

Figure 3-12: Successful amplification of DNA from *Fusarium* spp. using EF1α primers. Lane (M) contains a 100 bp molecular marker (GeneRuler 100 bp DNA Ladder, Thermo scientific), lanes 1 and 2 represent the *F. solani* isolate (CMW18782), lanes 3 and 4 represent the *F. oxysporum* isolate (CMW25503), lane 5 represents the *F. euwallaceae* isolate (CMW50555) and lanes 6 and 7 represent the *F. euwallaceae* isolate (CMW51808).

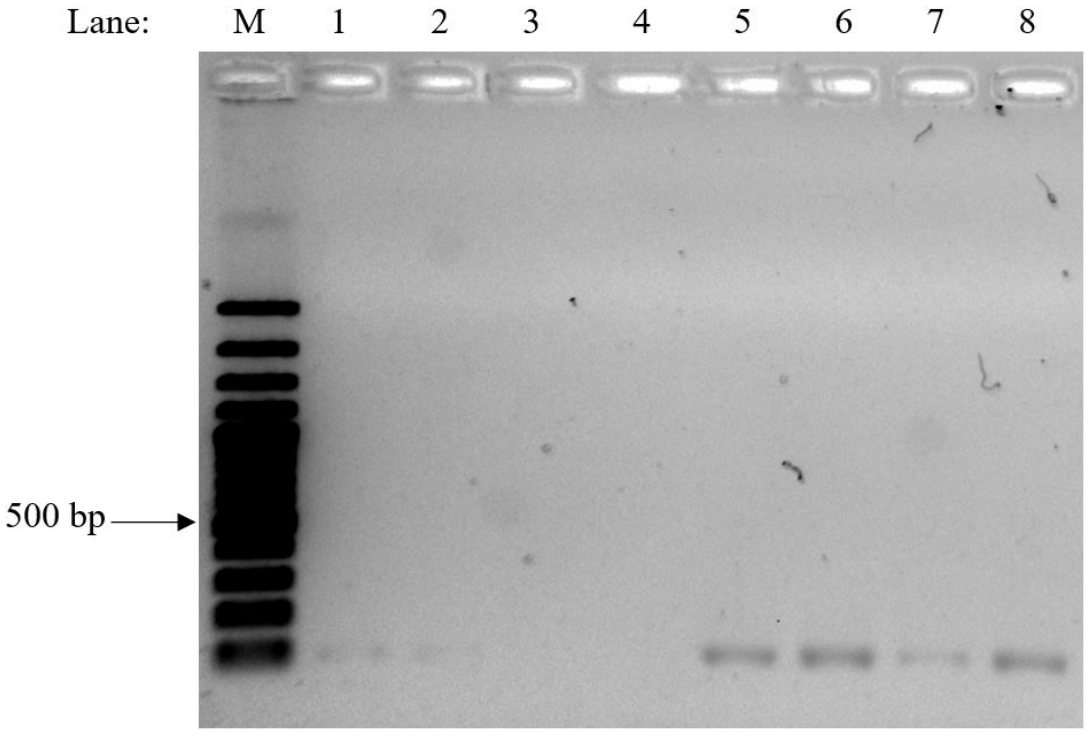

Figure 3-13: Successful amplification of DNA from *Fusarium* spp. using β-Tubulin primers (specific to *F. euwallaceae*). Lane 1 (M) contains a 100 bp molecular marker (GeneRuler 100 bp DNA Ladder, Thermo scientific), lanes 1 and 2 represent the *F. solani* isolate (CMW18782), lanes 3 and 4 represent the *F. oxysporum* isolate (CMW25503), lanes 5 & 6 represent the *F. euwallaceae isolate* (CMW50555) and lanes 7 and 8 represent the *F. euwallaceae* isolate (CMW51808).

3.4.5 Quantifying *F. euwallaceae* using real-time PCR

The Actin primers and the β-Tubulin-specific primers were tested on avocado and *F. euwallaceae* DNA respectively, at various temperatures and analysed via PCR to check the specificity of primers against the target gene. The Actin primers were optimal at all temperatures tested and the β-Tubulin specific primers demonstrated an optimal annealing temperature at and above 60˚C, as seen in **Figure 3-14** and **Figure 3-15**, respectively.

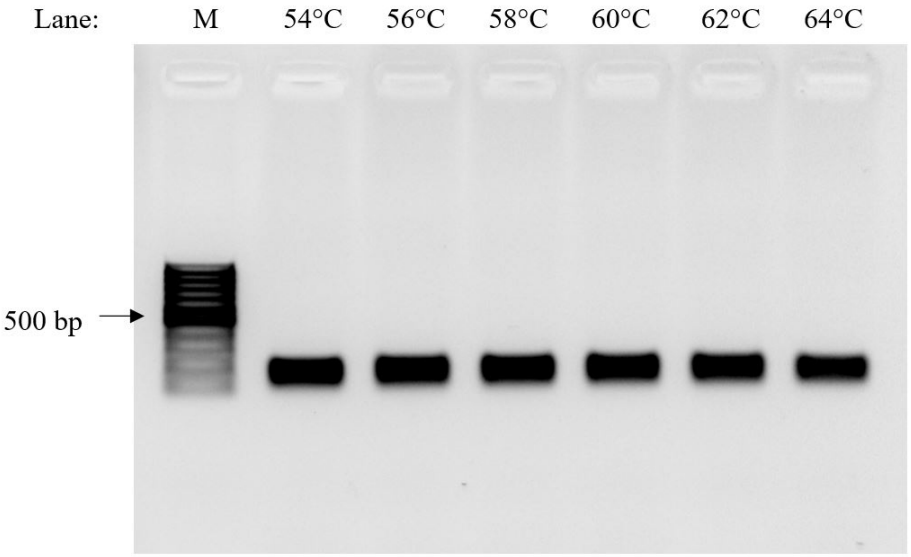

Figure 3-14: PCR optimisation of Actin primers using avocado DNA. Lane 1 (M) contains a 100 bp molecular marker (GeneRuler 100 bp DNA Ladder, Thermo scientific), the remaining lanes represent the temperatures tested from 54 °C to 64 °C. The PCR products were separated on a 1% agarose gel by electrophoresis.

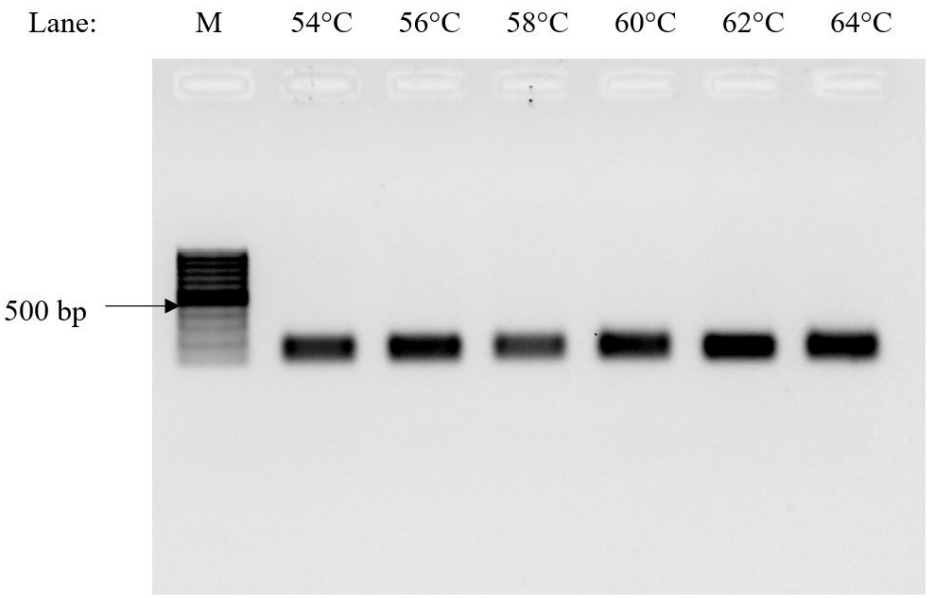

Figure 3-15: PCR optimisation of β-Tubulin primers using *Fusarium euwallaceae* DNA. Lane 1 (M) contains a 100 bp molecular marker (GeneRuler 100 bp DNA Ladder, Thermo scientific), the remaining lanes represent the temperatures tested from 54 °C to 64 °C. The PCR products were separated on a 1% agarose gel by electrophoresis.

Real-time primer pairs designed for Actin and β-Tubulin consistently amplified the expected single bands of 77 bp and 76 bp, respectively. No cross amplification was observed when Actin primers were tested on *F. euwallaceae* DNA (**Figure 3-16)** or when β-Tubulin primers were tested on avocado DNA (**Figure 3-17)**.

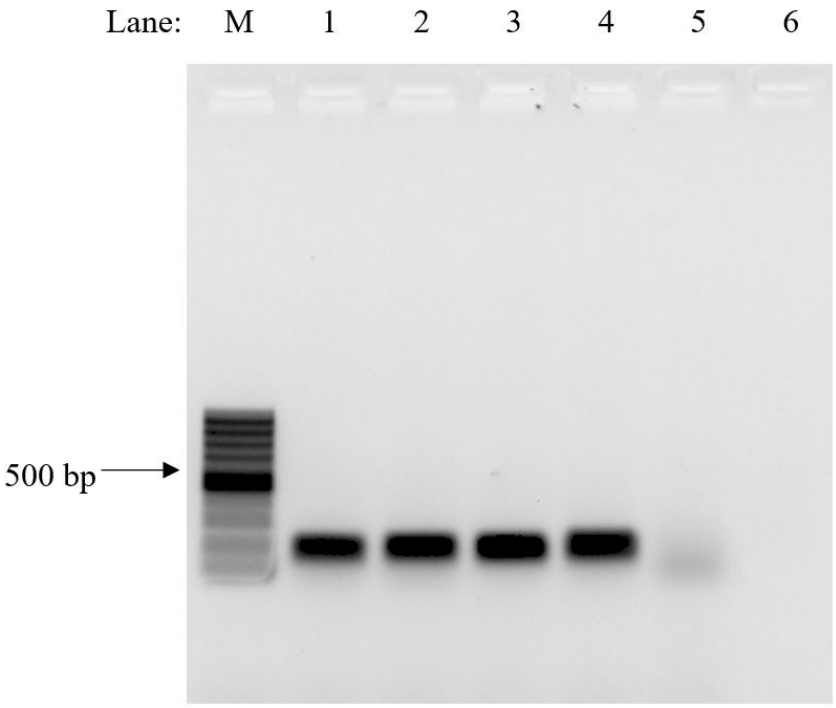

Figure 3-16: Agarose gel electrophoresis of DNA from avocado pathogenicity plantlet material and *Fusarium euwallaceae* using the Actin primer set. Lane M contains a 100 bp molecular marker (GeneRuler 100 bp DNA Ladder, Thermo scientific), lane 1 and 2 represents control avocado plantlet DNA, lane 3 and 4 represent infected avocado plantlet DNA, lane 5 represents *F. euwallaceae* DNA and lane 6 represents the negative PCR control, which did not contain a DNA template. The DNA samples were separated on a 1% agarose gel by electrophoresis. The control and infected avocado plantlet DNA showed successful amplification of the expected 77 bp length DNA region.

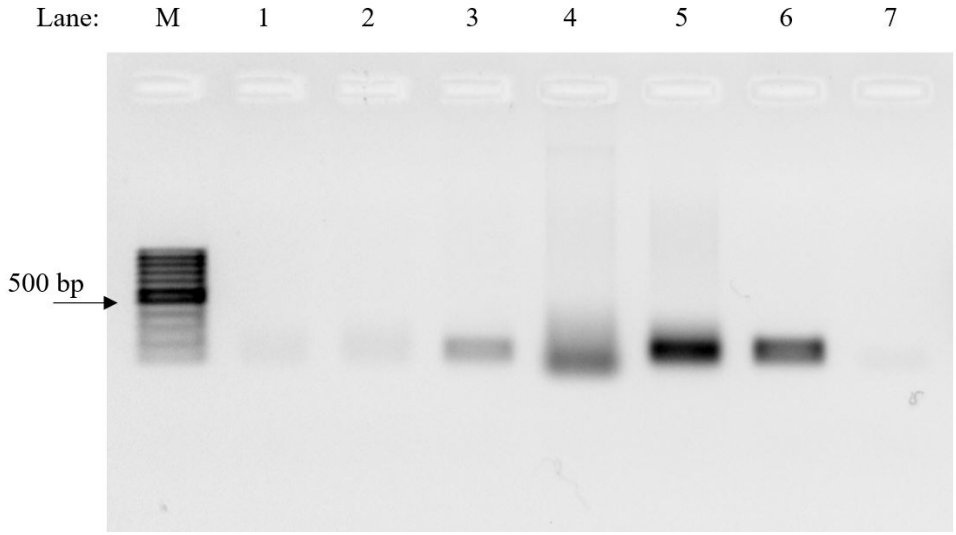

Figure 3-17: Agarose gel electrophoresis of DNA from avocado pathogenicity plantlet material and *Fusarium euwallaceae* using the β-Tubulin primer set. Lane M contains a 100 bp molecular marker (GeneRuler 100 bp DNA Ladder, Thermo scientific), lane 1 and 2 represents control avocado plantlet DNA, lane 3 and 4 represent infected avocado plantlet DNA, lane 5 and 6 represent *F. euwallaceae* DNA and lane 7 represents the negative PCR control, which did not contain a DNA template. The DNA samples were separated on a 1% agarose gel by electrophoresis. The infected avocado plantlet DNA and *F. euwallaceae* showed successful amplification of the expected 76 bp length DNA region.

The cycle threshold (Ct) values of known concentrations of 10-fold serially diluted DNA from *F. euwallaceae* and avocado were obtained and recorded (**Table 3-4)**. Linear standard regression lines were calculated from the cycle threshold values from the serial dilutions of known *F. euwallaceae* and avocado DNA concentrations. Primer pairs showed high qPCR efficiency rates with high linearity **(Figure 3-18)**. Standard regression curves revealed consistent amplification over the different concentrations of *F. euwallaceae* DNA. However, avocado DNA revealed consistent amplification, with the exception of the last order of magnitude (1:10,000 - 2 pg/µl).

Table 3-4: The cycle threshold (Ct) values generated by different concentrations of DNA from *Fusarium euwallaceae* and *Persea americana* during qPCR amplification using the primer/probe set FUEU- β-Tubulin and Actin, respectively. Technical replicates that deviated above 0.5 Cq were removed (-).

Fusarium euwallaceae **FUEU-β-Tubulin**		***Persea americana*** **Actin**	
DNA Concentration	**Ct values**	**DNA Concentration**	**Ct values**
35 ng/µl	17.53	20 ng/µl	22.60
	17.37		22.37
	17.27		22.39
3.5 ng/µl	20.82	2 ng/µl	25.53
	20.93		25.52
	20.74		25.53
350 pg/µl	24.15	200 pg/µl	29.03
	24.55		28.85
	24.34		28.94
35 pg/µl	27.80	20 pg/µl	32.80
	27.85		32.81
	27.90		-
3.5 pg/µl	-	2.0 pg/µl	NA
	31.32		
	31.59		

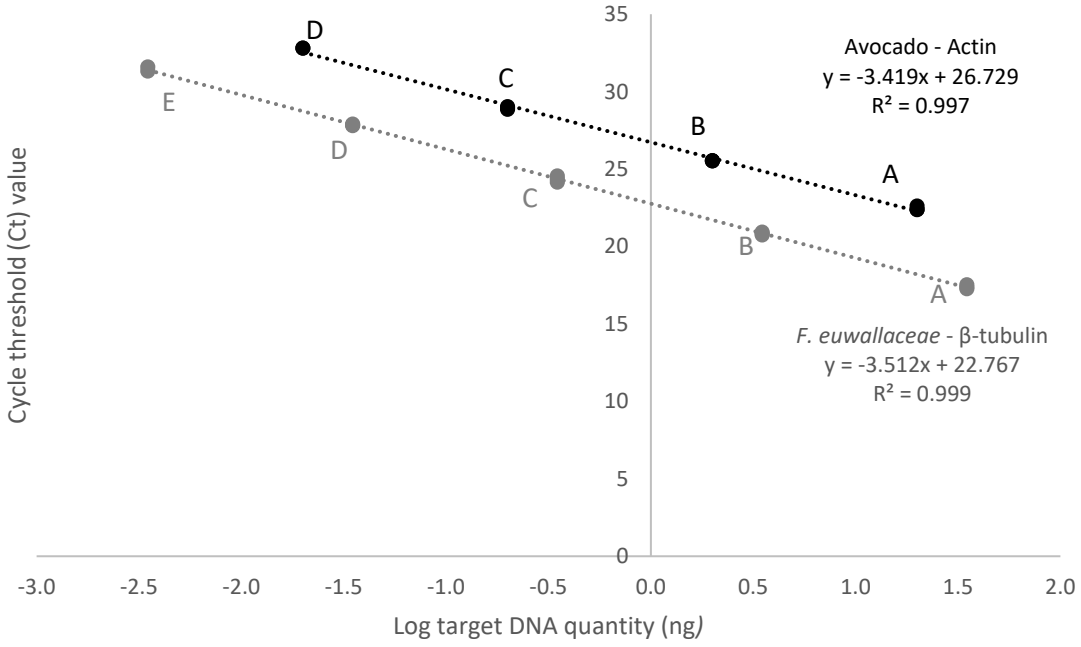

Figure 3-18: Standard regression curve plots to assess the sensitivity of the qPCR assay. The relationship between the log concentrations of DNA from a dilution series of avocado and *Fusarium euwallaceae* DNA and cycle threshold values tested for detection of target DNA using qPCR with Actin and β-Tubulin specific primers. Each datum point represents the means of triplicate samples of DNA at the standard concentrations spanning five orders of magnitude (1:0, 1:10, 1:100, 1:1,000, and 1:10,000).

All different concentrations (A-E) of DNA from *Fusarium euwallaceae* amplified (**Figure 3-19)**. No amplification was observed for the negative qPCR control, which contained sterile water in place of template DNA. Different concentrations (A-D) of DNA from avocado amplified (**Figure 3-20).** However, primer dimers were observed for the last order of magnitude (1:10,000 - 2 pg/µl). and for the negative qPCR control. This specific dilution and non-template controls were removed from analysis and graphical representations, and primer dimers were confirmed via gel electrophoresis.

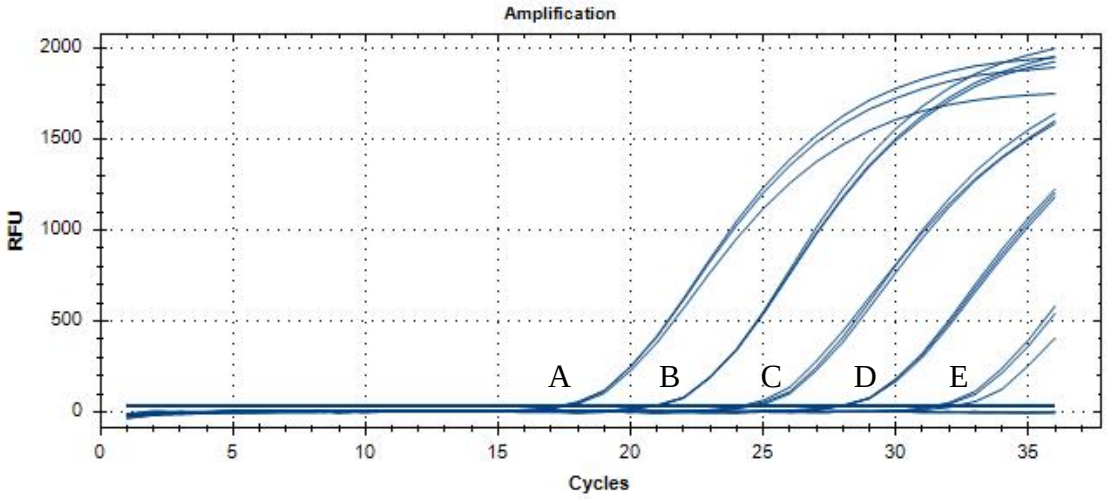

Figure 3-19: The Relative Fluorescence Units (RFU) generated per cycle from the qPCR reaction containing different concentrations (A-E) of DNA from *Fusarium euwallaceae*. No amplification was observed for the negative qPCR control, which contained sterile water in place of template DNA. All concentrations showed amplification. A: 35 ng, B: 3.5 ng, C: 350 pg, D: 35 pg, E: 3.5 pg.

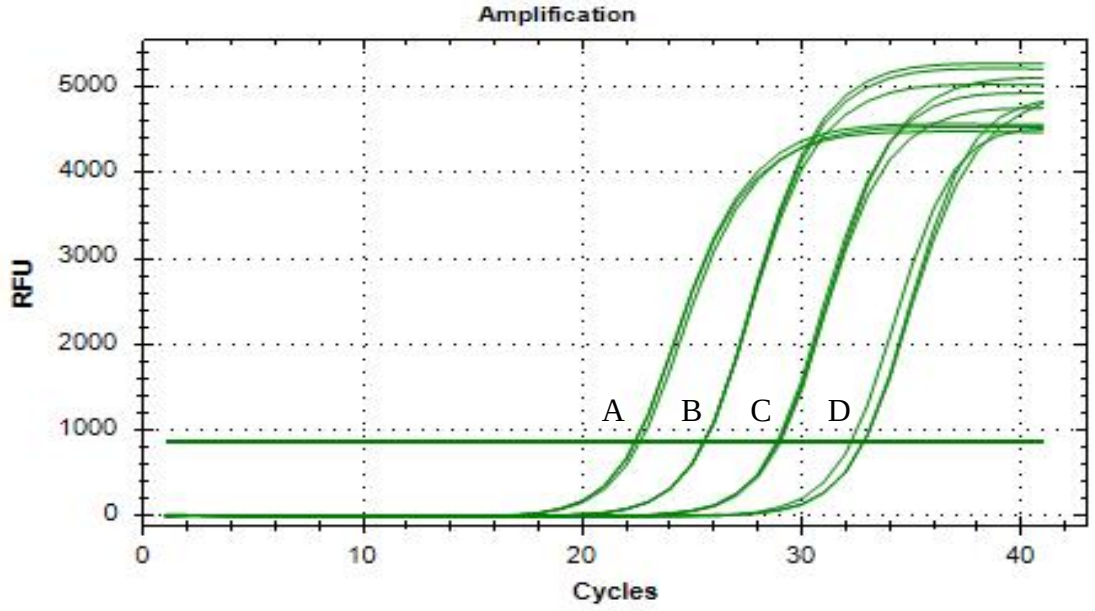

Figure 3-20: The Relative Fluorescence Units (RFU) generated per cycle from the qPCR reaction containing different concentrations (A-D) of DNA from avocado. All concentrations showed amplification. A: 20 ng, B: 2 ng, C: 200 pg, D: 20 pg.

The amount of plant DNA was quantified by using specific primers for the avocado Actin gene, and primer specificity was evaluated by a melt peak analysis, which displayed a single peak with specific melting temperatures for Actin (at 80.5°C), confirming that the primers were specific for their target sequences (**Figure 3-21)**. The qPCR was sensitive enough to detect the avocado DNA in the control and infected cultivar samples. No amplification was observed for the negative qPCR control, which contained sterile water in place of template DNA. The amount of pathogen DNA, *F. euwallaceae,* was quantified by using specific primers/probe set used for the β-Tubulin gene. The amplification plot indicates tight technical replicates that are evenly spaced (**Figure 3-22)**. The qPCR detected pathogen DNA in all infected samples. No amplification was observed for the control sample or for the negative qPCR control.

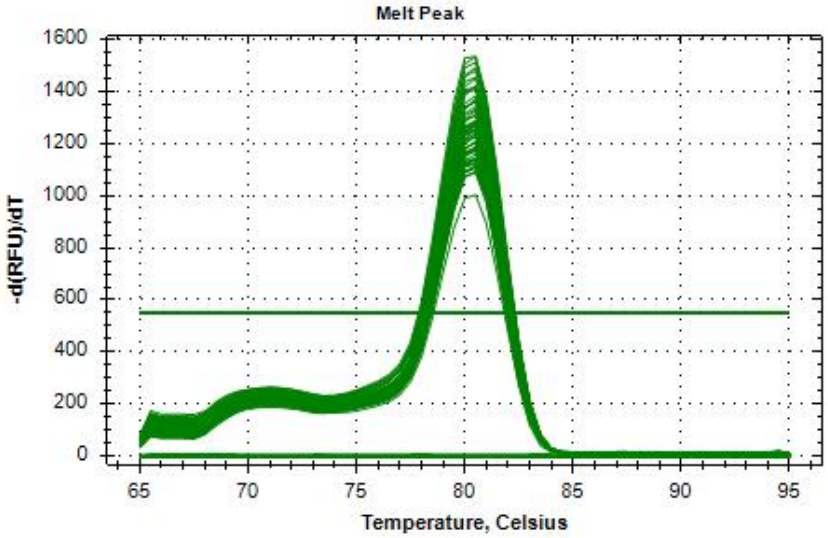

Figure 3-21: Melting curve analysis for Actin assayed by qPCR. The negative first derivative of the normalized fluorescence was plotted against the temperature to determine the melting temperature (Tm) of the amplicons generated during qPCR analysis.

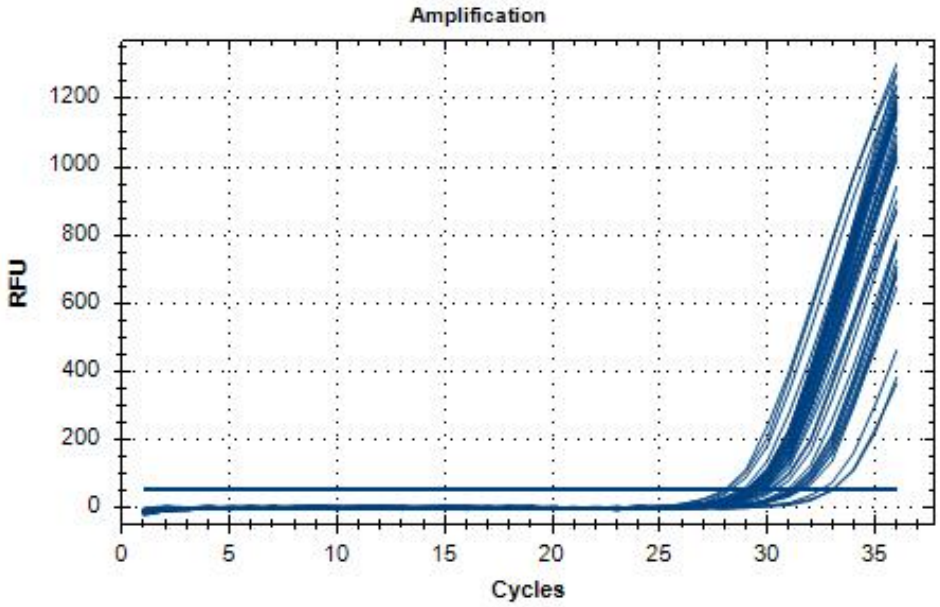

Figure 3-22: The Relative Fluorescence Units (RFU) generated per cycle from the qPCR reaction containing DNA from control and infected avocado plantlet samples using the β-Tubulin primers. No amplification was observed for the control samples or the negative qPCR control.

The qPCR success and specificity were assessed. Single qPCR products for the infected material were confirmed via gel electrophoresis (**Figure 3-23)**.

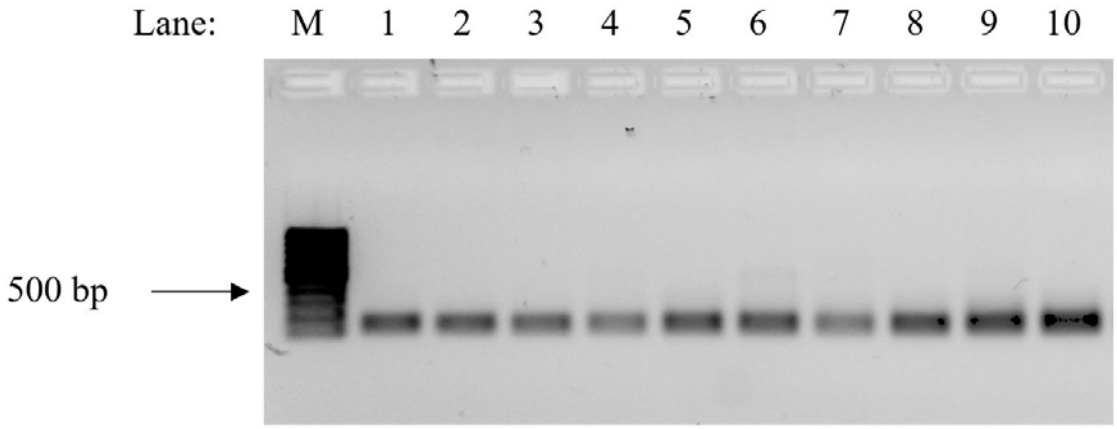

Figure 3-23: Agarose gel electrophoresis of qPCR products from infected avocado pathogenicity plantlet material using the β-Tubulin primer set. Lane M contains a 100 bp molecular marker and the remaining lanes (1-10) represent infected avocado material, which showed successful amplification of the expected 76 bp length DNA region.

The pathogen load within each cultivar was determined by comparing the amount of pathogen DNA with the amount of plant DNA. The trend of the ratio of *F. euwallaceae* DNA per plant DNA in the different cultivars was similar; with all pathogen ratios recorded under 0.3%. With 'Hass' at 0.248%, 'Maluma®' at 0.215% and the highest at 0.291% in 'Fuerte', as recorded in **Supplementary Table 3-17**. The statistical analysis for the artificially inoculated avocado pathogenicity trial compared the mean cycle threshold (Ct) values from infected samples from each cultivar. The statistical analysis indicated that the mean log target DNA quantity was not statistically significant across cultivars at $p = 0.05$ (**Figure 3-24)**. As 'Hass' is susceptible, and the pathogen loads were similar and not statistically significant, 'Maluma®' and 'Fuerte' are classified as susceptible to *F. euwallaceae*.

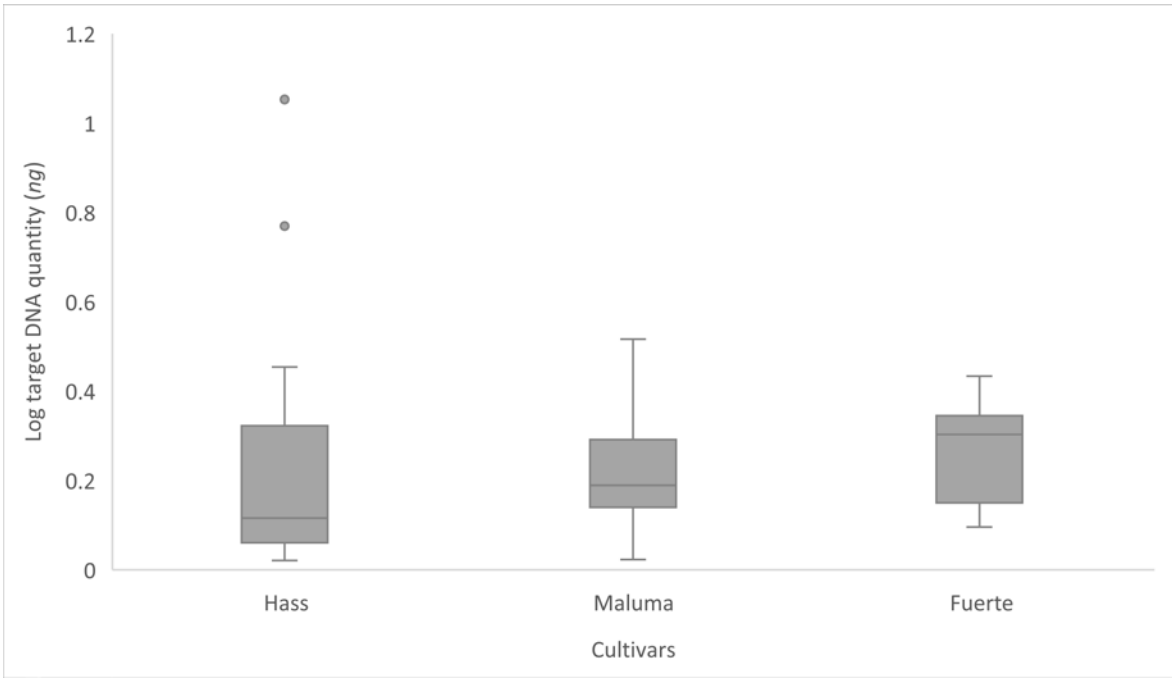

Figure 3-24: Statistical analysis of the three cultivars comparing the infected log target DNA quantity means for ‘Hass’, ‘Maluma®’ and ‘Fuerte’. * = significant difference at $p = 0.05$ across infected and control and • - significant difference at $p = 0.05$ across cultivars.

3.5 **Discussion**

The invasion of the PSHB and its fungal symbiont *F. euwallaceae*, in South Africa poses a significant threat to agriculture, particularly the avocado industry. The identification of potentially resistant cultivars would provide valuable information regarding the level of the threat posed by Fusarium dieback to existing avocado orchards. Currently, there is no susceptibility or resistance information available on popular South African avocado cultivars towards this pest-pathogen complex. Therefore, the objectives for this study were to perform a detached branch and live plantlet pathogenicity trial on avocado cultivars grown in South Africa, towards *F. euwallaceae*, with morphological and quantitative assessments to assign susceptibility or tolerance status.

The detached branch trial assessed the susceptibility of ten popular avocado cultivars and the statistical analysis of the lesion measurements indicated that majority of the screened cultivars were susceptible towards *F. euwallaceae*, with the most severely affected cultivar being ‘Hass’. In this study, ‘Pinkerton’ and ‘Fuerte’ cultivars were the least affected by the pathogen, indicating the potential resistance or tolerance towards *F. euwallaceae*. The susceptibility of ‘Hass’ is of concern since 70% of avocados produced by South African nurseries are ‘Hass’ (Donkin, 2007). Furthermore, Mendel *et al.* (2017) studied the interaction between popular avocado cultivars and the PSHB, which showed that ‘Hass’ displayed susceptibility (Mendel *et al.*, 2017). Although, Mendel *et al.* (2017) showed ‘Reed’, ‘Nabal’, and ‘Pinkerton’ to be visibly affected by the PSHB (Mendel *et al.*, 2017). The detached avocado branch trial used toothpicks (width of 2 mm) which simulated the size of the PSHB which is < 2.5 mm in size (Eskalen *et al.*, 2012). Additionally, it simulated the method of infiltration by the beetle, as the toothpick allowed for the similar

penetration of the stem. The main disadvantage of this toothpick method is the inconsistent growth of the pathogen on the surface of the toothpicks. The modification of placing one end of the branch in wax and placing the other end of the branch in moist Oasis® blocks, prevented desiccation and mimicked the water flow within live plants. Factors to consider with the detached branch trial, are the branches themselves. Firstly, branches have deformities, which could affect lesion measurements. Secondly, the thickness of the branches, and lastly, the age of the branches, which could affect the spread of the pathogen. A detached branch pathogenicity trial has limitations, as it is not a true reflection of a plantlet or in-field trial, however, it is a simple and conservative technique, that can be used to screen multiple cultivars using less space, time and money, as the wait for the plantlets are eliminated.

In this study, a live plantlet pathogenicity trial was also conducted to determine the susceptibility of three popular avocado cultivars, ‘Hass’, ‘Fuerte’ and ‘Maluma®’, towards *F. euwallaceae*, firstly by assessing lesion formation. The statistical analysis for the plantlet lesion measurements indicated that the right lesion was statistically significant across cultivars, between ‘Hass’ and ‘Fuerte’ and between ‘Hass’ and ‘Maluma®’. Therefore, reinforcing the susceptibility of the ‘Hass’ cultivar towards *F. euwallaceae*. The largest upper and lower, lesions were recorded at 20 cm (cultivar ‘Fuerte’) and 15.3 cm (cultivar ‘Hass’), respectively. Previously, van den Berg *et al.* (2018) reported average lesion lengths of 6 cm after 6 weeks on 18-month-old ‘Ettinger’ avocado plants. Whereas, Eskalen *et al.* (2012) reported lesions of 10 – 12 cm after 3 weeks on detached 1-year-old avocado shoots.

Additionally, the susceptibility of these cultivars, towards *F. euwallaceae* was determined by quantifying the amount of pathogen DNA present within each cultivar. As the amount of pathogen DNA can be correlated with the degree of tolerance or partial resistance in the host plant (Vandemark & Barker, 2003). The quantification of the amount of pathogen load was similar in all three cultivars and not statistically significant, however 'Fuerte' had highest ratio of *F. euwallaceae* DNA per plant DNA. As 'Hass' is susceptible based on available literature (Mendel *et al.*, 2017), all screened cultivars were also classified as susceptible to *F. euwallaceae*. The qPCR was developed that allowed detection limits as low as 3.5 pg of *F. euwallaceae* DNA in the stem tissues of avocado. A real-time PCR assay is a rapid, sensitive and specific technique that provides reliable data, therefore, this technique is widely used for the diagnosis of plant diseases (Gachon *et al.*, 2004).

The amount of pathogen DNA present in the cultivars were not statistically significant, this may have been affected by numerous factors. Firstly, the age and size of plantlets used in pathogenicity trials are factors to consider, as this could potentially affect pathogen progression. Previously, in avocado trees infected with *F. euwallaceae*, lesion density and gallery density index increased as branch diameter decreased (Mendel *et al.*, 2017), however, this was assessed in a mature plantation. Usually, young avocado plantlets are used for trials, therefore, the possible susceptibility of older and bigger trees towards pathogens can only be speculated during these pathogenicity trials. This, potentially explains the lack of statistical significance observed with lesion formation and quantification of pathogen DNA during the plantlet pathogenicity trial. In this study, the amount of pathogen DNA was directly normalized with the host plant DNA and, therefore, provided accurate and reliable results (Llorente *et al.*, 2010), which will contribute to a

better understanding of the interaction of avocado with *F. euwallaceae*. Furthermore, the artificial and natural inoculation of plants could also affect results. An artificial inoculation allows the study of the pathogen progression; however, it does not fully elucidate the pest-pathogen complex effect on avocado.

3.6 **Conclusion**

In summary, this study indicated that majority of the screened cultivars are susceptible to *F. euwallaceae*. Even though the qPCR analysis indicated that pathogen load was not statistically significant between cultivars, they could still be quantified. Thus, providing a potential screening tool against *F. euwallaceae* for the avocado industry. The threat of the PSHB and *F. euwallaceae* and the impact of Fusarium Dieback on avocado is serious, especially since its' detection in a commercial avocado orchard in South Africa. Complementation of this study with further beetle activity work is highly recommended as a holistic approach to a complex system is crucial in the assessment of potential risks. Future work would involve the repeat of the live avocado plantlet pathogenicity trial with older trees or seedlings and the inclusion of more cultivars. This study provides researchers and the industry with a valuable tool to quantify and screen the level of infection in avocado cultivars with varying levels of susceptibility or tolerance against *F. euwallaceae*.

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3.8 **Supplementary information**

Supplementary Table 3-1: Detached branch pathogenicity trial measurements, including the branch diameter, lesion lengths and widths for control material.

Measurement = mm	**Control 1**									**Control 2**								
		Site 1				Site 2					Site 1				Site 2			
Cultivars	Branch Diameter	Upper	Lower	Left	Right	Upper	Lower	Left	Right	Branch Diameter	Upper	Lower	Left	Right	Upper	Lower	Left	Right
DA8	30-35	5	7	0	1	8	11	0	0	25-30	10	7	1	0	10	8	1	1
Edranol	35-40	33	30	0	0	35	33	0	0	30	7	24	0	0	15	17	0	0
Fuerte	35-40	4	6	1	1	2	2	1	0	40-45	7	6	1	1	8	6	1	0
Hass	30-35	2	3	1	1	2	2	0	0	35-40	4	3	0	0	2	2	1	1
Hue	25-30	4	6	0	0	10	5	0	1	15-30	9	5	0	0	10	12	0	0
Marut	20-25	23	38	1	0	44	68	1	0	25-35	40	44	0	0	27	15	0	1
Maluma®	35	6	7	0	1	9	13	0	0	28	12	13	1	1	11	9	1	0
Pinkerton	55-60	1	1	0	0	0	0	0	0	30	3	4	0	0	3	3	1	0
Q19	25-30	1	2	0	0	2	3	1	0	25-30	4	2	0	0	1	1	1	0
Turner	35-40	5	3	1	0	6	7	1	1	35	8	6	1	0	7	8	1	1

Supplementary Table 3-2: Detached branch pathogenicity trial measurements, including the branch diameter, lesion lengths and widths for infected material.

Measurement = mm	**Infected 1**									**Infected 2**								
		Site 1				Site 2					Site 1				Site 2			
Cultivars	Branch Diameter	Upper	Lower	Left	Right	Upper	Lower	Left	Right	Branch Diameter	Upper	Lower	Left	Right	Upper	Lower	Left	Right
DA8	30-35	30	20	1	1	15	25	3	1	35	18	12	1	1	23	5	0	0
Edranol	35-40	20	18	0	0	9	49	3	2	50-55	33	25	1	1	50	32	1	1
Fuerte	45-50	15	9	1	0	4	3	0	0	37-40	4	7	0	0	5	4	0	1
Hass	40	5	5	1	1	7	12	2	1	35-40	35	35	11	7	30	35	11	12
Hue	30-50	8	11	1	2	6	10	1	2	35-50	15	10	2	2	12	7	1	0
Marut	40-50	33	24	1	1	34	25	1	1	40-45	27	29	1	1	44	30	1	1
Maluma®	45	25	18	1	0	30	23	1	1	35-40	27	24	2	0	50	62	1	1
Pinkerton	50	1	0	0	0	1	1	0	0	60	1	1	1	2	1	1	1	0
Q19	30-40	7	5	0	1	3	4	1	1	25-30	4	5	1	1	6	11	1	0
Turner	40	10	19	2	1	25	24	1	1	40-45	25	39	2	1	22	31	2	2

Supplementary Table 3-3: Detached branch pathogenicity trial measurements, including the branch diameter, lesion lengths and widths for infected material.

Measurement = mm	**Infected 3**								
		Site 1				Site 2			
Cultivars	Branch Diameter	Upper	Lower	Left	Right	Upper	Lower	Left	Right
DA8	35-40	33	35	0	1	23	19	1	1
Edranol	40-50	30	43	0	1	41	25	0	1
Fuerte	50	15	12	1	0	11	9	1	1
Hass	40-50	40	35	2	2	50	45	2	2
Hue	35-40	10	8	0	1	14	8	1	1
Marut	45-50	30	32	2	2	17	21	2	0
Maluma®	35-45	21	23	1	0	33	30	1	2
Pinkerton	45-60	2	2	0	0	1	1	0	1
Q19	30-40	8	11	1	1	9	6	1	1
Turner	35	55	39	1	1	53	40	1	1

Supplementary Table 3-4: The statistical analysis output from SAS for the detached branch pathogenicity trial, including the branch mean diameter with cultivar and infected/control as factors (Least Squares Means).

Cultivar	Mean Diameter - LSMEAN	Standard Error
DA8 - Control	30.000	10.129
DA8 - Infected	23.750	8.271
Edranol - Control	33.750	4.841
Edranol - Infected	45.000	3.953
Fuerte - Control	40.000	3.779
Fuerte - Infected	45.333	3.085
Hass - Control	35.000	2.635
Hass - Infected	40.833	2.152
Hue - Control	25.000	12.604
Hue - Infected	28.750	10.291
Marut - Control	26.250	13.199
Marut - Infected	30.750	10.777
Maluma® - Control	31.500	2.991
Maluma® - Infected	40.833	2.442
Pinkerton - Control	43.750	18.611
Pinkerton - Infected	38.417	15.196
Q19 - Control	27.500	2.500
Q19 - Infected	32.500	2.041
Turner - Control	36.250	2.320
Turner - Infected	39.167	1.894

Supplementary Table 3-5: The statistical analysis output from SAS for the detached branch pathogenicity trial, including the branch upper lesion with cultivar and infected/control as factors (Least Squares Means).

Cultivar	Upper Lesion- LSMEAN	Standard Error
DA8 - Control	8.250	2.807
DA8 - Infected	23.667	2.292
Edranol - Control	22.500	7.143
Edranol - Infected	30.500	5.832
Fuerte - Control	5.250	2.269
Fuerte - Infected	9.000	1.853
Hass - Control	2.500	7.189
Hass - Infected	27.833	5.870
Hue - Control	8.250	1.635
Hue - Infected	10.833	1.335
Marut - Control	33.500	4.677
Marut - Infected	30.833	3.818
Maluma® - Control	9.500	4.104
Maluma® - Infected	31.000	3.351
Pinkerton - Control	1.750	0.487
Pinkerton - Infected	1.167	0.397
Q19 - Control	2.000	1.013
Q19 - Infected	6.167	0.827
Turner - Control	6.500	7.194
Turner - Infected	31.667	5.874

Supplementary Table 3-6: The statistical analysis output from SAS for the detached branch pathogenicity trial, including the branch lower lesion with cultivar and infected/control as factors (Least Squares Means).

Cultivar	Lower Lesion- LSMEAN	Standard Error
DA8 - Control	8.250	4.139
DA8 - Infected	19.333	3.379
Edranol - Control	26.000	5.166
Edranol - Infected	32.000	4.218
Fuerte - Control	5.000	1.472
Fuerte - Infected	7.333	1.202
Hass - Control	2.500	6.179
Hass - Infected	27.833	5.045
Hue - Control	7.000	1.199
Hue - Infected	9.000	0.979
Marut - Control	41.250	6.868
Marut - Infected	26.833	5.608
Maluma® - Control	10.500	6.444
Maluma® - Infected	30.000	5.262
Pinkerton - Control	2.000	0.612
Pinkerton - Infected	1.000	0.500
Q19 - Control	2.000	1.275
Q19 - Infected	7.000	1.041
Turner - Control	6.000	3.579
Turner - Infected	32.000	2.923

Supplementary Table 3-7: The statistical analysis output from SAS for the detached branch pathogenicity trial, including the branch left lesion with cultivar and infected/control as factors (Least Squares Means).

Cultivar	Left Lesion- LSMEAN	Standard Error
DA8 - Control	0.500	0.468
DA8 - Infected	1.000	0.382
Edranol - Control	0.000	0.462
Edranol - Infected	0.833	0.377
Fuerte - Control	1.000	0.217
Fuerte - Infected	0.500	0.177
Hass - Control	0.50	1.903
Hass - Infected	4.833	1.553
Hue - Control	0.000	0.250
Hue - Infected	1.000	0.204
Marut - Control	0.500	0.270
Marut - Infected	1.333	0.220
Maluma® - Control	0.500	0.239
Maluma® - Infected	1.167	0.195
Pinkerton - Control	0.250	0.255
Pinkerton - Infected	0.333	0.208
Q19 - Control	0.500	0.239
Q19 - Infected	0.83	0.195
Turner - Control	1.000	0.217
Turner - Infected	1.500	0.177

Supplementary Table 3-8: The statistical analysis output from SAS for the detached branch pathogenicity trial, including the branch right lesion with cultivar and infected/control as factors (Least Squares Means).

Cultivar	Right Lesion- LSMEAN	Standard Error
DA8 - Control	0.500	0.239
DA8 - Infected	0.833	0.195
Edranol - Control	0.000	0.250
Edranol - Infected	1.000	0.204
Fuerte - Control	0.500	0.270
Fuerte - Infected	0.333	0.220
Hass - Control	0.500	1.766
Hass - Infected	4.167	1.442
Hue - Control	0.250	0.357
Hue - Infected	1.333	0.292
Marut - Control	0.250	0.293
Marut - Infected	1.000	0.239
Maluma® - Control	0.500	0.368
Maluma® - Infected	0.667	0.300
Pinkerton - Control	0.000	0.331
Pinkerton - Infected	0.500	0.270
Q19 - Control	0.00	0.161
Q19 - Infected	0.833	0.132
Turner - Control	0.500	0.239
Turner - Infected	1.167	0.195

Supplementary Table 3-9: Artificially inoculated avocado plantlet pathogenicity trail measurements, including the stem circumference, lesion lengths and widths for control material.

Measurement = mm	Hass					Fuerte					Maluma®				
	Stem Circumference	**Upper**	**Lower**	**Left**	**Right**	**Stem Circumference**	**Upper**	**Lower**	**Left**	**Right**	**Stem Circumference**	**Upper**	**Lower**	**Left**	**Right**
Control 1	25	53	13	1.5	1.5	37	34	30	2	2	30	78	56	2	3
Control 2	26	16	28	2.5	2	31	17	48	3	2	34	18	20	1	2
Control 3	30	16	22	2	1.5	29	39	20	2	2	33	55	58	2	2
Control 4	25	5	6	1.5	1.5	33	12	17	2	1.5	31	15	10	1.5	1.5
Control 5	36	28	22	2	2	29	16	16	1	2	29	24	31	1.5	3

Supplementary Table 3-10: Artificially inoculated avocado plantlet pathogenicity trail measurements, including the stem circumference, lesion lengths and widths for infected material.

Measurement = mm	Hass					Fuerte					Maluma®				
	Stem Circumference	**Upper**	**Lower**	**Left**	**Right**	**Stem Circumference**	**Upper**	**Lower**	**Left**	**Right**	**Stem Circumference**	**Upper**	**Lower**	**Left**	**Right**
Infected 1	20	31	36	2	1	41	36	37	2	1.5	41	58	40	2	2
Infected 2	29	37	23	2	1	32	28	35	1	2	32	60	37	2	2
Infected 3	42	96	88	2	1.5	29	25	13	3	3	36	55	38	1.5	2
Infected 4	28	35	64	2	2	24	77	42	1	2	34	25	22	2	2.5
Infected 5	37	76	68	1	2	49	57	40	1	1	35	45	26	2.5	1.5
Infected 6	31	76	53	1.5	2	34	200	49	2.5	2.5	33	51	60	3	3
Infected 7	33	5	62	2	1.5	32	43	56	2	2	28	48	62	1.5	1.5
Infected 8	39	64	97	2	2	33	39	56	3	2	34	52	55	2	2.5
Infected 9	35	20	31	1.5	2.5	33	148	109	3	2	31	31	19	2	2
Infected 10	22	11	4	2	1	34	32	91	3	3	30	54	49	2.5	2.5
Infected 11	36	116	153	2.5	1.5	31	17	21	2	2	31	25	30	2.5	2
Infected 12	30	18	12	2	2	34	102	42	2	2	34	55	33	3	2
Infected 13	44	98	147	3	2	31	32	65	2	2	33	51	74	1.5	2
Infected 14	37	20	46	1.5	2	49	27	49	2	2	38	28	30	2.5	2
Infected 15	33	18	33	2.5	1	26	23	79	3	2	38	94	98	1.5	2

Supplementary Table 3-11: The statistical analysis output from SAS for the artificially inoculated avocado pathogenicity trial, including the upper lesion with cultivar and infected/control as factors (Least Squares Means).

Cultivar	Upper Lesion- LSMEAN	Standard Error
Fuerte – Control	23.600	20.924
Fuerte – Infected	59.067	12.081
Hass – Control	23.60	14.818
Hass – Infected	48.067	8.555
Maluma® – Control	38.000	8.999
Maluma® - Infected	48.800	5.19

Supplementary Table 3-12: The statistical analysis output from SAS for the artificially inoculated avocado pathogenicity trial, including the lower lesion with cultivar and infected/control as factors (Least Squares Means).

Cultivar	Lower Lesion- LSMEAN	Standard Error
Fuerte – Control	26.200	10.421
Fuerte – Infected	52.267	6.017
Hass – Control	18.200	17.618
Hass – Infected	61.133	10.17
Maluma® – Control	35.000	9.669
Maluma® - Infected	44.867	5.582

Supplementary Table 3-13: The statistical analysis output from SAS for the artificially inoculated avocado pathogenicity trial, including the left lesion with cultivar and infected/control as factors (Least Squares Means).

Cultivar	Left Lesion- LSMEAN	Standard Error
Fuerte – Control	2.000	0.331
Fuerte – Infected	2.167	0.191
Hass – Control	1.900	0.209
Hass – Infected	1.967	0.121
Maluma® – Control	1.600	0.222
Maluma® - Infected	2.133	0.128

Supplementary Table 3-14: The statistical analysis output from SAS for the artificially inoculated avocado pathogenicity trial, including the right lesion with cultivar and infected/control as factors (Least Squares Means).

Cultivar	Right Lesion- LSMEAN	Standard Error
Fuerte – Control	1.900	0.201
Fuerte – Infected	2.067	0.116
Hass – Control	1.700	0.201
Hass – Infected	1.667	0.116
Maluma® – Control	2.300	0.208
Maluma® - Infected	2.100	0.120

Supplementary Table 3-15: The statistical analysis output from SAS for the artificially inoculated avocado pathogenicity trial, including the stem circumference with cultivar and infected/control as factors (Least Squares Means).

Cultivar	Stem Circumference - LSMEAN	Standard Error
Fuerte – Control	31.800	2.907
Fuerte – Infected	34.133	1.678
Hass – Control	28.400	2.821
Hass – Infected	33.067	1.629
Maluma® – Control	31.400	1.410
Maluma® - Infected	33.867	0.814

Supplementary Table 3-16: DNA concentrations of extracted avocado material performed in triplicate from the plantlet pathogenicity trial.

	SAMPLE ID	NUCLEIC ACID (NG/UL)	A260 (ABS)	A280 (ABS)	260/280	260/230
PURE MYCELIA	*F. euwallaceae (CMW51808)*	1368	2736	1295	211	100
	F. euwallaceae (CMW51808)	1646	3291	1576	209	160
CONTROL	*Hass C1 (1)*	74.7	1.493	0.730	2.05	1.31
	Hass C1 (2)	68.7	1.374	0.667	2.06	1.37
	Hass C1 (3)	27.4	0.548	0.252	2.17	2.10
	Hass C2 (1)	25.4	0.509	0.242	2.10	2.57
	Hass C2 (2)	52.9	1.058	0.496	2.13	2.14
	Hass C2 (3)	25.1	0.502	0.234	2.15	2.59
	Hass C3 (1)	46.7	0.934	0.430	2.17	2.38
	Hass C3 (2)	28.7	0.574	0.261	2.20	2.51
	Hass C3 (3)	54.2	1.084	0.506	2.14	2.33
	Hass C4 (1)	21.7	0.433	0.203	2.13	2.71
	Hass C4 (2)	31.7	0.635	0.286	2.22	2.48
	Hass C4 (3)	56.5	1.130	0.559	2.02	1.41
	Hass C5 (1)	30.7	0.615	0.307	2.00	2.06
	Hass C5 (2)	33.3	0.665	0.308	2.16	2.09
	Hass C5 (3)	35.4	0.709	0.319	2.22	2.29
	Maluma® C1 (1)	44.1	0.882	0.424	2.08	0.98
	Maluma® C1 (2)	45.6	0.911	0.423	2.15	2.39
	Maluma® C1 (3)	44.0	0.880	0.401	2.20	2.50
	Maluma® C2 (1)	40.0	0.800	0.362	2.21	2.29
	Maluma® C2 (2)	40.5	0.810	0.377	2.15	2.42
	Maluma® C2 (3)	67.2	1.344	0.643	2.09	1.84
	Maluma® C3 (1)	23.3	0.466	0.201	2.31	2.68
	Maluma® C3 (2)	22.3	0.447	0.202	2.21	2.37
	Maluma® C3 (3)	30.5	0.611	0.277	2.21	2.22
	Maluma® C4 (1)	48.0	0.959	0.450	2.13	1.88
	Maluma® C4 (2)	24.9	0.498	0.219	2.27	2.12
	Maluma® C4 (3)	62.0	1.241	0.612	2.03	0.96

	Maluma® C5 (1)	31.6	0.632	0.288	2.20	2.07
	Maluma® C5 (2)	53.4	1.068	0.502	2.13	1.86
	Maluma® C5 (3)	33.7	0.674	0.314	2.15	2.17
	Fuerte C1 (1)	55.0	1.101	0.549	2.01	1.29
	Fuerte C1 (2)	51.5	1.030	0.499	2.06	1.22
	Fuerte C1 (3)	31.5	0.630	0.283	2.22	2.38
	Fuerte C2 (1)	34.6	0.691	0.339	2.04	0.95
	Fuerte C2 (2)	39.9	0.798	0.376	2.12	0.89
	Fuerte C2 (3)	32.4	0.649	0.341	1.91	1.17
	Fuerte C3 (1)	39.8	0.796	0.399	2.00	1.21
	Fuerte C3 (2)	53.4	1.067	0.530	2.02	1.54
	Fuerte C3 (3)	36.9	0.737	0.387	1.91	1.22
	Fuerte C4 (1)	35.8	0.716	0.368	1.95	1.20
	Fuerte C4 (2)	26.0	0.521	0.261	1.99	1.16
	Fuerte C4 (3)	53.4	1.068	0.533	2.00	1.47
	Fuerte C5 (1)	44.5	0.889	0.459	1.93	1.08
	Fuerte C5 (2)	43.7	0.874	0.440	1.99	1.16
	Fuerte C5 (3)	40.3	0.807	0.405	1.99	1.21
INFECTED	*Hass I1 (1)*	30.0	0.600	0.291	2.06	1.99
	Hass I1 (2)	51.7	1.033	0.471	2.19	1.86
	Hass I1 (3)	53.8	1.076	0.521	2.07	2.05
	Hass I2 (1)	63.3	1.265	0.600	2.11	2.21
	Hass I2 (2)	59.0	1.181	0.562	2.10	2.28
	Hass I2 (3)	28.6	0.572	0.269	2.13	1.99
	Hass I3 (1)	34.9	0.698	0.353	1.98	0.12
	Hass I3 (2)	42.1	0.842	0.411	2.05	2.21
	Hass I3 (3)	35.3	0.706	0.339	2.08	2.28
	Hass I4 (1)	69.7	1.394	0.682	2.04	2.24
	Hass I4 (2)	60.1	1.202	0.572	2.10	2.28
	Hass I4 (3)	41.6	0.833	0.416	2.00	1.74
	Hass I5 (1)	46.3	0.925	0.448	2.07	2.23
	Hass I5 (2)	52.4	1.048	0.505	2.08	2.12
	Hass I5 (3)	50.2	1.005	0.490	2.05	1.81
	Hass I6 (1)	38.1	0.761	0.358	2.13	2.12

	Hass I6 (2)	30.8	0.616	0.303	2.03	2.15
	Hass I6 (3)	35.5	0.709	0.335	2.11	2.15
	Hass I7 (1)	53.2	1.063	0.514	2.07	1.99
	Hass I7 (2)	49.1	0.981	0.471	2.08	2.06
	Hass I7 (3)	45.1	0.902	0.429	2.10	2.14
	Hass I8 (1)	30.3	0.605	0.287	2.11	1.88
	Hass I8 (2)	39.5	0.790	0.373	2.12	1.94
	Hass I8 (3)	30.9	0.618	0.296	2.09	1.94
	Hass I9 (1)	27.9	0.558	0.275	2.03	2.22
	Hass I9 (2)	27.6	0.553	0.256	2.16	2.19
	Hass I9 (3)	23.0	0.460	0.226	2.04	1.17
	Hass I10 (1)	77.2	1.544	0.743	2.08	2.18
	Hass I10 (2)	70.1	1.401	0.715	1.96	2.04
	Hass I10 (3)	73.0	1.461	0.701	2.08	2.18
	Hass I11 (1)	42.0	0.841	0.403	2.08	2.09
	Hass I11 (2)	38.3	0.766	0.366	2.09	2.10
	Hass I11 (3)	44.6	0.893	0.438	2.04	2.00
	Hass I12 (1)	38.5	0.769	0.366	2.10	1.99
	Hass I12 (2)	75.6	1.511	0.745	2.03	1.30
	Hass I12 (3)	34.9	0.698	0.341	2.05	2.19
	Hass I13 (1)	48.9	0.978	0.490	2.00	1.86
	Hass I13 (2)	54.2	1.084	0.534	2.03	1.51
	Hass I13 (3)	40.0	0.800	0.392	2.04	1.66
	Hass I14 (1)	26.8	0.537	0.266	2.01	1.65
	Hass I14 (2)	46.5	0.930	0.450	2.07	1.78
	Hass I14 (3)	43.2	0.863	0.413	2.09	1.57
	Hass I15 (1)	51.6	1.032	0.501	2.06	1.74
	Hass I15 (2)	42.9	0.859	0.421	2.04	1.25
	Hass I15 (3)	40.6	0.812	0.405	2.00	0.99
	Maluma® I1 (1)	22.6	0.451	0.222	2.04	2.01
	Maluma® I1 (2)	27.3	0.545	0.271	2.01	1.90
	Maluma® I1 (3)	23.3	0.467	0.229	2.04	2.13
	Maluma® I2 (1)	45.9	0.917	0.433	2.12	2.27
	Maluma® I2 (2)	45.5	0.911	0.437	2.09	2.28
	Maluma® I2 (3)	53.3	1.066	0.502	2.12	2.22

	Maluma® I3 (1)	54.4	1.088	0.523	2.08	2.08
	Maluma® I3 (2)	53.1	1.062	0.523	2.03	1.10
	Maluma® I3 (3)	54.6	1.092	0.518	2.11	1.93
	Maluma® I4 (1)	28.3	0.565	0.288	1.96	1.97
	Maluma® I4 (2)	24.7	0.493	0.242	2.04	2.12
	Maluma® I4 (3)	29.0	0.580	0.282	2.06	2.13
	Maluma® I5 (1)	40.9	0.817	0.401	2.04	2.14
	Maluma® I5 (2)	37.4	0.748	0.363	2.06	2.12
	Maluma® I5 (3)	27.7	0.554	0.284	1.95	2.00
	Maluma® I6 (1)	44.8	0.896	0.441	2.03	2.11
	Maluma® I6 (2)	46.7	0.935	0.458	2.04	2.22
	Maluma® I6 (3)	45.0	0.900	0.440	2.05	2.25
	Maluma® I7 (1)	29.7	0.594	0.297	2.00	1.76
	Maluma® I7 (2)	50.1	1.002	0.530	1.89	0.96
	Maluma® I7 (3)	28.6	0.573	0.281	2.04	2.10
	Maluma® I8 (1)	46.0	0.919	0.448	2.05	2.06
	Maluma® I8 (2)	62.9	1.258	0.621	2.03	1.07
	Maluma® I8 (3)	62.8	1.256	0.626	2.00	1.21
	Maluma® I9 (1)	44.0	0.881	0.438	2.01	0.93
	Maluma® I9 (2)	33.2	0.664	0.318	2.08	2.17
	Maluma® I9 (3)	39.7	0.795	0.397	2.00	2.15
	Maluma® I10 (1)	42.3	0.846	0.410	2.06	2.04
	Maluma® I10 (2)	47.5	0.950	0.458	2.08	2.23
	Maluma® I10 (3)	43.3	0.866	0.413	2.10	2.17
	Maluma® I11 (1)	30.6	0.612	0.302	2.02	2.07
	Maluma® I11 (2)	35.1	0.703	0.339	2.07	2.08
	Maluma® I11 (3)	29.8	0.595	0.293	2.03	2.32
	Maluma® I12 (1)	21.5	0.431	0.226	1.90	2.09
	Maluma® I12 (2)	27.2	0.543	0.273	1.99	2.13
	Maluma® I12 (3)	35.0	0.701	0.352	1.99	1.88
	Maluma® I13 (1)	42.1	0.842	0.435	1.94	1.44
	Maluma® I13 (2)	29.9	0.599	0.309	1.94	1.13
	Maluma® I13 (3)	58.0	1.159	0.596	1.95	1.66
	Maluma® I14 (1)	32.6	0.651	0.326	2.00	0.72
	Maluma® I14 (2)	43.8	0.877	0.459	1.91	0.74

	Maluma® I14 (3)	24.6	0.493	0.265	1.86	0.86
	Maluma® I15 (1)	27.3	0.546	0.286	1.91	1.32
	Maluma® I15 (2)	29.4	0.589	0.307	1.92	0.98
	Maluma® I15 (3)	42.6	0.852	0.443	1.92	1.43
	Fuerte I1 (1)	48.3	0.966	0.495	1.95	1.13
	Fuerte I1 (2)	31.7	0.633	0.310	2.04	2.25
	Fuerte I1 (3)	27.1	0.543	0.264	2.05	1.93
	Fuerte I2 (1)	49.4	0.988	0.487	2.03	0.88
	Fuerte I2 (2)	29.2	0.584	0.289	2.02	0.72
	Fuerte I2 (3)	32.2	0.644	0.306	2.10	2.18
	Fuerte I3 (1)	43.1	0.863	0.416	2.07	2.10
	Fuerte I3 (2)	47.1	0.941	0.462	2.04	2.21
	Fuerte I3 (3)	34.8	0.695	0.345	2.01	2.18
	Fuerte I4 (1)	52.4	1.048	0.512	2.05	1.00
	Fuerte I4 (2)	77.4	1.549	0.761	2.03	1.56
	Fuerte I4 (3)	57.3	1.146	0.572	2.00	1.53
	Fuerte I5 (1)	78.0	1.561	0.784	1.99	1.59
	Fuerte I5 (2)	64.7	1.295	0.645	2.01	1.54
	Fuerte I5 (3)	58.1	1.161	0.560	2.07	1.75
	Fuerte I6 (1)	80.9	1.618	0.798	2.03	1.59
	Fuerte I6 (2)	61.8	1.235	0.626	1.97	1.18
	Fuerte I6 (3)	72.0	1.441	0.705	2.04	1.59
	Fuerte I7 (1)	74.5	1.491	0.741	2.01	1.60
	Fuerte I7 (2)	88.6	1.772	0.860	2.06	1.68
	Fuerte I7 (3)	67.8	1.357	0.672	2.02	1.52
	Fuerte I8 (1)	69.1	1.383	0.732	1.89	1.02
	Fuerte I8 (2)	35.8	0.715	0.356	2.01	1.16
	Fuerte I8 (3)	43.8	0.876	0.427	2.05	1.36
	Fuerte I9 (1)	83.1	1.663	0.810	2.05	1.75
	Fuerte I9 (2)	60.8	1.216	0.595	2.04	1.53
	Fuerte I9 (3)	48.2	0.964	0.490	1.97	1.11
	Fuerte I10 (1)	64.6	1.292	0.633	2.04	1.58
	Fuerte I10 (2)	55.4	1.107	0.552	2.01	0.99
	Fuerte I10 (3)	44.1	0.883	0.451	1.96	0.85
	Fuerte I11 (1)	37.8	0.757	0.362	2.09	1.48

	Fuerte I11 (2)	61.5	1.230	0.610	2.02	1.03
	Fuerte I11 (3)	73.4	1.469	0.721	2.04	1.34
	Fuerte I12 (1)	64.6	1.292	0.632	2.04	1.63
	Fuerte I12 (2)	70.9	1.419	0.700	2.03	1.44
	Fuerte I12 (3)	61.0	1.220	0.601	2.03	1.53
	Fuerte I13 (1)	88.6	1.773	0.846	2.09	1.45
	Fuerte I13 (2)	92.5	1.849	0.888	2.08	1.58
	Fuerte I13 (3)	63.7	1.275	0.607	2.10	1.36
	Fuerte I14 (1)	46.3	0.925	0.445	2.08	1.08
	Fuerte I14 (2)	41.4	0.827	0.401	2.06	0.99
	Fuerte I14 (3)	45.3	0.906	0.430	2.11	1.29
	Fuerte I15 (1)	53.9	1.078	0.518	2.08	1.29
	Fuerte I15 (2)	39.8	0.796	0.386	2.06	1.07
	Fuerte I15 (3)	56.1	1.121	0.557	2.01	1.20

Supplementary Table 3-17: The quantification data from control and infected avocado plantlet material. The cycle threshold (Cq), mean cycle threshold, the Log (x), the total DNA and the average pathogen DNA for each cultivar. The slope-intercept equation is: y = mx + c, having a gradient of m and a y-intercept of c.

Hass - Actin			y = mx+c c = 26.729 m = -3.419		**Hass - Pathogen**			y = mx+c c = 22.767 m = -3.512			**Average (Path DNA) = 0.248**
Well	**Cq**	**Mean Cq**	**x**	**Log(x)**	**Well**	**Cq**	**Cq Mean**	**x**	**Log(x)**	**Total DNA**	**Pathogen/Total DNA *100**
C1	23.979	23.998	0.799	6.293	**C1**						
C1	23.934				**C1**						
C1	24.080				**C1**						
C2	23.547	23.775	0.864	7.312	**C2**						
C2	23.736				**C2**						
C2	24.042				**C2**						
C3	24.358	24.342	0.698	4.991	**C3**						
C3	24.286				**C3**						
C3	24.382				**C3**						
C4	24.767	24.652	0.608	4.051	**C4**						
C4	24.560				**C4**						
C4	24.629				**C4**						
C5	24.805	24.730	0.585	3.843	**C5**						
C5	24.538				**C5**						

C5	24.848				**C5**						
NTC					**NTC**						
NTC					**NTC**						
NTC					**NTC**						
I1	24.319				**I1**	28.529					
I1	24.241	24.344	0.698	4.984	**I1**	28.585	28.539	-1.644	0.023	5.007	0.454
I1	24.472				**I1**	28.504					
I2	24.451				**I2**	27.580					
I2	24.433	24.433	0.671	4.693	**I2**	27.810	27.821	-1.439	0.036	4.729	0.769
I2	24.416				**I2**	28.072					
I3	24.530				**I3**	31.562					
I3	24.564	24.575	0.630	4.265	**I3**	-	31.577	-2.508	0.003	4.268	0.073
I3	24.632				**I3**	31.592					
I4	24.194				**I4**	32.217					
I4	24.031	24.111	0.766	5.831	**I4**	32.006	32.111	-2.661	0.002	5.833	0.037
I4	24.108				**I4**	-					
I5	24.039				**I5**	30.239					
I5	24.058	24.075	0.776	5.972	**I5**	30.336	30.291	-2.142	0.007	5.979	0.121
I5	24.130				**I5**	30.297					
I6	24.571				**I6**	31.596					
I6	24.670	24.587	0.626	4.230	**I6**	31.790	31.695	-2.542	0.003	4.233	0.068
I6	24.521				**I6**	31.699					
I7	24.081	24.094	0.771	5.900	**I7**	30.190	30.368	-2.164	0.007	5.907	0.116

I7	24.063				**I7**	30.636					
I7	24.137				**I7**	30.277					
I8	24.514				**I8**	-					
I8	24.703	24.582	0.628	4.246	**I8**	32.045	32.054	-2.644	0.002	4.248	0.053
I8	24.529				**I8**	32.064					
I9	24.793				**I9**	30.146					
I9	24.698	24.839	0.553	3.571	**I9**	30.339	30.173	-2.109	0.008	3.579	0.218
I9	25.027				**I9**	30.034					
I10	23.628				**I10**	28.463					
I10	23.627	23.658	0.898	7.911	**I10**	28.672	28.634	-1.671	0.021	7.933	0.269
I10	23.719				**I10**	28.767					
I11	24.243				**I11**	27.081					
I11	24.365	24.305	0.709	5.117	**I11**	27.337	27.206	-1.264	0.054	5.172	1.053
I11	24.307				**I11**	27.202					
I12	23.938				**I12**	32.704					
I12	23.744	23.846	0.843	6.972	**I12**	32.672	32.551	-2.786	0.002	6.974	0.023
I12	23.855				**I12**	32.277					
I13	23.914				**I13**	28.235					
I13	23.810	23.837	0.846	7.010	**I13**	28.425	28.308	-1.578	0.026	7.037	0.376
I13	23.788				**I13**	28.263					
I14	24.177				**I14**	31.129					
I14	24.206	24.242	0.727	5.337	**I14**	31.075	31.150	-2.387	0.004	5.341	0.077
I14	24.345				**I14**	31.246					

I15	24.100				**I15**	33.161					
I15	24.063	24.094	0.771	5.897	**I15**	-	33.000	-2.914	0.001	5.899	0.021
I15	24.119				**I15**	32.839					

Fuerte - Actin			y = mx+c		**Fuerte - Pathogen**			y = mx+c			**Average (Path DNA) =0.291**
			c = 26.729					c = 22.767			
			m = -3.419					m = -3.512			
Well	**Cq**	**Mean Cq**	**x**	**Log(x)**	**Well**	**Cq**	**Cq Mean**	**x**	**Log(x)**	**Total DNA**	**Pathogen/Total DNA *100**
C1	24.417				**C1**						
C1	24.318	24.376	0.688	4.878	**C1**						
C1	24.393				**C1**						
C2	24.755				**C2**						
C2	24.683	24.746	0.580	3.802	**C2**						
C2	24.799				**C2**						
C3	24.752				**C3**						
C3	24.695	24.706	0.592	3.906	**C3**						
C3	24.671				**C3**						
C4	24.756				**C4**						
C4	24.412	24.538	0.641	4.374	**C4**						
C4	24.446				**C4**						
C5	24.706	24.610	0.620	4.166	**C5**						
C5	24.467				**C5**						

C5	24.658				**C5**						
NTC					**NTC**						
NTC					**NTC**						
NTC					**NTC**						
I1	25.345				**I1**	29.856					
I1	25.180	25.291	0.421	2.634	**I1**	-	30.082	-2.083	0.008	2.643	0.313
I1	25.346				**I1**	30.307					
I2	25.242				**I2**	29.940					
I2	25.095	25.120	0.471	2.955	**I2**	-	30.004	-2.061	0.009	2.964	0.294
I2	25.024				**I2**	30.067					
I3	25.263				**I3**	32.098					
I3	25.275	25.294	0.420	2.629	**I3**	31.817	31.892	-2.598	0.003	2.632	0.096
I3	25.343				**I3**	31.763					
I4	-				**I4**	28.698					
I4	24.450	24.508	0.650	4.462	**I4**	28.789	28.779	-1.712	0.019	4.481	0.433
I4	24.567				**I4**	28.851					
I5	24.748				**I5**	31.026					
I5	24.643	24.688	0.597	3.953	**I5**	31.379	31.173	-2.394	0.004	3.957	0.102
I5	24.673				**I5**	31.114					
I6	24.701				**I6**	29.199					
I6	24.564	24.592	0.625	4.218	**I6**	29.153	29.167	-1.822	0.015	4.233	0.356
I6	24.511				**I6**	29.149					
I7	24.278	24.318	0.705	5.072	**I7**	32.168	32.131	-2.666	0.002	5.074	0.043

I7	24.311				**I7**	31.950					
I7	24.365				**I7**	32.273					
I8	25.069				**I8**	31.178					
I8	25.108	25.048	0.492	3.102	**I8**	31.056	31.091	-2.370	0.004	3.106	0.137
I8	24.967				**I8**	31.040					
I9	24.849				**I9**	30.495					
I9	24.590	24.782	0.569	3.711	**I9**	30.881	30.617	-2.235	0.006	3.716	0.157
I9	24.907				**I9**	30.475					
I10	25.157				**I10**	28.298					
I10	24.943	25.054	0.490	3.090	**I10**	28.583	28.445	-1.617	0.024	3.114	0.776
I10	25.062				**I10**	28.454					
I11	24.946				**I11**	30.760					
I11	24.780	24.807	0.562	3.649	**I11**	30.649	30.566	-2.221	0.006	3.655	0.165
I11	24.695				**I11**	30.291					
I12	24.483				**I12**	30.123					
I12	24.295	24.389	0.684	4.836	**I12**	30.125	30.104	-2.089	0.008	4.844	0.168
I12	-				**I12**	30.065					
I13	24.380				**I13**	28.720					
I13	24.201	24.276	0.717	5.218	**I13**	28.445	28.541	-1.644	0.023	5.240	0.433
I13	24.246				**I13**	28.458					
I14	24.331				**I14**	28.016					
I14	24.269	24.295	0.712	5.153	**I14**	27.578	27.757	-1.421	0.038	5.191	0.731
I14	24.284				**I14**	27.677					

I15	24.545				**I15**	30.170					
I15	24.347	24.441	0.669	4.668	**I15**	-	30.156	-2.104	0.008	4.676	0.168
I15	24.432				**I15**	30.141					

Maluma® - Actin			y = mx+c		**Maluma® - Pathogen**			y = mx+c			**Average (Path DNA) = 0.215**
			c = 26.729					c = 22.767			
			m = -3.419					m = -3.512			
Well	**Cq**	**Mean Cq**	**x**	**Log(x)**	**Well**	**Cq**	**Cq Mean**	**x**	**Log(x)**	**Total DNA**	**Pathogen/Total DNA *100**
C1	24.139				**C1**						
C1	24.103	24.152	0.754	5.671	**C1**						
C1	24.214				**C1**						
C2	23.951				**C2**						
C2	23.986	24.009	0.795	6.243	**C2**						
C2	24.092				**C2**						
C3	24.836				**C3**						
C3	24.949	24.939	0.524	3.338	**C3**						
C3	25.033				**C3**						
C4	23.964				**C4**						
C4	23.817	23.882	0.833	6.802	**C4**						
C4	23.867				**C4**						
C5	24.507	24.578	0.629	4.258	**C5**						
C5	24.383				**C5**						

C5	24.843				**C5**						
NTC					**NTC**						
NTC					**NTC**						
NTC					**NTC**						
I1	24.899				**I1**	30.816					
I1	24.887	24.991	0.508	3.223	**I1**	31.045	31.005	-2.346	0.005	3.227	0.140
I1	25.188				**I1**	31.156					
I2	24.498				**I2**	31.308					
I2	24.531	24.494	0.654	4.506	**I2**	31.339	31.344	-2.442	0.004	4.509	0.080
I2	24.453				**I2**	31.386					
I3	24.023				**I3**	30.316					
I3	24.224	24.183	0.745	5.556	**I3**	30.138	30.167	-2.107	0.008	5.564	0.140
I3	24.300				**I3**	30.048					
I4	24.697				**I4**	29.844					
I4	24.480	24.582	0.628	4.246	**I4**	29.566	29.710	-1.977	0.011	4.256	0.248
I4	24.569				**I4**	29.720					
I5	24.300				**I5**	29.369					
I5	24.541	24.483	0.657	4.540	**I5**	29.250	29.355	-1.876	0.013	4.553	0.292
I5	24.608				**I5**	29.447					
I6	24.167				**I6**	32.995					
I6	24.314	24.244	0.727	5.333	**I6**	-	32.998	-2.913	0.001	5.334	0.023
I6	24.249				**I6**	33.001					
I7	23.397	23.465	0.955	9.006	**I7**	29.005	29.031	-1.784	0.016	9.023	0.182

I7	23.535				**I7**	29.025					
I7	23.464				**I7**	29.064					
I8	23.887				**I8**	29.530					
I8	24.032	23.931	0.818	6.582	**I8**	29.591	29.454	-1.904	0.012	6.594	0.189
I8	23.874				**I8**	29.242					
I9	24.074				**I9**	28.121					
I9	23.998	24.129	0.760	5.760	**I9**	27.993	28.121	-1.524	0.030	5.790	0.516
I9	24.316				**I9**	28.249					
I10	24.094				**I10**	28.977					
I10	24.238	24.214	0.736	5.439	**I10**	29.086	29.070	-1.795	0.016	5.455	0.294
I10	24.311				**I10**	29.147					
I11	24.289				**I11**	29.065					
I11	24.429	24.374	0.689	4.884	**I11**	29.297	29.251	-1.846	0.014	4.898	0.291
I11	24.404				**I11**	29.392					
I12	24.299				**I12**	31.097					
I12	24.176	24.270	0.719	5.237	**I12**	31.161	31.266	-2.420	0.004	5.241	0.073
I12	24.336				**I12**	31.539					
I13	24.266				**I13**	-					
I13	24.225	24.288	0.714	5.175	**I13**	29.009	29.124	-1.810	0.015	5.191	0.298
I13	24.373				**I13**	29.240					
I14	23.471				**I14**	29.123					
I14	23.506	23.577	0.922	8.353	**I14**	29.066	29.111	-1.806	0.016	8.369	0.187
I14	23.754				**I14**	29.145					

I15	24.409				**I15**	29.443					
I15	24.612	24.550	0.637	4.338	**I15**	29.576	29.542	-1.929	0.012	4.350	0.271
I15	24.629				**I15**	29.606					

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CHAPTER 4 **Concluding remarks**

4.1 **Concluding discussion**

Avocado is an important economical crop, that has rapidly expanded both in South Africa and worldwide. Avocado breeding previously involved the identification and selection of new individuals with valuable physical characteristics, unfortunately, this is a lengthy process that can take over 20 years (Köhne, 2005). Over time as technology advanced, avocado breeding programmes began using molecular markers to determine genetic diversity (Ashworth & Clegg, 2003, Chen *et al.*, 2009, Gross-German & Viruel, 2013, Boza *et al.*, 2018), clarify genetic relationships (Lavi *et al.*, 1993, Mhameed *et al.*, 1997, Schnell *et al.*, 2003) and attempt to accelerate breeding cycles. This information provides insight into the genetic variation of avocado, as well as the mechanisms underlying improved performance against biotic and abiotic factors (Schaffer *et al.*, 2013). This is important as it ensures that the species can adapt to different environmental conditions and stressors in the form of resistance to existing and new threats. The South African avocado industry remains on high alert following the detection of a new threat - the Polyphagous Shot Hole Borer (PSHB) and its' fungal symbiont *Fusarium euwallaceae* - on a backyard avocado tree in 2018 (van den Berg *et al.*, 2018). However, in 2021, the PSHB was detected in a commercial orchard in the Western Cape (Unpublished data). This is of concern due to the limitations of control management strategies for the pest-pathogen complex, the susceptibility or resistance status of popular cultivars is vital to the avocado industry.

Currently, no genetic marker information or genetic population studies have been performed on avocado germplasms in South Africa. Therefore, the first aim of this study

was to address this knowledge gap. This was achieved by implementing molecular marker technologies for the validation of clonal material, verification of horticultural varieties and determining the genetic diversity and population structure of an avocado cultivar germplasm in South Africa. This study identified mislabelled individuals and validated clonal material; such is vital to prevent the future propagation of incorrect material. Additionally, the verification of horticultural variety of individuals and hybrids is useful as it is related to important horticultural traits that may be selected for in the future. Lastly, cultivar breeding efficiency may be improved by aiding in the selection of avocado with the ability to cope with changing environments and emerging pests and pathogens. Future work should involve the curation of more avocado germplasms in South Africa to provide a more accurate description and understanding of the population and create a link between the genotypic data and phenotypic data.

Likewise, very little phenotypic information is available on avocado cultivar resistance towards the PSHB and *F. euwallaceae*. Therefore, the secondary aim of this study was to perform a detached branch pathogenicity trial to evaluate ten fruiting cultivars towards *F. euwallaceae*, by assessing lesion measurements. Additionally, the final aim was to perform a plantlet pathogenicity trial to evaluate three fruiting cultivars using a molecular quantification tool for *F. euwallaceae*, in order to determine resistance of these accessions in the cultivar germplasm. This study revealed that majority of the screened cultivars were susceptible towards *F. euwallaceae*. Information on the susceptibility and resistance of cultivars towards this pathogen will aid in the selection of resistant accessions for future breeding, which is vital for the preservation and protection of the avocado industry worldwide. This study focused on the interaction of avocado and *F. euwallaceae* only;

however, we know that the PSHB carries two additional fungal symbionts, *Graphium euwallaceae,* and *Paracremonium pembeum*. Future work should include these additional fungal symbionts as well as their combined effect on the host plant. Additionally, further beetle activity work should be performed, as this would provide insight into the pathogen interactions as a whole. Future work should also involve the repeat of the live avocado plantlet pathogenicity trial with older trees or seedlings, thus providing a more complete assessment of this threat to established avocado orchards in the country. Lastly, this study provides researchers and the industry with a valuable tool to quantify and screen the level of susceptibility of avocado cultivars against *F. euwallaceae.*

Regrettably, this study could not link the *F. euwallaceae* susceptible phenotype to the genotypic SNP data for the screened cultivars, creating this link could aid in the breeding of resistance. Future work should include the screening of more cultivars, so that their resistance or susceptibility status can be determined and linked to the SNP genotypic data. To reiterate, the avocado industry in South Africa is currently under severe threat from this pest-pathogen complex, necessitating the urgent investment in molecular marker research and pathogenicity trials to better understand and identify potential sources of resistance.

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