Biocontrol of Postharvest Pathogens Infecting Avocado using Endophytic Trichoderma Species

By

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Dissertation Summary

Pre-harvest fungal infection causes both pre- and postharvest avocado (*Persea americana* Mill.) diseases. Some foliar diseases also cause avocado fruit spots, including those caused by *Pseudocercospora* and *Cladosporium* species. Pre- and postharvest rots of avocado fruit are largely caused by *Colletotrichum, Lasiodiplodia* and *Neofusicoccum* species, and these are also latent pathogens that usually infect the fruit in the orchards before harvest. These fungal pathogens cause crop losses in South African production in untreated fruit by about 50-90%. As a result, several agrochemicals including prochloraz, copper oxychloride, thiophanate-methyl and thiabendazole are being used by farmers for avocado disease management. However, the intensive use of chemicals does not prevent all diseases, and toxic residues affect the environment and consumers of fruit containing residues. As a result, there are growing restrictions on the use of agrochemicals being exported to the European Union (EU), due to reductions in the Maximum Residue Levels (MRLs) of most of these fungicides. The use of biological control agents (BCAs) such as *Trichoderma* species has been regarded as a promising and environmentally friendly approach to controlling plant diseases. *Trichoderma* species are opportunistic, avirulent plant symbionts and some strains have been developed commercially as BCAs for use on many crops against a wide range of plant pathogens. Their modes of action are complex and include antibiosis, mycoparasitism, producing bioactive secondary metabolites, inducing plant defensive mechanisms and promoting plant growth. Therefore, the overall objective of this study was to isolate and screen endophytic strains of *Trichoderma* species to control fruit diseases of avocado such as stem-end rot, anthracnose and leaf spot diseases.

Isolation and Identification of Fungal Pathogens

Avocado fruit from different supermarkets in Pietermaritzburg (KwaZulu-Natal, South Africa) that displayed symptoms of avocado fruit rot and fruit spot were collected and brought to the Plant Pathology Laboratory at the University of KwaZulu-Natal for fungal isolation. A total of forty-five isolates were isolated from symptomatic avocado fruit with typical characteristics of anthracnose, stem-end rot, Cladosporium spot, and Cercospora spot. The isolates were identified based on their cultural and morphological characteristics using light microscopy and scanning electron microscopy (SEM). Koch's postulates screening was performed on fresh avocado fruit by spraying the fruit with suspensions of the key fungal pathogens of anthracnose, stem-end rot, and leaf spot. Frequently isolated colonies causing anthracnose on avocado fruit were divided into 2 morphological groups (Cs1 and Cs2). Colonies of Group 3 (Ls1) constituted 35% of the fungal pathogens isolated from fruit displaying stem-end rot. Group 4 (Ls2) was a *Lasiodiplodia* species that was provided by Majola (2020) and freshly isolated colonies of this genus. In Group 5 were isolates of *Pseudocercospora* and *Cladosporium* species, which constituted about 20% of the cultures.

All the fungal isolates were subjected to a pathogenicity assay, conducted twice, to confirm the pathogenicity of each isolate. Anthracnose pathogens in Group 1 (Cs1) were the most virulent strains, while Group 2 (Cs2) were the least virulent. Stem-end rot pathogens in Group 3 (Ls1) were more virulent compared to Group 4 strains (Ls2). Some inoculated fruit did not show any symptoms, even after 14 days post inoculation (dpi). Morphological characterization showed that *Pseudocercospora* species and *Cladosporium* spp. were the least virulent strains. Molecular identification of the fungi was undertaken using ITS sequence analysis. Isolate Cs1 was identified as *Colletotrichum cobbittiense* and isolate Cs2 was identified as *Colletotrichum henanense.* The most virulent isolate causing stem-end rot was Isolate Ls1, which was identified as *Neofusicoccum parvum*, a pathogen that was not previously recognized as being of importance in KwaZulu-Natal, South Africa. This suggested that this fungus is probably causing severe postharvest losses for avocado farmers in KwaZulu-Natal. Isolate Ls2 was identified in a previous study as *Lasiodiplodia mahajangana* using ITS1 and ITS2 gene sequence analysis.

Isolation and Endophytic Screening of *Trichoderma* species

Avocado leaves were sampled from the five avocado trees of the cultivar Fuerte, which are growing at Ukulinga farm, University of KwaZulu-Natal, Pietermaritzburg. From these trees, sixty leaves were sampled, and five fresh avocado fruits from a local supermarket in Scottsville, Pietermaritzburg were used as plant material for the isolation of *Trichoderma* species. Colony colour differences and radial growth measurements were two characteristics that were used to differentiate between the strains of *Trichoderma* species. A screening for endophytic activity of the isolated *Trichoderma* strains was conducted on fresh avocado fruit and seedlings. Fifty-two avocado seedlings of the cultivar Edranol were transplanted into pots in a greenhouse and sprayed with benomyl to kill any natural endophytic *Trichoderma* spp. found in them.

The seventeen *Trichoderma* strains isolated previously were used to prepare suspensions with a concentration of $1x10^6$ conidia/ml^{-1}. The *Trichoderma* suspensions were sprayed on the seedlings and after two weeks the seedling leaves were sampled randomly and surface-sterilised using 2% sodium hypochlorite and sterile distilled water. Small segments were placed on *Trichoderma* selective medium (TSM) agar plates to check for the presence of the *Trichoderma*. During this study, only eleven strains of *Trichoderma spp.* were found to be

endophytic. Another screening was then done on fresh avocado fruits. The fruits were sprayed with suspensions of the eleven endophytic *Trichoderma* strains. After a seven-day waiting period, the fruit was surface-sterilized, and the skin of the fruit was sampled in ten places per fruit and plated onto TSM. Pure cultures were purified on Potato Dextrose Agar (PDA) and monitored every second day to record the growth. A total of nine isolates of *Trichoderma* demonstrated high endophytic ability.

Assessment of the Effect of Endophytic *Trichoderma* Strains on Anthracnose and Stem-end rot *in vivo*

An *in vivo* investigation was conducted to understand the antagonistic activity of endophytic *Trichoderma* as a potential biocontrol agent against anthracnose and stem-end rot. Fresh avocado fruit of the cultivar Hass were collected from a local farm in Richmond (KwaZulu-Natal, South Africa). The fruit were sprayed with *Trichoderma* isolates conidial suspensions at concentration $1x10^6$ conidia/ml^{-1} and a commercial *Trichoderma*-based product (Eco77®). The fruit were then air-dried and stored in boxes inside black plastic bags to create a high relative humidity at room temperature for 7 days. After 7 days post inoculation (dpi), the black plastic bags were removed and the fruit were inoculated with the pathogens by spraying the isolates Cs1, Cs2, Ls1, and Ls2 with concentration $1x10^6$ conidia/ml^{-1}. This experiment was conducted twice to confirm the antagonistic activity of the endophytic *Trichoderma* isolates. A rating scale of 1-3 was used to describe the disease severity caused by isolates Cs1, Cs2, Ls1, and Ls2 after the fruit were treated with endophytic *Trichoderma* strains. All the fruit inoculated with *Trichoderma* and the pathogen developed less anthracnose or stem-end rot symptoms than fruit inoculated with the pathogen only. Isolates UK1E, UK4C and Eco77® were able to reduce symptoms on the fruit inoculated with the pathogenic species isolated, including *C. cobbittiense, C. henanense, N. parvum* and *L. mahajangana*. Based on ITS1 and ITS2 gene sequence analysis, UK1E was identified as *Trichoderma asperellum* and UK4C as *Trichoderma koningiopsis*. These two strains have the potential to be commercialized as biocontrol agents against the Botryosphaeriaceae family associated with anthracnose and stem-end rot of avocado. The next research phase would be to undertake field trials to see if preventative inoculation of the best *Trichoderma* strains can provide seasonal protection of fruit from pre- and postharvest pathogens.

DECLARATION

I, LONDEKA MKHIZE, declare that

I. The research reported in this dissertation, except where otherwise indicated, is my original work.

II. This thesis has not been submitted for any degree or examination at any other university.

III. The thesis does not contain other persons "data, pictures, graphs, or other information unless specifically acknowledged as being sourced from other persons,

IV. This thesis does not contain other persons "work unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

   a. Where their words have been used, their writing has been placed inside quotation marks and referenced.

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Chapter 1 General Introduction

1.1 Introduction

Avocado (*Persea Americana* Mill.) belongs to the family Lauraceae of the order Laurales (Chen *et al.,* 2019). *Persea americana* Mill. gave rise to three horticultural races known as the Mexican (subtropical), Guatemalan (semi-tropical) and West Indian (tropical) races, respectively (Dabas *et al.,* 2013). Avocados have several cultivars such as Bacon, Fuerte, Hass, Pinkerton, Duke, Lyon, Lula, Ryan, Puebla, Reed and Zutano (Njuguna *et al.,* 2004). This species is said to have originated from Chiapas (Mexico) where the wild avocados are found and then scattered to the rest of the world (Chen *et al.,* 2019). Different countries produce different types of avocado cultivars. In South Africa (SA), approximately 80% of Hass cultivars are produced and 20% Fuerte, Ryana, Pinkerton and Reed (Kallideen, 2020). The main producing provinces are Limpopo, Mpumalanga, KwaZulu-Natal, Eastern Cape and Western Cape (Demoz, 2006). To get optimum yield and quality, avocado production requires good orchard management practices to be carried out (Schaffer *et al.,* 2013). It has been observed that many healthy trees produce few fruits, even when conditions are favourable (Shumeta, 2010). In the subtropical environment, where this crop is grown, diseases especially those encouraged by plant parasitic fungi, commonly cause important reductions in yield and quality of avocado fruit (Njuguna *et al.,* 2004). Pre-harvest fungal infection cause both pre- and postharvest avocado diseases (Mbaka *et al.,* 2020). Some of these foliar diseases lead to avocado stem-end rot caused by the fungus *Lasiodiplodia theobromae* (Adaskaveg *et al.,* 2013). This pathogen causes symptoms of decay, fruit discoloration and softening after harvest or fruit ripening (Kushalappa *et al.,* 2007). Anthracnose, caused by the fungus *Colletotrichum gloeosporioides*, is a serious disease of avocado fruit (Alakonya *et al.,* 2018). Fruit lesions start as circular, slightly sunken, brown to black spots (Aranda-Ocampo *et al.,* 2020). Cercospora leaf spot, caused by the fungus *Pseudocercospora purpurea* causes small, scattered, brown to black lesions on the skin of the fruit (Dann et al., 2017; Kallideen, 2020). Cladosporium leaf spot caused by the fungus *Cladosporium gloeosporioides* causes deformation of fruits (Bensch *et al.,* 2012; Pérez-Jiménez, 2008). These fungal pathogens cause about 50- 90% of avocado losses in South African production (Aoudou *et al.,* 2015). Several agrochemicals such as, prochloraz, copper oxychloride, thiophanate-Methyl and thiabendazole play a huge role in avocado disease management (Magwaza *et al.,* 2017). The exhaustive use of chemicals does

not prevent all diseases and the toxic residues cause harm to the environment (Majeed, 2018). Therefore, agrochemicals are being restricted due to the European Union (EU) legislation on maximum residue levels (MRLs) (Majeed, 2018). The use of biological control agents (BCAs) such as *Trichoderma* species has been regarded as a promising and environmentally friendly approach to controlling plant diseases (Butt *et al.,* 1999). *Trichoderma* spp. are opportunistic avirulent plant symbionts and are used as BCAs against several plant fungi due to their unique characteristics such as antibiosis, mycoparasitism, producing bioactive secondary metabolites, inducing plant defensive mechanisms and promoting plant growth (Arikit *et al*., 2020). Furthermore, *Trichoderma* spp. have also been reported as endophytes due to their ability to colonize internal plant tissues. The combination of antibiosis and the endophytic relationship plays an important role in the defense against several pathogens by releasing metabolites that act as antifungal compounds (Arikit *et* al., 2020).

1.2 Aim of the study

The aim of this study was to isolate and screen endophytic strains of *Trichoderma* spp. to control fruit diseases of avocado such as stem-end rot, anthracnose and leaf spot diseases.
The objectives of this study included the following:

- Isolation and identification of *Trichoder*ma strains
- Isolation of fungal pathogens causing the pre- and postharvest diseases (anthracnose, stem-end rot and leaf spot).
- Screening for endophytic activity of the isolated *Trichoderma* strains.
- Assessment of the pathogenicity of isolated anthracnose, stem-end and leaf spot pathogens on fresh avocado fruits.
- Screening for inhibition activity of endophytic strains of *Trichoderma* against anthracnose, stem-end rot and leaf spot *in vitro*.
- Assessment of the effect of endophytic *Trichoderma* strains on *Colletotrichum*, *Neofusicoccum,* and *Lasiodiplodia*.

1.3 Dissertation Layout

This dissertation consists of six chapters. The Plant Science Journal system of referencing was used in chapters unless indicated otherwise. Chapter 1 is the general introduction of the dissertation, contains the aims, objectives and provides the motivation for the study; Chapter

2 is a theoretical literature review with a critical analysis overview of previously published research on avocado production, *Trichoderma as a biocontrol agent* and plant fungal pathogens; Chapter 3 is the isolation and identification of *Trichoderma* species from avocado fruits and leaves; Chapter 4 is the isolation and identification of pathogens causing avocado fruit spot and fruit rot; Chapter 5 is the *in vitro* and *in vivo* screening for antifungal and antagonistic activity of endophytic *Trichoderma* isolates against fungal pathogens associated with anthracnose and stem-end rot of avocado in KwaZulu-Natal, South Africa; Chapter 6 is the last chapter that portrays of the major findings, conclusions and recommendations of the research.

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Chapter 2 Literature Review

2.1 Origin, history, and cultivation of avocado

Avocado (*Persea Americana* Mill.) belongs to the family Lauraceae of the order Laurales (Woolf & Yahia, 2011). This species is said to have originated from Chiapas (Mexico) where the wild avocados are found, and then was distributed to the south-eastern United States, West Indies, South America, Guianas, Colombia, Brazil, Ecuador, Peru, Bolivia, and Chile (Schaffer *et al.*, 2013). *Persea americana* produced three horticultural races; the Mexican (subtropical), Guatemalan (semi-tropical) and West Indian (tropical) races (Dabas *et al.,* 2013). Both the Mexican and Guatemalan races are named after the countries from which they originate from (Woolf & Yahia, 2011). However, the West Indian race is thought to have originated along the Pacific coast of Central America (Dabas *et al.,* 2013). Avocado only became a worldwide commercial crop in the early 1900s, it had only been cultivated commercially in South Africa and other countries from the 1920s (Kohne, 2005). There are a number of important avocado cultivars, including Bacon, Fuerte, Hass, Pinkerton, Lyon, Lula, Ryan, Puebla, Reed and Zutano (Njuguna *et al.,* 2004). In South Africa (SA), approximately 80% of the national production is of the Hass cultivar with Fuerte, Ryan, Pinkerton and Reed making up the remaining 20% (South African Avocado Growers' Association, 2020). To achieve optimum yield and quality, avocado production requires good orchard management practices to be carried out (Schaffer *et al.,* 2013). It has been observed that healthy trees may produce few fruits, even when conditions are favourable (Shumeta, 2010).

2.2 Classification and biology of avocado

True avocado "*americana*" was placed in a genus known as the *Persea,* which resulted in avocado being botanically labeled as *Persea americana* Mill. (Arzate-Fernandez *et al.*, 2008). This fruit is classified as follows: Kingdom: Plantae; Phylum: Magnoliophyta; Class: Angiospermae; Order: Laurales; Family: Lauraceae; Genus: *Persea*; Species: *Persea americana* Mill. (European and Mediterranean Plant Protection Organization (EPPO), 2021). This family includes *Laurus nobilis* (sweet bay) an evergreen tree or shrub. Most members of the Lauraceae family are tropical or subtropical, including the avocado (Bost *et al.,* 2018). Avocado is a vascular plant with two development phases, the juvenile and adult phases (Ahsan *et al.*, 2020). During the juvenile phase, floral development is not initiated because the plants do not sexually reproduce yet (Schaffer *et al.,* 2013). Fifteen years can pass before seedlings become flowering

trees. However, breeding programs have produced progeny that flower and set fruits within 2 years of seed planting (Schaffer *et al.,* 2013). Avocado flowering is stimulated by low temperature and short-day length. Avocado is characterized by a flowering mechanism called protogynous, diurnally synchronous, dichogamy (Malapana, 2016). Each flower opens twice. In the first opening, the flowers function as females with receptive stigma and anthers that have not dehisced (Davenport, 2011). In the second opening, the flowers function as males, with a desiccated stigma and anthers that dehisce 1-2 hours after opening (Davenport, 2011). Type A cultivars that are clonally propagated such as Hass open with functionally female flowers in the morning of the first day of anthesis and transform to male flowers in the afternoon of the second day of anthesis (Garner *et al.,* 2013). Type B cultivars such as Bacon, Fuerte and Zutano open first and function as females in the afternoon of the first day of opening, and then transform to male flowers on the morning of the second day of anthesis (Whiley, 2013). This result in an excellent system of cross-pollination due to anther dehiscence occurring in one flowering type and the other flowering type being receptive (Davenport, 1986). Farmers are advised to interplant Type A and Type B cultivars to increase pollination and avoid self-pollination.

2.3 Consumption and usage of avocado

Avocado is healthy, energetic and nutritious fruit rich in carbohydrates, proteins, fats, minerals and wide range of vitamins (A, B, C, D, E and K) (Borges *et al.,* 2016). The avocado pulp contains different oil levels and bioactive compounds; thus, it is used across the world in pharmaceutical and cosmetic industries (Borges *et al.,* 2016; Davenport and Dreher, 2013). Bioactive compounds found in avocado such as the β-sitosterol which has a special effect on immunity and phytosterols which reduces the total LDL-cholesterol and triglycerides (Davenport and Dreher, 2013). β-sitosterol contributes to the treatment of cancer by suppressing carcinogenesis; HIV by strengthening the immune system and studies show that β-sitosterol support weight control in hypercholesterolemic clinical trials by reducing fat accumulation in the abdominal area (Borges *et al.,* 2016; Chauhan et al., 2018). Mexico has the largest avocado market in the world, with more than 70% of their production geared for local consumption. Peru has the second largest avocado local market as they use 60-70 % of their production for consumption while Hass is exported (Naamani, 2007; Sibulali, 2020). In South Africa, 38,000 tons a year of avocado are produced for the export market and a similar volume is left for the local market. In Chile, the domestic market demand is more than 60,000

tons and 35-40% of the entire production is absorbed by the local market for consumption. Mexicans use the leaves of the Mexican race of avocado as a spice for barbacoa (stewed meat) and for beans (Bost *et al.,* 2013). Avocado fruits are used to make the famous Mexican dip guacamole, which is a mixture of mashed avocado pulp, lime juice, minced onion, tomatoes, and peppers (Bobroff and Simonne, 2018). In the Caribbean avocados are used to prepare various dishes such as chilled avocado soup and fruit salads (Bost *et al.*, 2013). In Brazil and most of Asia, avocados are mixed with sugar and condensed milk to make ice cream and milkshakes (de Arriola *et al.,* 2012). In Java, mashed avocado is mixed with coffee, and in Indonesia, they produce avocado-chocolate shakes (Bost *et al.,* 2013). Avocado oil is extracted from mashed avocados in Mexico, Israel, and South Africa (Betti and Costagli, 2015). The oils and purẻe are exacted from the mash of wastes using bleaching, solvents, and deodorizing to make cosmetics with high vitamin E and emollient properties (Eyres *et al.,* 2001). In New Zealand, avocado is used to manufacture extra-virgin cold-pressed oil from Hass fruit (Requejo-Jackman *et al.,* 2010). These oils contain high pigment levels, a strong flavour, and excellent health benefits (Eyres *et al.,* 2001). California also produces cold-pressed avocado oil such as emerald, and green oil which is famous for its full rich flavour when used as a salad dressing (Eyres *et al.,* 2001).

2.4 Agronomy and economic importance

2.4.1 Propagation

Desired clonal rootstocks can be propagated by a method known as the etiolation technique (figure 2.1) (Hayward *et al.,* 2017). The largest seeds are planted in 5 litres cans and the seedlings are then grafted onto a root rot tolerant clonal scion (Bender and Whiley, 2002). When the stem of the graft reaches about 6.35 mm in diameter, the top is cut off leaving a whorl of buds just above the graft. Black tar paper is formed into an extension of the can and filled with vermiculite and placed in a dark box with high temperature and humidity (Hayward *et al.,* 2017). When growth is some 76–100 mm above the vermiculite, the plant is removed into the light where the upper portion quickly becomes green in colour (Bender *et al.,* 2013). The tar paper is then moved, the shoot is cut from the seed and then placed in propagating frames or cases where the cuttings are rooted in the normal manner (Bender and Whiley, 2002). Any seed may also be used for rootstock, but study shows that the Mexican race makes the strongest growth and are the most often used (Bender and Whiley, 2002). The seedling plants are ready to bud the following year. Budding is done when suitable buds are available

(Hayward *et al.*, 2017). Larger stocks are generated by bark grafts in the spring and scions are collected after the buds are well-formed (Bender *et al.*, 2013). The graft is then painted and covered with a moistened plastic bag.

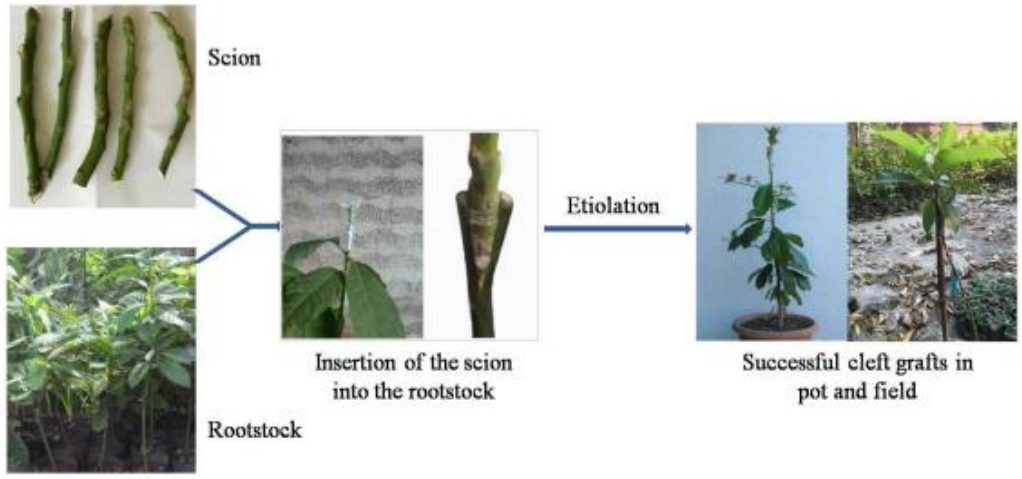

Figure 2.1: Different steps of avocado cleft grafts using the etiolation technique (Gomasta and Sarker, 2024).

2.4.2 Fertilisation

The application of manures and fertilizers are based on the soil fertility, tree age, growth, and yield (Cohen *et al.*, 2018). Young trees are fed after one year of growth, using a balanced fertilizer, four times a year. Older trees benefit from feeding with nitrogenous fertilizer applied in late winter and early summer (Awachare and Selladurai, 2020). Various micronutrients (iron (Fe), zinc (Zn), Boron (B)) have a great influence on tree growth and the yield of avocado (Awachare and Selladurai, 2020). The demand for mineral nutrition is higher in places where the avocado is grown on shallow, light, or rocky soils (Cohen *et al.*, 2018). Integrated nutrient management is advocated for avocados. In South Africa, a soil test is conducted to determine the nutrient and pH levels to help create a tailored fertilisation plan (Kuntashula *et al.*, 2006). For young trees, applying balanced fertilisers such as nitrogen (N)- phosphorus (P)-Potassium (K)-10-10-10 or NPK fertiliser 15-15-15, every 2-3 months and splitting the nitrogen application into two or three doses during the growing season for matured trees is practiced (Kuntashula *et al.*, 2006, Mohammed, 2015). Zinc foliar sprays and other micronutrients are applied when deficiencies are detected and compost manure is incorporated to improve nutrient availability in the soil (Mohammed, 2015). In India, many of the soils are deficient

in N, Zn and B. Therefore, the application of urea fertiliser is carried out in two split doses, before the prevailing rains in March/April and September/October (Awachare and Selladurai, 2020). Foliar application of zinc sulphate may be undertaken in April-May, and other fertilizers applied in the soil during March-April (Begane *et al.,* 2014). Chlorosis is corrected by a chelated foliar spray containing iron. In the Philippines, young avocado trees require only nitrogenous fertilizer (Sotto, 2000). Farmers apply 100-200 g of $(NH_4)_2SO_4$ twice a year (Sotto, 2000). As the trees bear fruit, 500 g of complete fertilizer is applied, twice a year. For full-bearing trees, 2 kg of complete fertilizer is applied per year. A supplemental application of organic fertilizers such as animal and poultry manure and compost are also given (Sotto, 2000).

2.4.3 Training and pruning

There is little tree training practiced on avocados during their establishment years apart from tipping the central shoot to encourage branching (Mathumbu *et al.*, 2000). Tipping the growing points of trees in their second year to increase tree complexity is carried out by some growers but there is little evidence of benefits (Armendariz-Arnez *et al.*, 2022). Controlling tree growth in mature orchards remains the biggest challenge of orchard management. Being terminal flowering trees, avocados need to grow each year to remain productive, hence trees become larger (Armendariz-Arnez *et al.*, 2022). To maintain orchard access between rows, limbs are periodically removed. Likewise, to contain tree height major limbs are removed from the top of the tree when necessary (Mathumbu *et al.,* 2000). In some orchards, trees are not pruned at all until their size is so big that they are uneconomical to manage (Mathumbu *et al.*, 2000). They are then cut back to approximately one meter high and allowed to re-grow. After this treatment, they are back in full production within two years (Chernoivanov *et al.*, 2022). The best results are obtained by using plastic mesh fencing around the trees for the first two to three years, as shading helps protect crops from cold stress (Chernoivanov *et al.*, 2022). In South Africa, it is now a standard practice to prune trees annually (Stowell and Thorp, 2001). Branches exposed to the sun by defoliation are extremely susceptible to sunburn. Therefore, such branches should always be whitewashed (Stowell and Thorp, 2001).

2.4.4 Economic importance

The avocado trees are of great importance as they produce fruits that are eaten worldwide (Van Rooyen, 2011). South Africa currently produces approximately 17 500 ha of avocado, with

approximately 360 commercial growers (members and non-members of the South African subtropical Growers' Association (Subtrop)), 62 emerging growers (members of Subtrop), 1 000 to 1 500 ha annual production, 115 000 ton which is approximately 104 326 245 kg of annual production and approximately R1,85 billion annual value (Genis, 2020). Of these 13% are Fuerte, 33% are Hass, and 12 to 41% are Ryan and Pinkerton are exported to the European markets (Van Rooyen, 2011). The Limpopo province respectively has the highest percentage of avocado production at 61% with 9 401 ha, followed by Mpumalanga with 30% (4 554 ha) and KwaZulu-Natal with 8% (1319 ha) (Randela, 2018). Production is challenged by poor management practices, pests and diseases and excessive vegetative growth, all of which can cause economic losses (Schoeman, 2005). Foliar diseases can reduce crop yields by up to 50% (Bara and Laing, 2019).

2.5 Pests and diseases

2.5.1 Pests

Thrips (*Pseudophilothrips perseae* Watson (Thysanoptera: Aeolothripidae)) are small insects that survive on foliage, by scraping and sucking the superficial cells, thereby causing the appearance of discoloured spots leaves and fruits, which are silver, white at first, and later turn dark (Bara and Laing, 2019). In South Africa the major fruit damage caused by pests is when thrips feed on young fruits, producing crest-shaped malformations of the exocarp (Bara and Laing, 2019). However, they can also be found on shoots, buds, and flowers. The damage produced by pests such as *Liothrips perseae* can cause the crop to lose 50% of their commercial value (Aluja *et al.*, 2013).

Scale (Hemiberlisia lataniae Signoret (Hemiptera: Diaspididae) are less than 5 mm in length and their taxonomy is based on the cuticle of the adult females. This pest has been causing severe outbreaks of avocado scale in South African orchards where it weakens or kills the trees by attacks leaves, twigs, branches and fruits (Grové, 2022; Dhalin et al., 2020). The fruit gets damaged by slightly changing in colour, damaging the aesthetic and quality of the fruits to be sold in the market (Grové, 2022).

Small seed weevil (***Conotrachelus perseae* Barber (Coleoptera: Curculionidae))** is found in the eastern central region of Mexico, northern parts of Central America, Guatemala, and Panama. The larvae are yellowish white in colour with a dark cephalic capsule and reach a

length of 6 mm (Aleman *et al.*, 2015). They tunnel, forming a long hole throughout the pulp until they arrive at the seed, which is usually destroyed (Chamé-Vázquez *et al.*, 2015). Highly infected areas can affect up to 85% of the fruit, destroy the seeds and affect the production (Chamé-Vázquez *et al.*, 2015).

Seed moth, **(*Stenoma catenifer* Walsingham (Lepidoptera: Elachistidae))** Seed moths can penetrate fruits of any size and destroy the seed completely. The hole they form inside the branches cause withering, reduce flowering of the tree, and up to 90% of the production (Cabrai *et al.*, 2021).

2.5.2 Diseases

Phytophthora root rot - Avocado trees are very susceptible to root rot, which is caused by the soil-borne fungus *Phytophthora cinnamomi* var. *cinnamomi* (Rands) (Dann *et al.*, 2018). Controlling this disease is the highest priority for avocado growers as it is ever-present and requires constant attention. Without root rot management the tree will lack an adequate root system and will not be able to function (Dann *et al.*, 2018).

Anthracnose (*Colletotrichum gloeosporioides* (Penz) Penz. & Sacc.) causes a latent infection, that cannot be identified at harvest, and is usually only discovered at the point of consumption by the consumer. In particular, the variety Hass is vulnerable to anthracnose, but it is difficult to detect the disease until it is cut because its thick, dark skin masks the disease symptoms (Aranda-Ocampon *et al.*, 2020).

Scab (*Sphaceloma perseae* Jenk.) Circular, brown, scabby areas are found on mature fruit whilst leaves develop brown spots and become crinkled (Everette *et al.*, 2011). The disease can become a problem if there is cool, moist weather when fruit and leaf tissue is young. Fungicides can control the disease and should be applied when flower buds appear, near the end of the main bloom period, and 3-4 weeks after that (Bara and Laing, 2019).

2.6 Diseases under study

2.6.1 Avocado leaf and fruit spot

2.6.1.1 Cercospora spot

Cercospora spot is caused by a fungal plant pathogen called *Pseudocercospora purpurea* (Cooke) Deighton (Reina-Noreña *et al.*, 2020*).* This pathogen belongs to the Kingdom: Fungi; Phylum: Ascomycota; Class: Dothideomycetes; Order: Capnodiales; Family: Mycosphaerellaceae; Genus: *Pseudocercospora*; Species: *Pseudocercospora purpurea* (EPPO, 2021). Cercospora spot is one of the severe pre-and postharvest diseases of avocado fruits in South Africa (Pérez-Jiménez, 2008). The economic importance of the disease was noted by Brodrick et al. (1974), who reported that untreated orchards produced only 20% exportable fruit, while 85-90% of the fruit was exportable from trees regularly sprayed with fungicides. *Pseudocercospora purpurea* affects all commercial cultivars; but cultivars Fuerte and Ryan are extremely susceptible (Kallideen, 2020).

Life and Disease cycle

Pseudocercospora purpurea reproduces only by means of conidia. A brunch of conidiophores emerges either through stomata or ruptured epidermis (Figure 2.2.B) (Aptroot *et al.*, 2000, Braun et al., 2013). Conidiophores are clear to dark brown, septate or aseptate, straight 20 - 200 µm long and each conidiophore form single conidium acrogenously at its apex (Figure 2.2.O) (Braun et al., 2013, Korsten and van Eeden, 2013). The growth of the conidiophore is renewed after the formation of first conidium and conidia are long, cylindrical and multi-septate (Figure 2.2.P). They vary in size but always have a width, length ratio of 1: 10 − 1: 150. Conidia germinate by giving rise to one or more germ tubes (Kallideen, 2020). Each germ tube develops an appressorium, infects the leaf, and develop new mycelium inside the leaf, creating a distinct leaf lesion, Cercospora spot (Aptroot *et al.*, 2000). Conidia germinate during favourable warm temperatures (Marais, 2004). The fungus is difficult to isolate, but once isolated it grows readily on nutrient media, producing a tufted leathery growth, which is first greyish in color, later becoming brown or blackish brown (Marais, 2004). New cycles of infection and sporulation occur every seven to 10 days during warm, wet weather (Reina-Noreña *et al.*, 2020). The pathogen is readily disseminated by wind, and splashing rain or irrigation water, and insects (Marais, 2004).

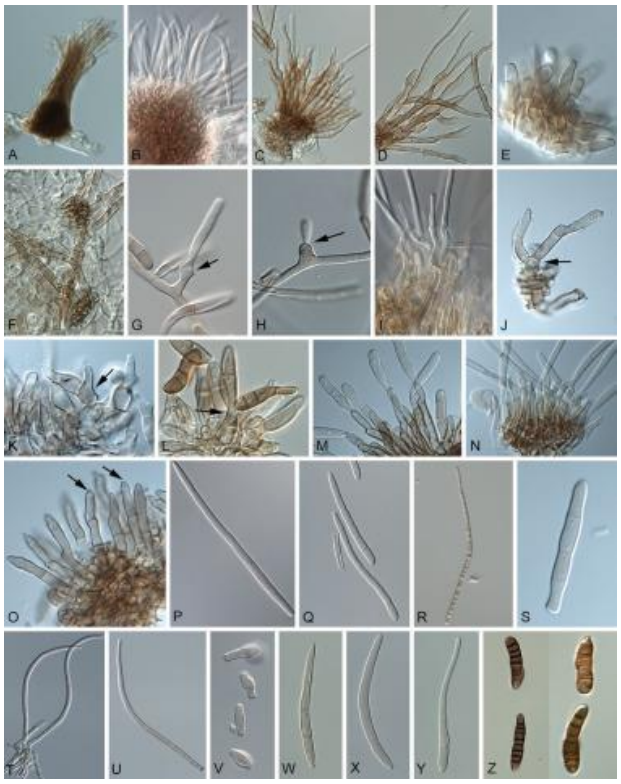

Figure 2.2: Morphological structure of Pseudocercospora species. A. Synnemata (brunched conidiophore). B. Dense fascicle of conidiophore with well-developed brown stroma. C. Fascicles of conidiophore with moderately developed brown stroma. D. Fascicles of conidiophore with poorly developed brown stroma. E. Conidiogenous cells. F. Fascicles of conidiophores from stomata. G-H. Conidiogenous cells on superficial hyphae. I. Geniculate conidiophore with truncate apical locus. J-K. Branched conidiophores. L. Percurrent proliferations from conidiogenous cells. M-N. Conidiophores with sympodial proliferation. O. Conidiophores with Conidiogenous cells. P. Subcylindrical conidium with subacute apex. Q. Conidia with constrictions at septa. R. Conidium with guttules. S. Cylindrical Conidium with obtuse apex and truncate base. T. Undulate conidia. U. Curved conidium. Aseptate to 1-septate conidia. V. 1-septate conidia. W-X. Obclavate conidia with obconical base. Y. Obclavate conidium with short obconical base. Z. Dark brown, muriform septate conidia (Crous *et al*., 2013).

Pseudocercospora purpurea small angular spots on leaves and fruit that appear as small, scattered, brown, slightly sunken spots that have a definite outline but irregular shape (Menge and Ploetz, 2003). When conditions are favourable, lesions continue to grow rapidly after their initially appearance, until most parts of the host are symptomatic, a process called lesion expansion (Amorim *et al.*, 1997). Fruits tend to become infected during the wettest part of the

growing season. Cercospora spot does not damage the avocado fruit flesh, but it causes unsightly fruit that is unsaleable. Furthermore, the lesions provide an infection site for more destructive flesh pathogens (Darvas and Kotzé, 1979).

Symptoms

Lesions on young fruits appear as small greenish white spots which develop into scattered, brown, slightly sunken spots that have a definite outline but irregular shape (Dann *et al.,* 2017). Greyish spore-bearing structures of the fungus appear on the spots, which are 3 to 6 mm in diameter, and later develop cracks, which permit the entry of other fungi that cause fruit decay (Figure 2.3.A). On young leaves, *P. purpurea* causes greenish-white spots. On mature leaves (Figure 2.3.B) it causes small purple to brown angular spots (1–5mm in diameter) and is generally surrounded by a chlorotic yellow halo (Menge and Ploetz, 2003).

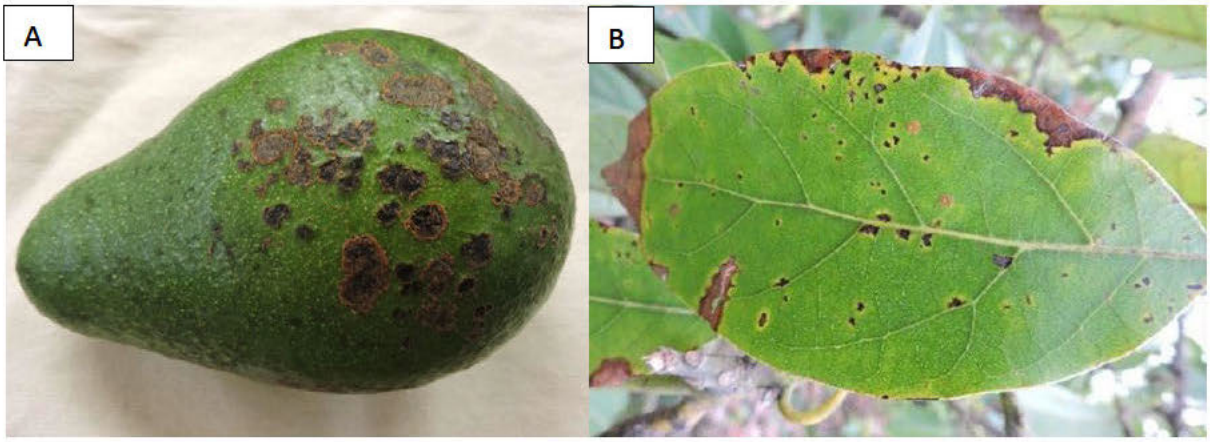

Figure 2.3: A) Avocado fruit and b) Avocado leaf showing lesions caused by Cercospora spot (Kallideen, 2020).

The lesions first appear on the underside of the leaf and as the infection progresses, it appears on both sides (Kallideen, 2020). Lesions may coalesce to form large dead areas on the leaf (Pernezny and Marlatt, 2000). Conidial are produced in abundance on infected leaves in warm, rainy weather and germinate readily in a film of water (Dann *et al.,* 2017).

2.6.1.2 Cladosprorium rot

*Cladosporium cladosporioides (*Fresen.) G. A. de Vries, causes Cladosporium rot associated with black spots on avocado fruits (Coertzen and Fourie, 2018). *C. cladosporioides* is a fungal plant

pathogen that is classified as follows: Kingdom: Fungi; Phylum: Ascomycota: Class: Dothideomycetes; Order: Cladosporiales; Family: Cladosporiaceae; Genus: *Cladosporium;* Species: *cladosporioides* (EPPO, 2021). *C. cladosporioides* is now found worldwide affecting leaves and fruits as it is not host specific and it can survive severe temperatures (Bensch *et al.*, 2012). *C. cladosporioides* causes a leaf spot on avocado but it is typically found as a secondary invader of physical or chilling injuries (Pérez-Jiménez, 2008).

Life and disease cycle

Species of *Cladosporium* are characterized by reproducing asexually and being an exclusively anamorphic species. Colonies are olive-green to olive-brown and appear velvety or powdery (Figure 2.4 A) (Bouziane *et al.*, 1988). *C. cladosporioides* produces conidiophores that arise from hyphae, bearing branched ramoconidia 10 µm long, and chains of conidia (Figure 2.4 B and C). The conidia are oval to spherical in shape and separated ramoconidia have a dark scar at one end or both ends (Figure 2.4 D) (Bouziane *et al.*, 1988). The fungi cause a sooty mold and are very common in the atmosphere and can survive harsh conditions such as conidia or mycelium on a wide range of materials (Goyal and Prasad, 2010). They do not require living host material to survive. After overwintering in crop debris, *C. cladosporioides* produces conidia that are blown by air onto plant material (leaves or fruit). The pathogen then colonizes the plant and causes disease symptoms (Goyal and Prasad, 2010).

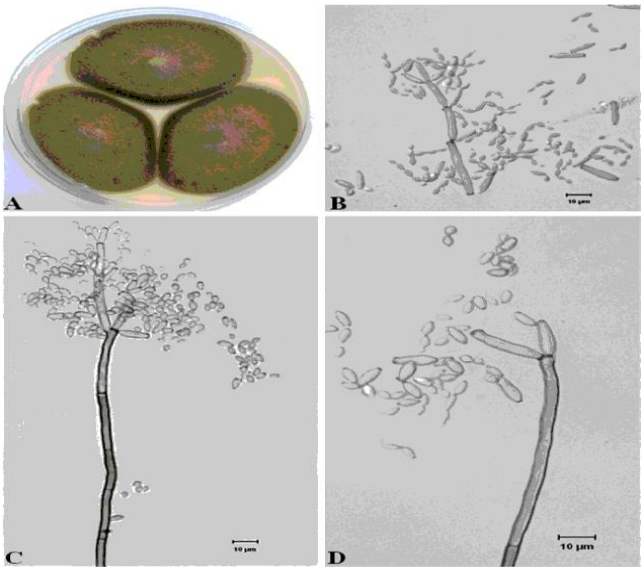

Figure 2.4: (A-D) *Cladosporium cladosporioides* colonies growing on a Potato dextrose agar (PDA) plate; and conidia and conidiophores viewed under a light microscope (Bouziane *et al.*, 1988).

C. cladosporioides is xerophilic and aerotolerant, meaning it is capable of growth at or below a water activity (a_w) of 0.85. It is also psychrophilic as demonstrated by its ability to grow at freezing temperatures (Boland and Hunter, 1988). It can grow slowly at temperatures between of -10°C and -3°C on Potato Dextrose Agar and Malt Extract Agar. *C. cladosporioides* grows optimally at a temperature range of 18-28°C and favours moist areas for growth such as buildings where the indoor humidity is higher than 50%, in damp areas such as basements and carpet pads of buildings that have been exposed to excess moisture, and buildings with poor ventilation (Boland and Hunter, 1988). *C. cladosporioides* also commonly occur as saprotrophs on plant vestiges and cause diseases of plants (Miluch *et al.*, 2012).

Symptoms

Disease symptoms on plants consist of lesions on leaves and fruits. Infection of flowers leads to necrosis of the entire flower, or parts of it, as well as to the production of small and deformed fruits and green-grey sporulation on the stigma (Pérez-Jiménez, 2008). There is a higher proportion of deformed fruits due to *C. cladosporioides* infections. Lesions are dark circular to oval-shaped, and on fruit, they are approximately 1 to 14 mm in diameter (Figure 2.5). Symptoms on leaves include yellow irregular lesions on the surface, whereas intense greyish brown sporulation developed on the undersides of the lesions (Goyal and Prasad, 2010).

Figure 2.5: Avocado fruit showing black spots caused by *C. cladosporioides* (Pérez-Jiménez, 2008).

2.6.1.3 Current control

Cultural controls

Cultural practices such as avoiding overlapping crops, having windbreakers to avoid wind from carrying the fungus to another orchard should be practiced. Sanitation should be maintained to avoid disease spread, where this is feasible (Derie *et al.,* 1997).

Before planting:

Avoid overlapping crops, preventing spores from older crops infecting new ones (plant the newer crop far from the infected ones). Windy and rainy conditions favor the fungus, so shield the orchard with windbreaks (Zentmyer, 1953).

During plant growth:

Maintain proper sanitation to get rid of insects that also spread the disease If previously infested, avoid intercropping the orchard with bean, beetroot, capsicum, okra, carrot, and coffee, which are alternate hosts of the disease (Reddy, 2010). Remove dead twigs and branches as they can host the fungus in readiness for attack, remove all fallen fruits from the surroundings as they harbor insects that transmit the fungus and weed regularly (Zentmyer, 1953). In commercial plantings, use drip irrigation. If using overhead irrigation, time irrigation events so that plants dry out rapidly after watering (Reddy, 2010).

After harvest: Collect and burn, plough in or compost trash (Zentmyer, 1953).

Chemical controls

Certain fungicides such as Dithane DF (mancozeb) and Bravo 720 (chlorothalonil), when used preventively help minimize the onset of infection and development of Cladosporium leaf spot (Maciel *et al.,* 2016). It is important to use them early in the season to protect healthy tissue from infection as they are not effective once the disease becomes established (Derie *et al.,* 1997). Protectant fungicides are used before rainy weather that is highly favourable for the development of the disease and is applied regularly to ensure adequate coverage as the plant grows (Derie *et al.,* 1997). Fungicides may reduce the spread of the disease cannot cure the crop once infected. Avoid spraying chemicals during the harvesting/ ripening stage to avoid

leaving residues in the fruit (De Villiers *et al.,* 1997*).* Cercospora spot can be controlled by timely applications of copper sprays and benomyl to developing leaves and fruit (Darvas, 1982). In South Africa, the timely applications are based on J.M. Darvas's predictive model which uses weekly temperature and rainfall as criteria. The equation of the model is:

$$Z = -58.99 + 3.22 \text{ (Mean weekly temp.)} + 0.18 \text{ (Weekly rainfall in mm)}$$

If Z>20 (the number of conidia) there is a high chance of infection. Recommendation in South Africa are for farmers to spray copper oxychloride as a full cover spray at 30- and 60-day interval when Z>15, and if Z exceeds 20, the second spray should be replaced by benomyl (Manicom, 2001). An application of copper in early May followed by another in early June gives effective control on cultivars maturing in summer and autumn (De Villiers *et al.,* 1997). On winter-maturing cultivars, a third application about mid-July is necessary for adequate disease control on the fruit. Although there are different types of chemical sprays that growers can use, it is important to follow their restrictions (Table 2.1) (De Villiers *et al.,* 1997).

Table 2.1: Chemical sprays to control leaf spot along with their restrictions.

Chemical sprays	Restrictions
Thiophanate-methyl (Topsin M, Topnet, Topguard, Alert) Frac. B1. Thiophanate	World Health Organization (WHO) class U. Foliar spray is unlikely to present acute health hazards in normal use. Maximum of 3 applications. Interval 10 days. Do not spray on fruits.
Captan (Merpan, Captan) Frac M4 phthalimide	WHO class U. Not to be applied on fruits. Moderately harmful to pollinators so avoid spraying during flowering or daytime hours. Toxic to fish, avoid use near water bodies. Max 3 applications. 10 days interval.

Copper oxychloride (Cuprocafaro, Green Cop, Primer, Trinity Gold, Colonizer) FRAC M3. Dithiorcarbamates	WHO class II. Maximum of two applications, 14 days intervals. Toxic to fish, avoid spraying near water bodies, Harmful to earthworms. Reduce the number of applications to avoid soil accumulation.
Copper hydroxide. (Vitra, Champflo), FRAC M3	WHO class II. Maximum of two applications, 14 days intervals.

Crop protection is also in a transitional phase even though it is still largely achieved by applying chemical products. There is a gradual integration of new practices such as the use of biocontrol agents to minimise the negative impact of pollution on the environmental and chemical residue build up associated with the use of synthetic chemicals (Barka *et al*., 2022).

Biological controls

Biocontrol agents suppress the damaging activities of another organism and have been shown to play a pivotal role in the improvement of soil quality, plant growth and health through different mechanisms (Kapoor et al., 2013). Baker and Cook (1974) described biocontrol as the reduction of inoculum disease with activities of a pathogen in its active state by one or more organisms through producing endotoxins, antibiotics, bacteriocins, antagonism and mycoparasitism (Barka *et al.*, 2022). For example,

- Use of *Bacillus spp. (Bacillus subtlis isolate, BacB*) as a biocontrol agent of *Cercospora*

  leaf spot (Collins and Jacobsen, 2003.)
- Use of *Trichoderma* isolates to control *Cercospora beticola* Sacc (Burzi *et al.*, 2008).

Isolate T39 of *Trichoderma harzianum* Rifai (TRICHODEX) is a commercial biocontrol agent (Etebarian *et al.*, 2000). It controls *Botrytis cinerea* Pers. (grey mould) in greenhouse crops and in vineyards, *Sclerotinia sclerotiorum* (Lib.) de Bary (white mould) in various greenhouse and field crops, *Cladosporium fulvum* (Cooke) (leaf mould) in tomato, and the powdery mildews

Podosphaera fusca (Fr.) Braun & Shishkoff. in cucurbits and *Leveillula taurica* (Lév.) G. Arnaud in pepper (Elad, 2000).

2.6.2 Avocado fruit rot

Botryosphaeriaceae species are known to infect crops in all tropical and sub-tropical growing regions, causing dieback, cankers, shoot blight, leaf spot and fruit rots such as anthracnose and stem-end rot in a wide range of plant hosts (Adikaram and Karunanayake, 2020).

2.6.2.1 Anthracnose

Anthracnose is an avocado disease that cause severe economic loss as it affects the shelf life, fruit quality and marketability of avocado (Alakonya *et al.,* 2018). It is caused by a plant fungal genus called *Colletotrichum* that is classified as follows: Kingdom: Fungi; Phylum: Ascomycota; Class: Sordariomycetes; Order: Phyllachorales; Family: Glomerellaceae; Genus: *Colletotrichum.* Several species may cause anthracnose in avocado, including *C. gloeosporioides* (EPPO, 2021). *C. gloeosporioides* causes anthracnose on many hosts including avocado, tomato, pepper, green bean, almond, coffee, apple, cassava, mango, sorghum, and strawberry (Alakanya, 2018). Anthracnose has been reported as one of most severe postharvest diseases in the tropics and the sub-tropics growing regions such as South Africa, Mexico, New Zealand, and Hawaii (Nelson, 2008). In South Africa, this disease causes up to 37% losses on fresh fruit and vegetables (Marais, 2004).

Life and Disease Cycle

Figure 2.6 summarises the anthracnose disease cycle (Zakaria, 2021). Infection begins in the orchard during wet conditions. Leaves, twigs, and fruits serve as sources of inoculum (Agrios, 2005). When a *C. gloeosporioides* conidium lands on the surface of a fruit, it germinates and produces a germ tube that develops a terminal appressorium (Zakaria, 2021). Infection takes place and the pathogen penetrates less than 1.5 µm into the outer wax layer and cuticle of the skin, where it remains quiescent until the ripening stage of the fruit (Than *et al.,* 2008). The latent infections are a response to unfavourable conditions in the host tissues for the pathogen because they lack critical nutrients and have a range of antifungal compounds (Michereff and Silva, 2013). The presence of ethylene in the ripening process of avocado is important (Than *et al.,* 2008). When the fruit reaches peak maturity, hypha grow into the surrounding fruit tissue, causing a severe post-harvest disease (Maymon *et al.,* 2017).

Anthracnose is an important disease in avocado trees, causing serious damage during the postharvest stage (Korsten *et al.*, 2013). The inoculum is present throughout the year. Wet periods and the presence of fruit are necessary for infection to take place. Spores can infect all the parts of the plant, but the fruits are more susceptible to the pathogen (Michereff and Silva, 2013). *C. gloeosporioides* spread by rain splash, wind, high moisture, and warm temperatures (Zakaria, 2021). At low temperatures, the fungus is almost completely inhibited, whereas symptoms develop rapidly at 24 - 28°C (Kotze, 1978). The pathogen has facultative saprophytic characteristics, and it can survive on dead branches, leaves, and fruit remaining in the soil, from which it sporulates during favourable conditions (Michereff and Silva, 2013). Studies show anthracnose can cause losses of up to 80% of avocado production when correct management measures are not applied (Bosse *et al.*, 2013).

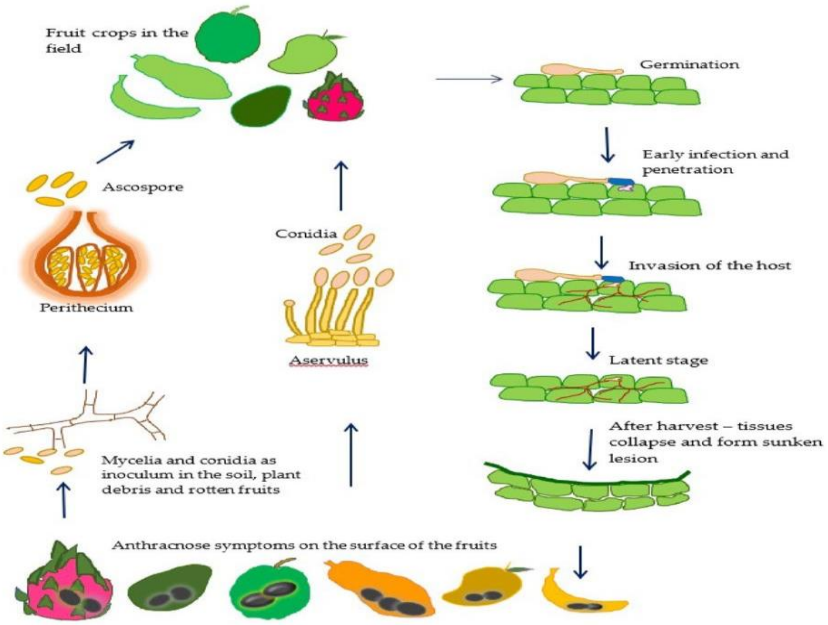

Figure 2.6: Anthracnose disease cycle of tropical fruits including avocado (Zakaria, 2021).

Symptoms

Anthracnose symptoms may occur at any stage between fruit set and harvest (Bento *et al.*, 2014). The initial latent infections cause no visible symptoms as the fungus is quiescent. As the fruit ripens, lesions develop as small, light brown and circular. These progress to being dark-brown or black sunken spots on the fruit skin (Figure 2.7) (Nelson, 2008). The dark spots enlarge rapidly on the skin and lead to the rotting of the plant tissues (Sarkar, 2016). The lesions may develop acervuli covered with pink spore masses. Symptoms may develop on fruits

while they are still on the trees, which may result in premature fruit drop (Dann *et al.*, 2013). It is more difficult to detect on cultivars with dark-coloured skins, e.g., 'Hass' fruit (Coates *et al.*, 2013). Leaf and stem symptoms only appear under very humid conditions (Barbanti and Sanoubar, 2017). This disease is associated with severe losses of avocado fruits both in field and after harvest (Alakanya, 2018). All avocado cultivars are susceptible, but Fuerte is very susceptible (Kotze, 1978).

Figure 2.7: An avocado anthracnose lesion that appeared after ripening (Nelson, 2008).

2.6.2.2 Avocado stem-end rot

In South Africa, *Thyronectria pseudotrichia* (Berk. & M.A. Curtis) Wollenw., *Dothiorella aromatica* (Saccardo) Petrák & Sydow, *Pestalotiopsis versicolor* (Speg.) Steyaert, *Lasiodiplodia theobromae* (Pat) Griff. & Maubi, *Rhizopus stolonifer* (Ehrenb.) Vuill. has been reported to cause stem-end rot (Darvas and Kotze, 1987). Stem-end rot (SER) causes fruit loss in all avocado-growing regions (Alkan *et al.*, 2020). This study will focus on avocado SER caused by a fungal pathogen called *L. theobromae*, formerly known as *Botryodiplodia theobromae* Pat., (Lodhi *et al.*, 2014). It is classified as: Kingdom: Fungi; Phylum: Ascomycota; Class: Dothideomycetes; Order: Botryosphaeriales; Family: Botryosphaeriaceae; Genus: *Lasiodiplodia*; Species: Lasiodiplodia *theobromae* (Pat.) Griff & Maubi (Lodhi et al., 2014). *L. theobromae* is a key pathogen causing postharvest decay in over 500 different host plants (Zhang, 2014), including citrus, papayas, mangos, guava, peach, and

bananas in tropical and subtropical growing regions (Zhang, 2014). SER is one of the most severe postharvest diseases worldwide, causing significant postharvest losses of fruit in storage affecting the shelf life, quality, and marketability (Ni *et al.,* 2012).

Life and Disease Cycle

Lasiodiplodia theobromae relies on cracks, grooves, pedicels, and twigs to complete its life cycle (Marais, 2004). The fungus grows and sporulates in these dead tissues and overwinters as both mycelia and conidia, subsequently infecting the fruit, shoots and leaves of avocado (Aiello *et al.,* 2016). This pathogen enters the fruit via wound openings such as the pedicels and stem-end scars of the fruit, penetrating the host tissue during the post-harvest period (Marais, 2004). Once the conidia germinate on the unripe host fruit surface, they remain as latent infections until the fruit ripens (Mbaka *et al.,* 2020), after which disease symptoms become visible as the fungal mycelium ramifies through the ripening host tissues. The fungus has a sexual stage that produces ascospores that disperse with the conidia (Amatulli *et al.,* 2012). The fungus also forms specialised chlamydospores under unfavourable conditions (Munirah *et al.,* 2017). The chlamydospores germinate during favourable conditions and form specialised hyphae that infect host tissues through wounds (Munirah *et al.,* 2017). The germination tube forms an appressorium that forms a penetration peg that penetrates host wounds. Once in a wound, the fungus develops haustoria for feeding on the fruit nutrients (Aikaram and Karunanayake, 2020). On PDA, *L. theobromae* colonies initially develop as a whitish mycelium with a grey centre, but after few days, the white turns into a grey-black pigment (Munirah *et al.,* 2017). At maturity, these colonies produce pycnidia aggregated into black stromatic masses (Fig. 2.8A) (Aikaram and Karunanayake, 2020). Under the microscope, the pycnidia produce hyaline, ovoid to ellipsoidal, dark brown longitudinal conidia (Figure 2.8) (Adikaram and Karunanayake, 2020). The paraphyses produced from the pycnidia tissues are presented as hyaline, cylindrical and non-septate up to 50-65 um long (Munirah *et al*., 2017).

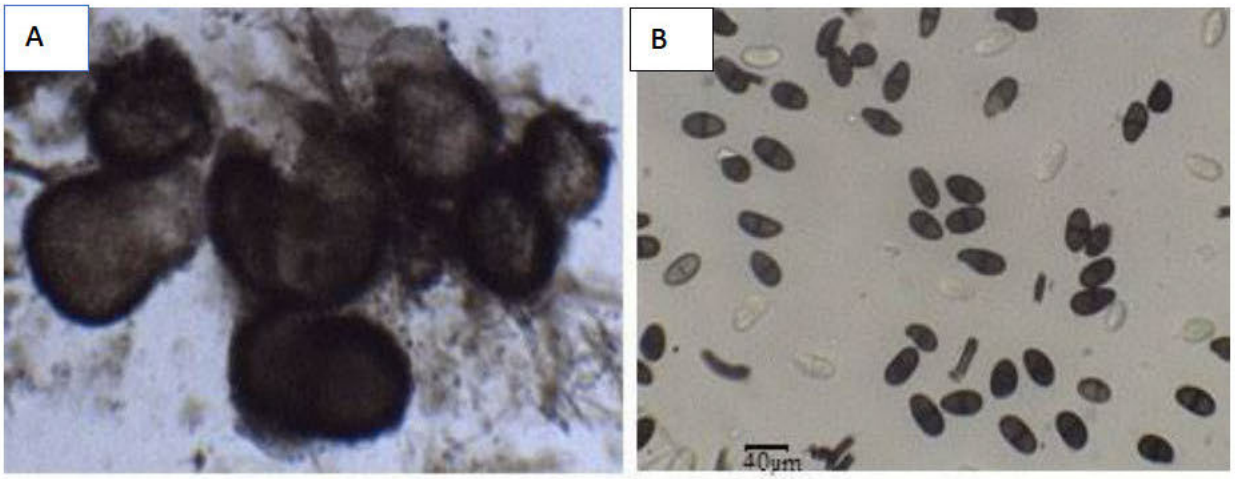

Figure 2.8: *Lasiodiplodia theobromae* pycnidia on PDA medium (B) and mature conidia (B) (Adikaram and Karunanayake, 2020).

Crop losses may be severe when harvesting of fruit occurs during rainy periods since conidia and ascospores of the pathogen are transmitted by rain splash and wind to the surface of unripe fruit from twigs, leaves, pedicels, living and dead branches (Adaskaveg *et al.,* 2013). Fruits get infected via wounds or natural openings such as stem-end scars that result during harvest. Spore transmission and disease infection occur during harvest due to shaking branches and by cutting tools (Adaskaveg *et al.,* 2013). After transmission, the disease remains latent and does not cause any symptoms until ripening and senescing of the fruit (Zhang, 2014).

Symptoms

SER symptoms on fruit usually do not appear before harvest but symptoms develop as fruit ripens in the packhouse, marketing or at storage (Aiello *et al.,* 2016). After the fruit is removed from the tree, slight softening and discolouration develops at the union where the fruit was attached (Latorre *et al.,* 2019). Fungal mycelia and conidia may become visible on the abscission scar. The earliest sign of infection on the fruit is a shriveling, dark brown to black rot development at the stem-end, followed by decay, discoloration and softening (Figure 2.9) (Adikaram & Karunanayake, 2020). As the fruit ripens, well-defined dark rot margins develop downwards from the stem-end, covering most of the fruit. Eventually the internal vascular bundles turn dark brown (Adaskevey *et al.,* 2013). The infected fruit then becomes soft, water-soaked and pale in colour (Figure 2.9B).

Figure 2.9: External (A) and internal symptoms (B) of SER on ripe avocado fruit (Adikaram and Karunanayake, 2020).

2.6.2.3 Current Control

Cultural control

Pruning dead twigs and branches from the avocado trees before the fungus produces spores is essential for both anthracnose and stem-end rot (Marais, 2004). Removing dead leaves from the orchard floor regularly is important as they play an important role in pathogen epidemiology (Kotze, 1978). Pruning and harvesting only during dry conditions and minimising fruit contamination and injury are important to prevent the spread of diseases and fungal infections, maintaining the quality and shelf life of avocado (Coates *et al.,* 2013; Marais, 2004). The use of cold storage soon after harvest is important as it helps to slow down ripening, reduce decay, maintain quality, and prevent physiological disorders but refrigeration is costly (Adikaram and Karunanayake, 2020).

Chemical control

Pre-harvest fungicidal treatments are applied to reduce inoculum levels in the field include the application of copper oxychloride before periods of high rainfall, when inoculum is dispersed (Korsten *et al.,* 2000). Pre-harvest applications of benomyl are currently restricted if used on fruit being exported to certain markets. In South Africa, prochloraz and thiabendazole are registered for post-harvest treatment of avocados (Kimaru *et al.,* 2020). Due to the toxic residues affecting human health and the environment, alternative methods aimed to prevent and suppress disease symptoms such as biological control are being investigated (Adikaram

and Karunanayake, 2020).

Biological control

The use of antagonistic microorganisms to control pathogens and pests, is a method for disease and pest management that is eco-friendly (Kimaru *et al*., 2020). In South Africa, a biocontrol agent (*Bacillus subtilis* B246; Avogreen®) was introduced to avocado growers in 1987 and was used briefly by growers, before being withdrawn due to difficulties with its commercialization (Korsten and Van Eeden, 2013). The application of *Bacillus* spp. can be used to control avocado postharvest diseases when applied as a postharvest dip. More effective disease control is achieved by mixing *B. subtilis* in wax (Kimaru *et al.,* 2020). *Trichoderma* spp. such as *Trichoderma harzianum* has been used a biocontrol agent to control anthracnose and *Trichoderma viride* has been used as a biocontrol agent to control SER of avocado (Alam *et al.*, 2013; Alemu *et al.,* 2019).

2.7 Endophytes

The term endophytes applies to fungi capable of symptomless occupation of healthy plant tissue (Polishook *et al.,* 2004). This definition includes the entire spectrum of symbiotic interactions in which fungi and plants participate and includes parasitism, commensalism, and mutualism (Franken *et al.,* 2006). Many of the fungi commonly reported as endophytes are regarded as minor or secondary pathogens by forest pathologists (Begoude *et al.,* 2011). Endophytic fungi are usually present as internal, unseen, microscopic hyphae; their presence is revealed only when they sporulate, usually a seasonal and ephemeral event (Polishook *et al.,* 2004). Endophytes have been described as mutualists that protect plants against insects and pathogens, and many of those fungi produce biologically active secondary metabolites (Boyle *et al.*, 2002). More than 100 years of research have contributed to the knowledge we have today of fungal endophytes. Previous studies show that endophytic fungi are divided into two groups, clavicipitalean and non-clavicipitalean endophytes (Sieber, 2007). Clavicipitalean also known as grass endophytes were first discovered in the late 19th century by European scientists in seeds of *Lolium* species where animals that consumed infected seeds experienced toxic syndromes (Arnold *et al.,* 2009). However, this was just a hypothesis until Bacon *et al.* (1977) found that the cause of the widespread fescue toxicosis syndrome is a fungus identified as *Neotyphodium coenophialum,* which systemically colonized the grass leaves and stems improving their deference mechanism (Zabalgogeazcoa, 2008; Arnold *et al.,* 2009). Clavicipitalean endophytes have a narrow host range, they systemically colonize shoot, rhizome, and not the roots

(Bruggmann *et al.,* 2016). In contrast, non-claviciptalean endophytes are highly diverse, their colonization is non-systemic, isolated from all parts of the host; shoot, root, rhizome and they represent a wide range of comycetes families (Arnold *et al.,* 2009; Sieber, 2007). Non-claviciptalean endophytes are classified into different functional groups based on their host range, tissue colonization, *in planta* colonization, biodiversity and transmission (Arnold *et al.,* 2009). This study will focus on endophytic *Trichoderma* species and their abilities to enhance plant resistance to pathogens, potentially controlling plant diseases and increasing crop yields (Bae *et al*., 2008; Doni *et al.,* 2021).

Trichoderma as a biological agent: discovery and mode of action

A well-known class of biological control agents is represented by the fungi belonging to the *Trichoderma* genus (family Hypocreaceae) (Cerato, 2008). Persoon (1794) first proposed the genus Trichordema in Germany, and he proposed four species; *Trichoderma viride, T. nigroscens, T. aureum and T. roseum* (Pandya, 2017). *Trichoderma* species are versatile, non-pathogenic fungi often found in soil, as well as in plants and the potential value of Trichoderma as a biocontrol agaent was first reported by Weingling in 1932 (Bae *et al*., 2008; Baldwin *et al.,* 2020; Pandya, 2017). Several strains of *Trichoderma* have been developed into biological control products, which are used all over the world to control fungal plant diseases on various crops using several mechanisms such as antibiosis, parasitism and induced resistance (Chet *et al.,* 2004; Bae *et al.,* 2011). Most of these biological control products are from the species *T. harzianum, T. viride and T. atroviride* (Cerato, 2008). Apart from being used as a biocontrol agent, *Trichoderma* species also promote nutrient uptake, plant growth and yield increase (Baldwin *et al.,* 2020). *Trichoderma* species have been isolated from healthy plants such as grapevine wood, suggesting that they may be present inside plants without causing any symptom or disease, as endophytic fungi (Crous *et al.,* 2003). These species have evolved into opportunistic, avirulent, non-pathogenic, and symbiotic symbionts of plants (Crous *et al.,* 2003; Bae *et al.,* 2011).

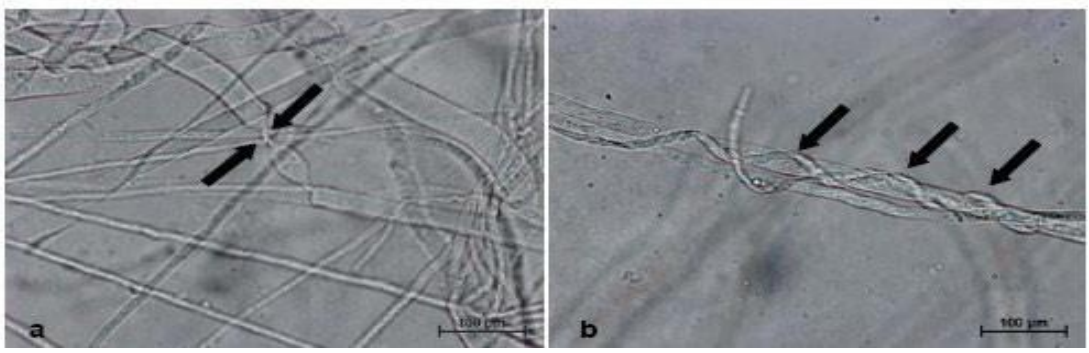

Figure 2.10: Some of the interactions such as mycoparasitism and antibiosis that can be observed under a microscope when the BCA *Trichoderma* is growing together with a fungal pathogen (Brotman *et al*. 2010)

Endophytic *Trichoderma* protects host plants by attacking fungal pathogens directly by producing toxins that activate defence mechanisms such as salicylic acid and jasmonic acid (Baldwin *et al.*, 2020). *Trichoderma* species also produce enzymes that cause shriveling, hyphal disintegration and ultimately death of the pathogen, a phenomenon called antibiosis (Figure 2.8a) (Brotman *et al.*, 2010). *Trichoderma* are also parasites of other fungi (mycoparasites) in which case they attach to, coil around and penetrate the hyphae of the host fungi (Figure 2.8b) (Brotman *et al.*, 2012).

2.8 Conclusion

Avocado has become an important tropical fruit exported and consumed globally. Avocado farmers are challenged by a range of pre- and postharvest diseases of avocado trees, leaves and fruit. These pre-and postharvest diseases include Cercospora leaf spot, Cladosporium leaf spot, anthracnose, and stem-end rot which can cause severe economic losses. Several BCAs such as *Trichoderma* spp. and *Bacillus* spp. that are effective against avocado diseases like root rot caused by *Phytophthora cinnamomi* have been identified (Merfield, 2012). However, more studies are required to understand the ecological impact and effect of biocontrol agents on the avocado agroecosystem. This study will investigate a wider range of potential *Trichoderma* spp. that will be biocontrol agents for avocado diseases such as anthracnose, stem-end rot, Cladosporium spots, and Cercospora spots. More studies will be conducted to investigate the persistence, long-term efficacy, and adaptability of the isolated *Trichoderma* spp. in field trials.

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Chapter 3 Isolation and Identification of *Trichoderma* species from avocado fruits and leaves

Abstract

Fungal pathogens are a major cause of plant diseases and result in significant crop losses. Avocado (*Persea americana* Mill.) production is affected by pre-harvest fungal infections, which cause both pre- and postharvest avocado diseases. Agrochemicals are being limited to the avocado industry due to maximum residue levels (MRLs) being reduced in many countries, including those of the European Union (EU), a major market for South African fruit exports. The effective use of biological control agents (BCAs) such as *Trichoderma* is an important component of reducing environmental and health risks, sustainable agriculture, and the promotion of plant growth. This study aimed to isolate and screen endophytic strains of *Trichoderma* spp. for their ability to control key fungal pathogens such as *Colletotrichum*, *Pseudocercospora*, *Lasiodiplodia*, and *Cladosporium*. A total of 17 *Trichoderma* spp. was isolated from avocado fruits and leaves. Single colonies of *Trichoderma* strains were established with samples from the Trichoderma Selective Medium (TSM) petri dishes onto Potato Dextrose Agar (PDA) plates. For cultural morphology, different mycelial radial growth of the colonies of each strain was recorded on days 3 and 5 on PDA plates. Endophytic screening was conducted by preparing *Trichoderma* conidial suspensions at a concentration of 1×10^6 conidial ml^{-1} from fresh cultures of each strain grown on PDA plates and sprayed on fresh avocado fruits and seedlings. Sterile distilled water was sprayed onto the control fruits. After 7 days *Trichoderma* strains were re-isolated from ten different spots on the fruit using TSM. This experiment was repeated twice. A total of 9 isolates out of 17 *Trichoderma* strains demonstrated high endophytic ability of 70 to 100%. Isolate UK1E and UK4C showed 100% endophytic ability.

Keywords: avocado; biocontrol agents; endophytes; fungal pathogens; Trichoderma

3.1 Introduction

Avocado (*Persea americana* Mill.) is a subtropical/tropical fruit widely produced and consumed worldwide (Belmonte-Herrera *et al.,* 2020). In South Africa, avocados are mostly exported or consumed locally as fresh fruits or processed to extract avocado oil and create guacamole or cosmetic products (Chang *et al.*, 2021). Several fungi infect avocado leaves and fruit pre-harvest, which cause both pre- and postharvest avocado diseases, resulting in significant crop losses (Alvarez, 2004). Historically, agrochemicals have been used to manage most avocado plant diseases. However, public sentiment has turned against agrochemicals due to residues in food, and the damage that they are perceived to cause to the environment (Majeed, 2018). Therefore, agrochemicals are being lost due to the minimum accepted residue levels (MRLs) being reduced in many countries, including those of the EU, a major market for South African fruit exports (Majeed, 2018). In this respect, the use of biological control agents (BCAs), such as *Trichoderma* species, has been regarded as a promising and environmentally friendly approach to controlling plant diseases (Butt *et al.,* 1999). The potential use of *Trichoderma* species as BCAs of plant diseases was first recognized in the early 1930s by Weindling (Howell, 2003). *Trichoderma* species are opportunistic, avirulent plant symbionts and are used as BCAs against plant pathogens due to their multi-mode of action against pathogens, including antibiosis, mycoparasitism, production of induction of plant defensive mechanisms and promotion of plant growth (Arikit *et al*., 2020). Furthermore, some strains of *Trichoderma* spp. have been found to be symbiotic endophytes, due to their ability to colonize internal plant tissues (leaves, roots and fruits). The combination of biocontrol activity and endophytic behaviour plays an important role in the defence that they could provide against pre-harvest pathogens (Arikit *et al.,* 2020). Latent infection is the main problem faced by many farmers in transportation and storage due to pathogens that infected the fruits in the field. Therefore, the application of endophytic biocontrol agents in pre-harvest stages is to prevent pathogen colonization on the surface of the fruit in order for the wounds made during harvest to be colonised by the antagonist agent (de Carmen Orozco-Mosqueda *et al.,* 2021). Fungal endophytes are found living within the intercellular or intracellular spaces of host plants, without causing apparent disease symptoms, in symbiotic relationships with their host plants (Bae *et al.,* 2019). They produce secondary metabolites that produce major benefits to their host plants (Hosseyni- Moghaddam and Soltani, 2014). *Trichoderma* has long been associated with plant root systems due to its saprophytic abilities of inhabiting soil (Garrett, 1950). More recent studies have indicated that the interaction between *Trichoderma* and plants is now more

intimate and, complex, involving direct contact and internal colonization of a range of plant tissues (Bailey and Melnick, 2013). The isolation of *Trichoderma* strains from inside plant tissues has resulted in the discovery of new *Trichoderma* species (Bailey and Melnick, 2013).

The aim of this study was to isolate *Trichoderma* spp. and the endophytic screening of the isolated *Trichoderma* strains.

3.2 Materials and Methods

3.2.1 Plant material used for isolation of potential endophytic Trichoderma species

Avocado leaves were sampled from five avocado trees of the cultivar Fuerte growing at Ukulinga Farm, University of KwaZulu-Natal, Pietermaritzburg. On the 1st February 2021, twelve leaves: four young leaves (recently emerged, lighter in colour, softer and not yet fully expanded to their mature size); four old leaves (matured leaves, darker green with more chlorophyll, tougher and have reached maximum) and four diseased leaves (with colour changes, spots and lesions, wilting and curling) were sampled from each tree, resulting in a total of sixty leaves (Bower and Cutting, 1988). Five fresh avocado fruits with no disease symptoms or physical damage were collected from a local supermarket in Pietermaritzburg, Scottsville. The fruits and leaves were washed using tap water, then surface sterilized using 2% sodium hypochlorite for three minutes, rinsed three times in sterile distilled water and air-dried before use.

3.2.2 Isolation, storage and identification of potential endophytic *Trichoderma* species

Trichoderma species were isolated from the leaf material by cutting small fragments of the leaves which were placed onto *Trichoderma* Selective Media (TSM) in Petri dishes. Five fragments from each leaf were placed onto TSM per Petri dish, four Petri dishes per leaf, sealed using parafilm and incubated at 25°C for 7 days (Abdoulaye *et al.,* 2022). *Trichoderma* spp. was also isolated from the fresh avocado fruits. Small pieces of the fruit flesh were randomly cut and placed on TSM medium, sealed with parafilm and incubated at 25°C for 7 days. Pure cultures were purified on Potato Dextrose Agar (PDA) medium and incubated at 25°C for 3 to 7 days. For visual or macro-morphology observation, the mode of mycelia growth, and colour for each PDA isolate were examined every day for the identification of *Trichoderma* species. For micro-morphology, a slide culture technique was used to examine the shape, size, development of mycelia, conidiophores and conidia under a light microscope. Isolates were

then stored at 4°C for short-term and current use. For long term storage, the *Trichoderma* strains in microcentrifuge tubes were stored at -80°C in 30% glycerol solution.

3.2.3 Conidial suspension preparation of *Trichoderma* strains

Trichoderma conidial suspensions were prepared from fresh cultures grown on PDA plates. Each plate was surface-washed with 10 ml^{-1} of sterile distilled water and gently scraped with a glass hockey stick. The concentrations of the conidial suspensions were adjusted to 1 x 10^6 conidia/ ml^{-1} using a hemacytometer (Marienfeld Superior™, Germany) under a light microscope (Zeiss Axiophot, Germany).

3.2.4 Endophytic screening of *Trichoderma* isolates

Fifty-two avocado seedlings (Edranol cultivar) were sprayed with benomyl to kill any natural endophytic *Trichoderma* spp. found in them and there was a waiting period of two weeks. The *Trichoderma* strains isolated in section 3.2.2. were used to prepare suspensions with a concentration of $1x10^6$ conidia/ml^{-1}. The avocado seedlings were sprayed with the *Trichoderma* suspensions and after fourteen days, seedling leaves were sampled randomly. The sampled leaves were surfaced and sterilised using 2% of sodium hypochlorite and sterile distilled water. Small fragments of the leaves were placed onto TSM Petri dishes, sealed using parafilm, and incubated at 25°C for seven days as described in section 3.2.2. The endophytic *Trichoderma* strains isolated from the seedlings were then used to prepare suspensions to inoculate fresh avocado fruits for the second endophytic screening. Fresh avocado fruits of the cultivar Pinkerton were collected from a local farm in Pietermaritzburg. The fruits were first washed using tap water, and then surface sterilized using 2% sodium hypochlorite as described in Section 3.2.1. The fruits were left to air-dry overnight. On day two, each fruit was sprayed with the *Trichoderma* conidial suspension at a concentration of 1 x 10^6 conidia/ ml^{-1} as prepared in Section 3.2.3, each *Trichoderma* strain had six replicates. Fruits sprayed with only sterilised distilled water served as a control. The fruits were then air-dried and stored in boxes covered with black plastic bags for relative humidity at room temperature for 7 days.

The plastic bags were removed after 7 days and the fruits were washed using tap water, surface sterilized using 2% sodium hypochlorite for three minutes, rinsed three times in distilled water and air-dried prior to use. *Trichoderma* spp. was re-isolated following the same technique described in Section 3.2.2. The strains were isolated from ten different spots of the fruit to

check where it colonised and protected the fruit. Those *Trichoderma* isolates showing the highest endophytic ability were screened in a second experiment performed in the same way to confirm the results. *Trichoderma* isolates with the highest endophytic ability in both experiments were considered as endophytic *Trichoderma* spp. and were then used in subsequent studies to their biocontrol capabilities.

3.3 Results

3.3.1 Isolation and morphological identification of potential endophytic *Trichoderma* species

A total of 17 *Trichoderma* species were isolated from avocado fruits and leaves. *Trichoderma* fungal growth on TSM Petri dishes were purified on PDA plates as demonstrated in Figure 3.1A and 3.1B. After 3-7 days, all the pure cultures (Figure 3.1C) were differentiated by observation of colour, odour, and measurement of mycelial radial growth. The isolated strains were first labelled randomly, then at a later stage, each isolate was labelled according to the source of *Trichoderma*. All the strains obtained from the trees at Ukulinga farm were labelled UK1A (UK- Ukulinga; 1A- tree number one, replicate A). All the strains obtained from the five avocado fruits were labelled TsF1A (Ts- *Trichoderma* strain; F- fruit; 1A- fruit number one replicate A). Table 3.1 demonstrates the different radial growth of the colonies of each strain at day 3 and day 5 on a PDA Petri dish. Isolate UK1C, UK2G, TsF2B, TsF3G and TsF5C were the only isolates that grow faster mean > 6 cm at day 3 (Table 3.1). The colonies of *Trichoderma* strains grew rapidly and were initially woolly but became compact with time (Figure 3.1 C). Formicro-morphologyy, a slide culture technique was used to examine each *Trichoderma* strain under a light microscope (Zeiss Axiophot, Germany) to observe the shape, size, development of mycelia, conidiophores and conidia (Figure 3.1 D).

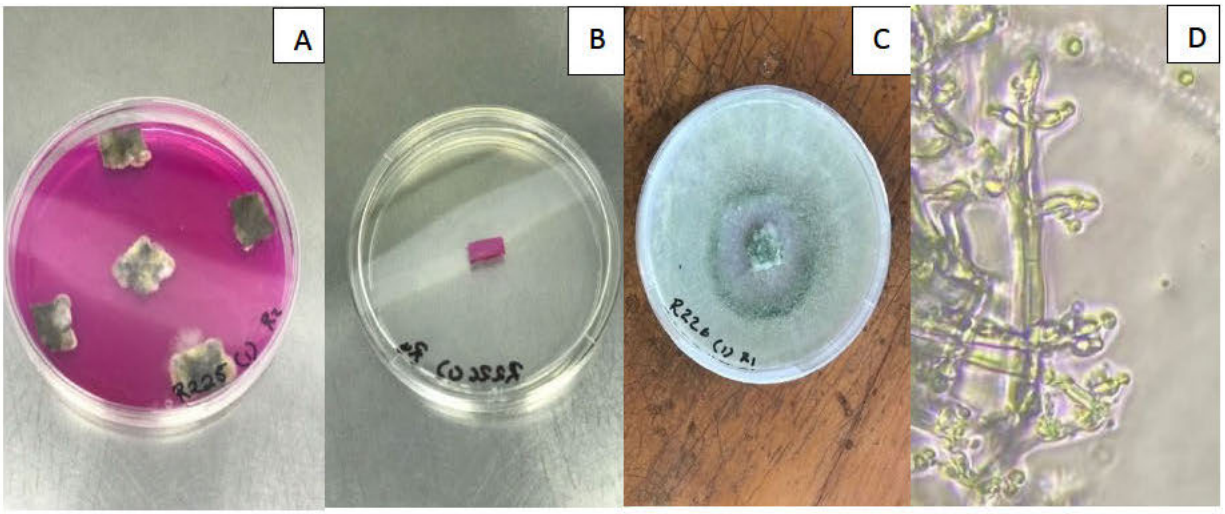

Figure 3.1. Photograph show colonies of *Trichoderma* mycelium (A) on TSM (B) on a PDA plate, (C) *Trichoderma* pure culture after 7 days and (D) conidiophore with conidia under a light microscope (40x).

Table 3.1. Fungal growth of *Trichoderma* spp. isolated from avocado leaves and fruits on PDA plates after day 3 and day 5.

Trichoderma strains	Day 3 (cm)	Day 5 (cm)
UK1C	6	8.5
UK1E	5	8.5
TsF2B	6.8	8.3
UK2C	4.1	7.9
UK2E	4.8	8.1
UK2F	5.2	8.4
UK2G	6.5	8.5
UK2H	4.5	7.6
TsF3A	4	7
UK3B	4.4	7.3
TsF3E	5	8.1
TsF3F	4.9	8.5
TsF3G	6.2	8.5
UK4C	4.5	7.7
UK4G	5.8	8.1
UK5A	6	8.5
TsF5C	6.1	8.5

3.3.2 Endophytic screening of *Trichoderma* spp.

In this study, a total of eleven strains of *Trichoderma* demonstrated high endophytic ability on avocado seedling and nine isolates of *Trichoderma* demonstrated high endophytic ability in both seedlings and avocado fruits experiments described in Section 3.2.4. *Trichoderma* isolates described in Section 3.3.1 were inoculated by spraying conidial suspension onto each fruit. After 7 days the *Trichoderma* strains were re-isolated from ten different positions spaced around the fruit and the presence of the strain in each spot was recorded as demonstrated in Table 3.2. The positive (+) sign represented the growth of the *Trichoderma* strain, and the negative (-) sign represented the absence of the *Trichoderma* strain. The nine strains showed endophytic abilities of 70 to 100%.

Table 3.2. Endophytic screening of *Trichoderma* spp. Isolated from avocado leaves and fruits.

Trichoderma strains	Endophytic ability
UK1C	++++++++--
UK1E	++++++++++
TsF2B	+++++++---
UK2C	++++------
UK2E	+++-------
UK2F	++--------
UK2G	+++++++---
UK2H	++++------
TsF3A	++--------
UK3B	+++++-----
TsF3E	++++++++--
TsF3F	+++-------
TsF3G	+++++++---
UK4C	++++++++++
UK4G	++++++++--
UK5A	+++-------
TsF4A	+++++++---

(+) growth of *Trichoderma* strain; (-) absence of *Trichoderma* strain

Table 3.3. Macro and micro characterisation of endophytic *Trichoderma* species

Isolate designation	Macro-/Micro characteristics
UK1C	The colony initially observed to be whitish to light green. At a later stage the central part colony turned watery white to dark green. Concentric rings present. (Figure 2A)
UK1E	At the early stage (2-4 days) the colony was observed to be watery white, turned bright green and finally at a later stage mycelium at the central part of the PDA turned dark green with branched conidiophores. (Figure 2B)
TsF2B	Watery white mycelium with denser dark to dull green conidial production at the centre and then towards the margins. Conidiophores were septate and highly branched. Conidia ellipsoid to oval in shape. (Figure 2C)
UK2G	Colony white to pale green and gradually turned dark green at the centre of the PDA plate as well as at the margins. Round-shaped conidia. (Figure 2D)
TsF3E	Watery white center to dark green colony. Hairy and mycelia uniformly grown on PDA after 7 days. (Figure 2E)
TsF3G	The colony colour was first observed to be whitish to light green and watery in the center. The colony gradually became a deep green and appeared soft and leathery. The conidiophores were smooth, erect and had asymmetrical branches. Conidia sub-globose to ellipsoid. (Figure 2F)
TsF4A	The colonies first appeared whitish to pale green. After 4-7 days colonies turned dark green and conidia sub-globose to ovoidal. (Figure 2G)
UK4C	Watery white colony and dark green colony at the central parts of PDA. Conidia smooth and sub-globose round. (Figure 2H)
UK4G	White mycelia with orange-green or yellow-green patches that become visible. Conidial globose to sub-globose and arranged in divergent groups. (Figure 2I)

The endophytic *Trichoderma* strains were characterised in Table 3.3 according to their visual or macro-morphology such as the mode of mycelia growth, colour, and the changes of medium colour on each PDA isolate. This was examined on Days 1- 7 for the identification of the endophytic *Trichoderma* species. For micro-morphology, culture slides were prepared to examine the shape, size, development of mycelia, conidiophores and conidia under a light microscope (Figure 3.2A-I). Results demonstrated that all isolated were initially whitish in colour and later became different shades of green (Figure 3.2A-I). The conidiophores were highly branched for isolates UK1E and TsF2B (Figure 3.2 A-B) and the conidia was initially sub-globose for most isolates such as TsF3G, TsF4A, UK4C and UK4G (Figure 3.2 F-I).

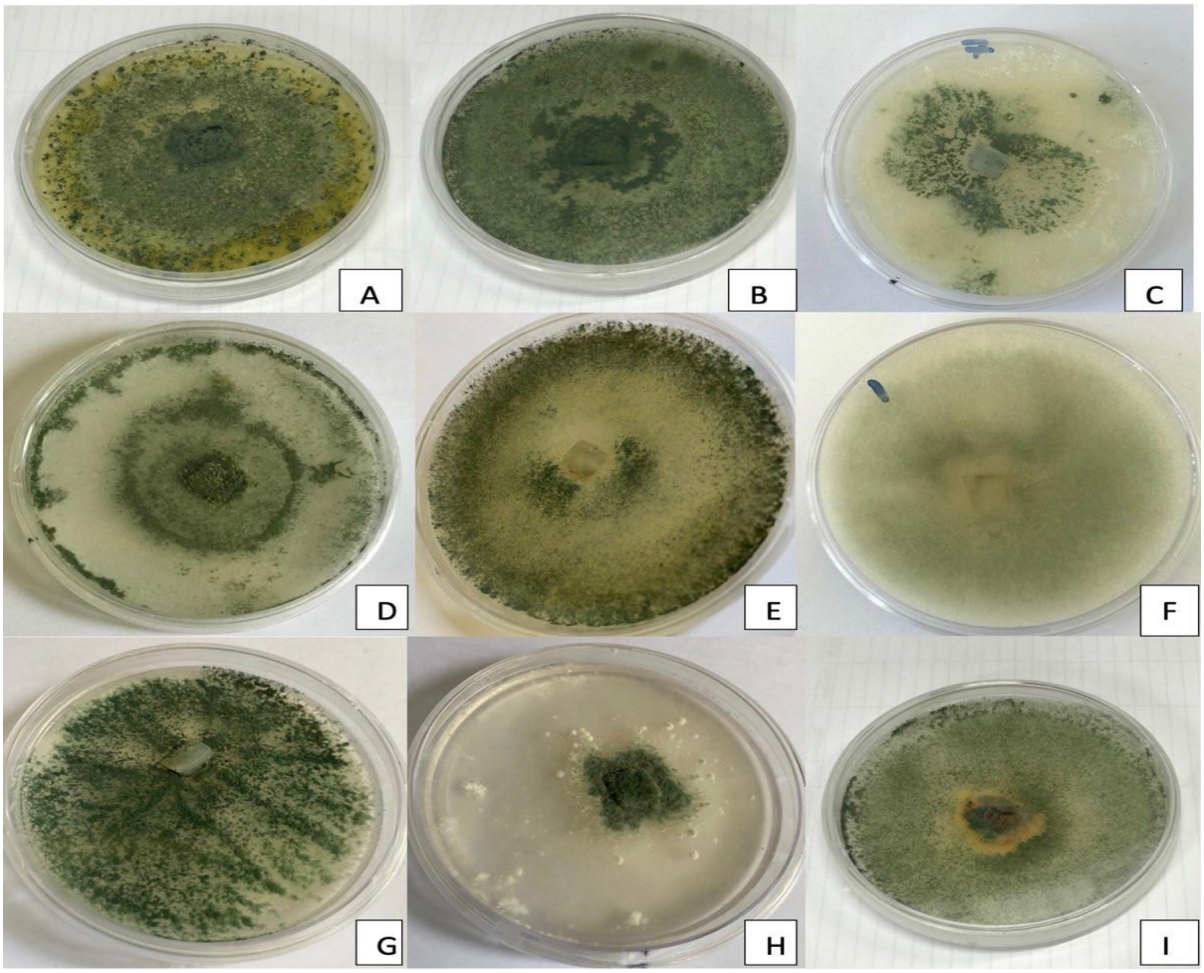

Figure 3.2. Colonies of endophytic *Trichoderma* strains isolated from avocado leaves and fruits on PDA plates: (A) UK1C, (B) UK1E, (C) TsF2B, (D) UK2G, (E) TsF3G, (F) TsF3G, (G) TsF4A, (H) UK4C and (I) UK4G

3.4 Discussion

Trichoderma species are worldwide in occurrence and known for being easily isolated from soil, decaying wood, and other plant organic matter (Howell, 2003). In this study, seventeen strains of *Trichoderma spp.* were isolated from avocado fruit and leaf material. It took 4-7 days for the different strains to grow fully and to change colour from whitish to light or dark green. Previous studies show that fungal species belonging to the *Trichoderma* genus grow at a rapid rate in culture and produces numerous conidia that vary in shades of green (Kotasthane and Shalini, 2007). The isolates were first grown on Trichoderma Selective Medium (TSM) which was developed by Elad *et al., (*1981) and later improved by Elad and Chet (1983) who added Captan to TSM (Askew and Laing, 1993). The addition of Captan was to prevent other fungi such as *Fusarium* spp. from growing in TSM while selecting *Trichoderma* species (Askew and Laing, 1993). Askew and Laing (1993) modified the medium further by using Previcur® (propamocarb) to control oomycete fungi. For culture purification, mycelia growth on TSM was sub-cultured to PDA plate which promotes, rapid growth for a wide range of fungi (Adhikary *et al.,* 2013). The different strains were differentiated based on colony colour and morphology, and conidial structure. A total of eleven strains out of seventeen *Trichoderma* strains were isolated from the leaf materials, which is 65% greater than the *Trichoderma* strains isolated from the fruit materials (7 isolates) (Table 3.1.). *Trichoderma* spp. are free-living fungi common in soil and root ecosystems (Chet *et al.*, 2004). Avocado trees are therefore exposed to *Trichoderma* spp. living in the soil from the seedling stage up until the mature stages of the tree. Mature trees would be constantly showered with conidia released from *Trichoderma* colonies living in the soil or on organic matter or in other trees. These fungi stimulate plant growth by providing useful growth substances such as glucose oxidase and growth-stimulating compounds (Amol *et al.,* 2019). When the tree starts reproducing fruits, endophytic strains of *Trichoderma* would already be established in many tissues. The endophytic screening study showed that eleven of seventeen strains were endophytic on avocado seedling leaves and nine of eleven *Trichoderma* isolates had endophytic abilities on avocado fruits. Isolate UK1E and UK4C, were the only two strains that were able to colonize the entire fruit, demonstrating the presence of the *Trichoderma* strain inoculated onto the fruit on all ten random spots of isolation (Table 3.2). The nine endophytic strains were able to colonize avocado internal tissues without causing harm to them, similar to the results obtained by Bailey and Melnick (2013). *Trichoderma* species have been described as

opportunist avirulent plant symbionts for their abilities to benefit from their host and to provide benefits to their host in direct interaction. Macro and micro-morphology of the endophytic strains (Table 3.3) demonstrated that most *Trichoderma* species produce prodigious conidia and submerged mycelium (Figure, 3.1D). These endophytic *Trichoderma* strains are potential biocontrol agents to be used in further studies to control pre-harvest infections of avocado fruits.

3.5 Conclusion

Out of seventeen *Trichoderma* strains isolated from this study, only nine *Trichoderma* isolates demonstrated endophytic abilities. 65% of the endophytic strains were isolated from avocado leaves and 25% were isolated from avocado fruits. The *Trichoderma* strains with endophytic ability are potential biocontrol agents for avocado fungal diseases as antagonist endophytes help colonise the fruits before the pathogens if applied in pre-harvest stages (del Carmen Orozco-Mosqueda *et al.,* 2021). The endophytic *Trichoderma* spp. will be identified by molecular techniques analysis and the biocontrol capabilities test *in vitro* and *in vivo*.

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Chapter 4 Isolation and identification of pathogens causing avocado fruit spot and fruit rot

Abstract

Avocado (*Persea americana* Mill.) is one of the most sought-after food sources worldwide, produced in more than 30 countries around the world, with growing demand. However, competition in the market is high and production is limited by the presence of diseases, and the high costs associated with crop losses and the costs of controlling these diseases. To date, postharvest fruit diseases are responsible for most of the avocado losses in South Africa, especially those caused by plant pathogenic fungi. The objectives of this study were to isolate and identify fungal pathogens that cause fruit and leaf spot and fruit rot on avocado fruit in KwaZulu-Natal (KZN), South Africa. A total of 45 isolates were recovered from fruit displaying typical symptoms of anthracnose, stem-end rot, Cladosporium spot and Cercospora spot. Pure cultures were grown on Potato dextrose agar (PDA), and the fungi were tentatively identified according to their cultural and morphological characteristics, observed using light and scanning electron microscopy. Pathogenicity tests of the isolates on non-symptomatic avocado fruit were performed through a non-wounding inoculation method, following surface sterilization of the fruit. Out of 45 isolates, *Colletotrichum* species were the most frequently isolated genus. These isolates could be divided into two morphological groups (Cs1 and Cs2). *Lasiodiplodia* spp. was the second most isolated genus (Ls1) and another strain of *Lasiodiplodia* spp. (Ls2) was obtained from a previous study. About 20% of the fungal genera, *Pseudocercospora* (Pp1) and *Cladosporium* (Cc1) were isolated rarely. The fungal pathogens associated with anthracnose Cs1 and stem end rot isolate Ls1 appeared to be more virulent than the other morpho-groups. The anthracnose isolate Cs1 was identified as *Colletotrichum cobbittiense* and Cs2 as *Colletotrichum henanense,* and the stem-end rot isolate Ls2 was identified as *Lasiodiplodia mahajangana*. However, isolate Ls1 was identified as *Neofusicoccum parvum*. This study revealed that *N. parvum* is currently a serious problem in KwaZulu-Natal as it was the most virulent postharvest pathogen isolated during this investigation.

Keywords: avocado, Cercospora spot, Cladosporium spot, Colletotrichum cobbittiense, Colletotrichum henanense, Lasiodiplodia mahajangana, Neofusicoccum parvum.

4.1 Introduction

Avocado (*Persea americana* Mill.) is a highly sought-after food source worldwide due to its high nutritional value and its use in the cosmetic, health, and pharmaceutical industries (Marais, 2004; Pérez-Jiménez, 2008). This crop is produced in more than 30 countries and global production continues to grow (Marais, 2004). However, its production is limited by diseases and the high costs of controlling them. Pre- and post-harvest fruit diseases cause significant losses of avocado crops in South Africa, largely due to biotrophic plant pathogenic fungi (Kulshrestha and Sharma, 2015; Gashaw *et al.,* 2019). These fungi infect host plants without causing cell death, feeding on the host biotrophically, but at some point, they become necrotrophic and cause a variety of fruit rots that result in crop yield and quality loss (Green and Perfect, 2001). The genus *Colletotrichum* is one of the top 10 plant fungal pathogens that cause significant economic damage worldwide to a wide range of crops (Dean *et al.*, 2012). Anthracnose caused by *Colletotrichum* species, and stem-end rot caused by *Lasiodiplodia* species, are two of the major constraints in the avocado industry globally due to their high incidence, and their severe impact on the shelf-life and marketability of infected fruit (Obianom and Sivakumar, 2018). Avocado fruit infections occur in the field but remain dormant until after harvest, developing during the ripening, storage, or shelf stages (Marais, 2004). Stem-end rot of avocado, usually caused by members of the family Botryosphaeriaceae, has also been reported to cause serious pre-harvest crop losses during rainy seasons (Adaskaveg *et al.,* 2013). The most common cause of stem-end rot is *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. Symptoms first appear as a shriveling at the scar of the fruit left after stem removal during harvest (Adaskaveg *et al.,* 2013). This is followed by decay, discoloration, and softening of the interior of infected fruit (Latorre *et al.,* 2019). Most stem-end infections occur when the pathogen produces conidia during periods of high humidity, which are disseminated by rain splash, wind, and harvesting tools (Adaskaveg *et al.,* 2013). Avocado trees are also attacked by various fungal pathogens causing fruit and leaf spots, such as *Pseudocercospora* species that causes Cercospora spot (black spot), and *Cladosporium* species that cause Cladosporium spot (brown spot) (Korsten and van Eeden, 2013; Coertzen and Fourie, 2018). Cercospora spot is of economic importance in South Africa as it results in severe crop losses in untreated orchards (Kallideen, 2020). *Cladosporium* is considered a secondary invader that results in dark, circular- to-oval shaped lesions on fruit called avocado brown spot (Alok and Jai, 2015).

The aim of this study was to isolate and analyse the cultural morphology of the fungal pathogens from avocado fruits with typical symptoms, such as mycelium growth and colour, spore size, shape, and presence of the conidia.

The objectives of the study were:

1. To isolate and identify fungal pathogens causing avocado fruit spots and fruit rot.
2. Pathogenicity screening on healthy avocado fruit to verify Koch's postulates.

4.2 Material and Methods

4.2.1 Plant material used for isolation of postharvest avocado pathogens

Avocado fruits that displayed symptoms of avocado fruit rot and fruit spot were purchased from several supermarkets in Pietermaritzburg, KwaZulu-Natal (KZN). The fruit was brought to the Plant Pathology Laboratory at the University of KwaZulu-Natal, Pietermaritzburg campus for fungal isolation. The fruit was first washed in running tap water, surface sterilized using 2% sodium hypochlorite for three minutes, rinsed three times in sterile distilled water, and air-dried on a laminar floor bench before use.

4.2.2 Isolation and storage of postharvest pathogens

Avocado black spot

Small pieces (3 to 10 mm^2) of discoloured tissue from fruit with typical Cercospora spot were cut out and placed on small quantities of petroleum jelly (Vaseline) placed the underside of the lids of Petri dishes containing water agar, sealed with parafilm and incubated at 25°C for 7- 14 days. Pure cultures were isolated and transferred onto potato dextrose agar (PDA) medium for identification of the fungus and incubated at 25ºC for 7 days. For long term use, cultures were grown on PDA agar slants that were stored in a fridge at 10˚C.

Avocado brown spot

Small pieces (3 to 10 mm^2) of disinfected tissue of avocado fruit displaying symptoms of Cladosporium spot were removed using a sterile scalpel and placed on PDA medium, sealed with parafilm and incubated at 28°C for 7 days. After 7 days, the mycelium of fungal colonies was subcultured onto PDA medium, which was sealed with parafilm and incubated at 28°C to create

pure cultures. The cultures were grown on PDA agar slants and stored in a fridge at 10˚C. Agar plugs were transferred to a new PDA medium every three weeks to create fresh colonies.

Avocado anthracnose

Sections of fruit showing sunken lesions were cut into 5 mm^2 squares from disinfected avocado fruit with anthracnose symptoms. The squares were placed on PDA medium in Petri dishes, which were sealed with parafilm and incubated at 25°C for 7 days. Mycelium and conidial masses of *Colletotrichum* were observed and transferred onto freshly prepared PDA medium for culture purification and incubated at 28°C. Pure cultures were grouped according to colony colour, mycelium morphology and conidial presence. Isolates were stored at 4°C for short term and current use and stored in 30% glycerol solution and stored at -80°C for long-term use.

Avocado stem-end rot

5 mm^2 squares of symptomatic flesh of the fruit from the margins of decay were placed on PDA medium in Petri dishes, sealed with parafilm and incubated at 28°C for 7 days. A *Lasiodiplodia* strain had been isolated and identified in a previous study (Majola, 2020). This strain was provided for this study on a slant bottle and sub-cultured on freshly prepared PDA medium, after which the Petri dishes were sealed with parafilm and incubated at 28°C for 7 days.

4.2.3 Preparation of Conidial Suspensions of Fungal Pathogens

The conidial suspensions were prepared from fresh cultures of fungal pathogens grown on PDA medium. The pure cultures on each PDA plate were surface-washed with 10 mL of sterile distilled water and gently scraped with a glass hockey stick to release the conidia. The concentration of the conidial suspensions was adjusted to 1×10^6 conidia/ml^{-1} using a hemacytometer (Marienfeld Superior®, Germany) (Neubauer IMP kit 0.1 mm) under a light microscope (Zeiss Axiophot, Germany).

4.2.4 Cultural and Morphological Evaluation

Fresh pure cultures of each isolate were grown on PDA medium and incubated at 25-28°C. Four cultures of each isolate were evaluated for colony characteristics, and the colony diameter and colour were recorded for 7 days. After 14 days of growth on PDA, conidial suspensions of each isolate were prepared as described in Section 4.2.3. A drop of the suspension was placed on a

glass slide, covered with a glass cover slip, and viewed under a light microscope (Zeiss Axiophot, Germany). The colony size, colour of the conidial masses, and zonation were observed and recorded for identification. The cultures were also studied under scanning electron microscopy (SEM) (Zeiss, EVO LS 15). The cultures of each pathogen were fixed in 3% glutaraldehyde for 3 hours, washed in 0.05M sodium cacodylate buffer, and dehydrated for 10 minutes in ethanol (30%, 50%, 70%, 80%, 90%, and 3 times in 100%). The specimens were mounted on SEM stubs, transferred into a critical point dryer basket under 100% ethanol, and dried in a Quorem K850 critical point dryer. The specimens were coated with gold in a gold-palladium sputter coater and examined.

4.2.5 Pathogenicity Test of the Pathogens

Fresh Pinkerton avocado fruit was collected from a local farm in Pietermaritzburg (KwaZulu-Natal, South Africa). The fruit was first washed under running tap water, then surface-sterilised using 2% sodium hypochlorite for three minutes, rinsed three times in sterile distilled water, and air-dried overnight on a laminar flow bench. All isolated strains were subjected to a pathogenicity assay, with six fruits being inoculated per isolate. The inoculation method was non-invasive and involved spraying the entire fruit with a conidial suspension of 1×10^6 conidia/ml^{-1}. For stem-end rot, the fruits were sprayed directly onto the stem-end. The isolates of each species were kept in different boxes and immediately covered with black plastic bags to avoid cross-contamination. The inoculated fruit were maintained at 25°C with high relative humidity until symptoms appeared. A control treatment was sprayed with sterile distilled water, and a second control treatment consisted of uninoculated fruit. After the disease symptoms were observed, the pathogens were re-isolated as described in Section 4.2.2, in order to verify Koch's Postulates on the identification of pathogens. The pathogens were identified using cultural and morphological approaches. This experiment was conducted twice independently to confirm the disease incidence and severity of each isolate.

4.2.6 Molecular identification of fungal pathogen isolates associated with anthracnose and stem-end rot

Fresh cultures of the fungal pathogen isolates Cs1, Cs2 and Ls1 were sent to Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa) for DNA extraction, Polymerase Chain Reaction (PCR) and sequencing. Genetic DNA was extracted from the fungal isolates using the Quick-DNA™ Fungal/Bacterial Miniprep kit (Zymo Research, Catalogue No. D6005). Internal transcribed spacer (ITS) target region (ITS1 and ITS2) was amplified using Primers ITS1 and ITS4 (Table 4.1).

Table 4.1: ITS Primers sequences used for the identification of the pathogenic fungal isolates.

Name of Primer	Target	Sequence (5' to 3')	Reference
ITS1	Internal Transcribed Spacer Region	TCCGTAGGTGAACCTGCGG	Bruns *et al.,* 1990
ITS4	Internal Transcribed Spacer Region	TCCTCCGCTTATTGATATGC	Bruns *et al.,* 1990

The PCR parameters used under this investigation were NEB OneTaq 2X MasterMix with Standard Buffer (Catalogue No. M04825), Genomic DNA (10-30ng/µl), forward primer (10 µM), reverse primer (10 µM) and nuclease-free water (catalogue No. E476). The PCR protocol followed as the initial denaturation at 94C for 5 min, followed by 35 cycles (94°C for 30 sec, 50°C for 30 sec, 68°C for 1 min), with a final step at 68°C for 10 min and on hold at 4°C. The amplified PCR amplicons was examined in 1% agarose gel (CSL-AG500, Cleaver Scientific Ltd) stained with EZ-vision® Bluelight DNA Dye. The NEB Fast ladder was used on gels (N3238) as the size standard. The fragments were purified using the ExoSAP procedure (NEB M0293L; NEB M0371). Amplicons were purified for sequencing (Zymo Research, ZR-96 DNA Sequencing Clean-up kit™, Catalogue No. D4050), and sequenced in a forward and reverse direction (Nimagen, BrilliantDye™ Terminator Cycle Sequencing kit V3.1, BRD3- 100/1000) using an AB1 3730*x*/ Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific).

The sequences were submitted to the GenBank database, and BLASTn analysis was performed on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine if a sequence in the database matched the query sequence above a certain threshold (99% query coverage; 99% identity) (Dubey *et al.*, 2021).

4.3 Results

4.3.1 Isolation and Morphological Identification of Fungal Pathogens Causing Fruit Spot and Fruit Rot on Avocado

In this study, a total of 45 isolates (Cs1 + Cs2 = 20; Ls1 + Ls2= 16; Pp1 + Cc1= 9) were isolated from symptomatic avocado fruit with typical characteristics of anthracnose, stem-end rot, Cladosporium spot, and Cercospora black spot. The isolates were identified based on their cultural morphology as described in Section 4.2.4 (Table 4.2) (Cheesbrough, 2006). *Colletotrichum* spp. was the most frequently isolated genus causing anthracnose on avocado fruit (Figure 4.2), and these could be divided into 2 morphological groups (Table 4.2). *Lasiodiplodia* spp. was also divided into 2 morphological groups, as it was the second most isolated species and another isolate of *Lasiodiplodia* spp. was provided by Majola, (2020) (Table 4.2). *Pseudocercospora* spp. and *Cladosporium* spp. constituted 20% of the isolated cultures (Table 4.2, Figure 4.2, Figure 4.3).

Table 4.2: Cultural and morphological characteristics of *Colletotrichum, Lasiodiplodia, Pseudocercospora* and *Cladosporium* strains isolated from infected avocado fruits

Group	Morphological characteristics			Morpho-group
	Isolate name	Culture colour	Conidial shape	
1	Cs1	White to grey	Hyaline, cylindrical, Obtuse to slightly rounded ends	*Colletotrichum* spp.
2	Cs2	White to pink, orange	Fusiform with obtuse ends	*Colletotrichum* spp**.**
3	Ls1	White to light grey	Hyaline, aseptate rod shape	*Lasiodiplodia* spp.
4	Ls2	Cotton white, hyaline grey to dark grey	Ellipsoid to slightly rod-shaped conidia (Majola, 2020)	*Lasiodiplodia* spp.
5	Pp1	White, grey to dark grey black	Conidia are long cylindrical, obclavate, multi-septate. Conidiophores are hyaline to dark brown.	*Pseudocercospora purpurea*
	Cc1	Olive green to Dark green	Conidiophores arise from hyphae, bearing branched ramoconidia.	*Cladosporium spp.*

Cs- *Colletotrichum* species; Ls- *Lasiodiplodia* species; Ps- *Pseudocercospora* species; Cs- *Cladosporium* species

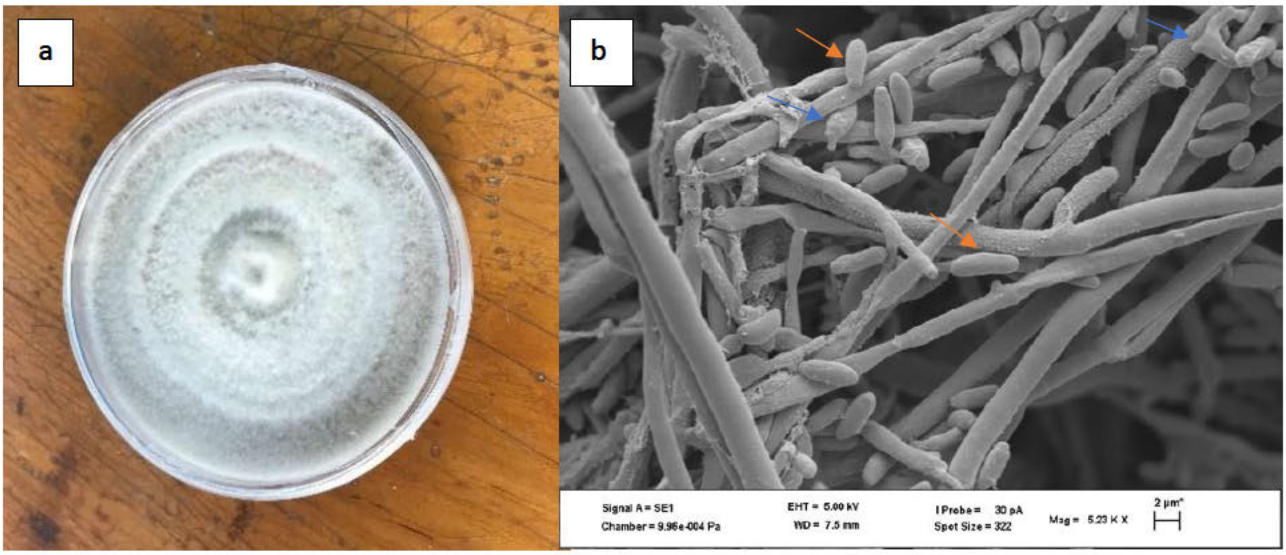

Figure 4.1: Photographs displaying the cultural and morphological characteristics of *Colletotrichum* spp.: (a) a pure culture growing on PDA, top view; (b) Conidiophores with conidia viewed by scanning electron microscopy (SEM).

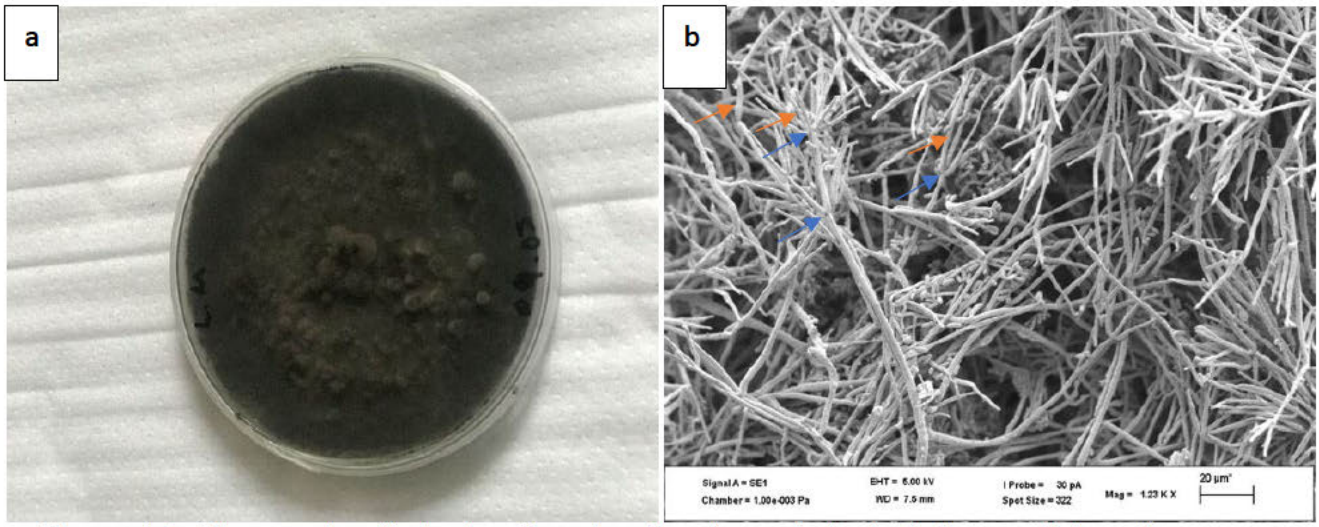

Figure 4.2: Photographs displaying the cultural and morphological characteristics of *Pseudocercospora* spp.: (a) Pure culture growing on PDA, top view; (b) Conidiophores with conidia under SEM.

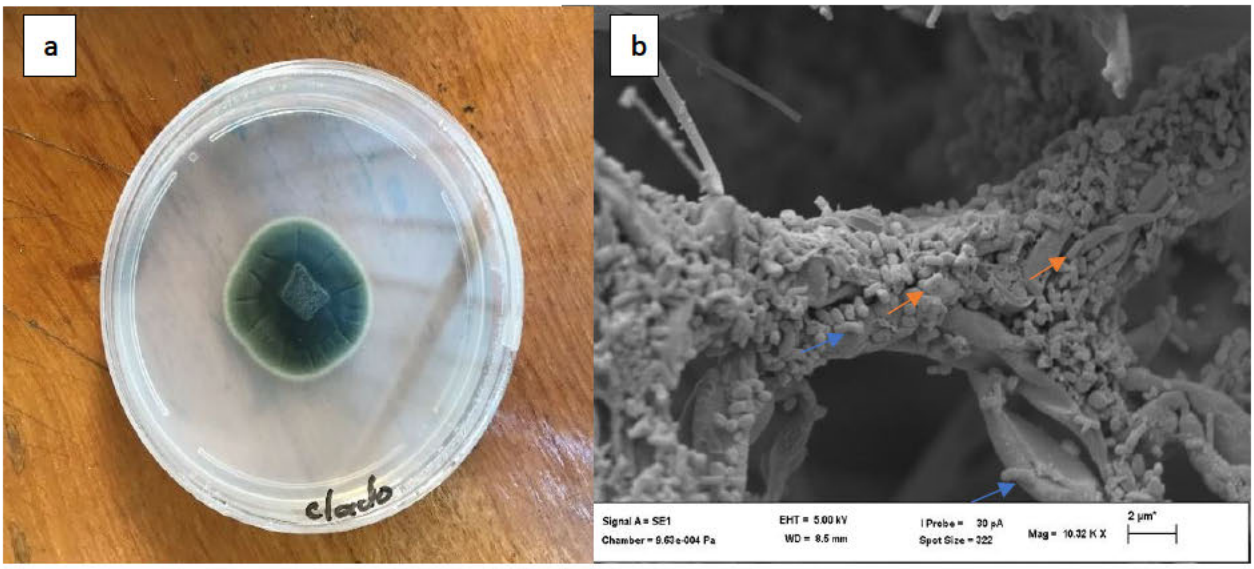

Figure 4.3: Photographs displaying the cultural and morphological characteristics of *Cladosporium* spp.: (a) Pure culture growing on PDA, top view; (b) Conidiophores with conidia under SEM.

4.3.2 Pathogenicity Test

In this study, there were six replicates per isolate, which resulted in a total of 270 inoculations. 80% of the *Colletotrichum* isolates caused typical symptoms of anthracnose five to seven days post-inoculation (dpi), suggesting that the isolates were highly pathogenic (Figure 4.4a). The inoculated fruit started to develop small, light brown lesions that matured into dark brown-black, sunken necrotic lesions, which were covered with pink-orange conidial masses. To validate Koch's postulates, pathogens were re-isolated onto PDA medium from the infected fruit tissue, as described in Section 4.2.1 (Ávila-Quezada and Silva-Rojas, 2011). Members of *Colletotrichum* isolates in Group 1 (Cs1) were the most virulent strains, while members of Group 2 (Cs2) were the least virulent (Table 4.2). The control fruit did not show any signs of anthracnose.

After inoculation with *Lasiodiplodia* spp., at 10 dpi, white fungal mycelia were observed on the abscission scar of the fruit, which was later followed by shriveling, slight softening of the flesh, and discoloration at the stem-end of the inoculated fruits (Figure 4.4b). The fruit were surface-sterilized, and small pieces of infected fruit tissue were placed on PDA medium for re- isolation of the pathogen. The identity of the

pathogens was confirmed through morphological characterization, as described in Section 4.3.1. that the isolates of *Lasiodiplodia* spp. caused the most severe stem-end rot symptoms, thus confirming Koch's postulates (Table 4.2) (Alves *et al.*, 2021). Members of *Lasiodiplodia* isolates Group 3 (Ls1) were more virulent than the isolates in Group 4 (Ls2).

Fruit with dark brown-black spots on the skin tissue after 7-14 dpi were surface-sterilized and small pieces of infected fruit tissue cut out and placed on PDA medium. Some inoculated fruit did not show any symptoms, even after 14 dpi. Morphological characterization showed that *Cladosporium* spp. and *Pseudocercospora* isolates were less aggressive than *Colletotrichum* and *Lasiodiplodia* isolates (Table 4.2). Fruit inoculated with *Cladosporium* spp. developed black spots, as well as dark brown, sunken lesions (Figure 4.4c). After re-isolation of pathogens from these lesions, isolates of both *Cladosporium* and *Colletotrichum* species were discovered. Only 20% of *Pseudocercospora* spp. isolates were found to be infectious (Figure 4.4d), while 80% of the isolates caused no symptoms (Table 4.2).

Figure 4.4: Symptoms on avocado fruit artificially inoculated with isolates of (a) *Colletotrichum* spp.; (b) *Lasiodiplodia* spp.; (c) *Cladosporium* spp.; (d) *Pseudocercospora* spp., previously isolated from avocado fruit.

Table 4.3: Disease incidence and severity of lesions caused by isolates inoculated to healthy avocado fruits

Group	Isolate replicates	Disease incidence				Severity
		Day 4	Day 7	Day 10	Day 14	Non-wound inoculation
1. *Colletotrichum*	Cs1A	Yes	Yes	Yes	Yes	3
	Cs1B	No	Yes	Yes	Yes	3
	Cs1C	Yes	Yes	Yes	Yes	3
	Cs1D	Yes	Yes	Yes	Yes	3
	Cs1E	No	Yes	Yes	Yes	2
	Cs1F	Yes	Yes	Yes	Yes	3
2. *Colletotrichum*	Cs2A	No	Yes	Yes	Yes	2
	Cs2B	No	Yes	Yes	Yes	2
	Cs2C	Yes	Yes	Yes	Yes	3
	Cs2D	No	No	Yes	Yes	1
	Cs2E	Yes	Yes	Yes	Yes	3
	Cs2F	No	No	Yes	Yes	2
3. *L*asiodiplodia	Ls1A	Yes	Yes	Yes	Yes	3
	Ls1B	Yes	Yes	Yes	Yes	3
	Ls1C	No	No	Yes	Yes	2
	Ls1D	No	No	Yes	Yes	2
	Ls1E	Yes	Yes	Yes	Yes	3
	Ls1F	No	Yes	Yes	Yes	3
4. Lasiodiplodia	Ls2A	No	No	Yes	Yes	2
	Ls2B	No	No	Yes	Yes	2
	Ls2C	No	Yes	Yes	Yes	3
	Ls2D	No	No	Yes	Yes	2
	Ls2E	No	Yes	Yes	Yes	3
	Ls2F	No	Yes	Yes	Yes	3
5. Pseudocercospora	Ps1A	No	No	No	No	1
	Ps1B	No	No	No	No	1
	Ps1C	No	No	Yes	Yes	2
	Ps1D	No	No	No	No	1
	Ps1E	No	No	No	Yes	2
	Ps1F	No	No	No	No	1
6. Cladosporium	Cs1A	No	No	Yes	Yes	2
	Cs1B	No	No	No	No	1
	Cs1C	No	No	Yes	Yes	2
	Cs1D	No	No	Yes	Yes	2
	Cs1E	No	No	No	No	1
	Cs1F	No	No	Yes	Yes	2

Severity scale: 1= perfect fruit; 2= fruit infected but largely edible; 3=fruit inedible

4.4 Molecular identification of fungal pathogen isolates associated with anthracnose and stem-end rot

Approximately 600 bp product of the ITS region was amplified from the pathogenic isolates Cs1, Cs2, and Ls1. Primers ITS1 and ITS4 were used in this investigation. The ITS gene region was highly polymorphic with enough informative taxonomic units to separate samples up to a species level (Bruns *et al.,* 1990). Results revealed that the two pathogenic isolates that caused anthracnose symptoms in avocado fruits were identified as *Colletotrichum cobbittiense* (Cs1), *and Colletotrichum henanense* (Cs2) (Table 4.4). Isolate Ls1 that caused stem-end rot symptoms in vivo was identified as *Neofusicoccum parvum* (Table 4.4).

Table 4.4: The primers and GenBank accession used for the identification of pathogenic isolates that cause anthracnose and stem-end rot on avocado fruits in KwaZulu-Natal region

Isolate name	Species name	Name of Primer	GenBank accession
Cs1	*Colletotrichum cobbittiense*	ITS1,	Mn85625.1
Cs2	*Colletotrichum henanense*	ITS4	ON793152.1
Ls1	*Neofusicoccum parvum*	ITS1,	KU997560.1
		ITS4	
		ITS1,	
		ITS4	

4.5 Discussions

In this study, the primary objective was to isolate and identify fungal pathogens causing avocado fruit rot and fruit spot. The identification was based on morphological features and the validation of Koch's Postulates for pathogenicity. The colours and shapes of conidia and appressoria were observed using light microscopy and scanning electron microscopy as described in Section 4.2.4. A total of 45 isolates were recovered from symptomatic avocado fruit, with *Colletotrichum* and *Lasiodiplodia* species being the most frequently isolated genus, and less commonly *Pseudocercospora* and *Cladosporium.* Most *Colletotrichum* isolates produced white mycelial colonies on PDA medium, which changed colour as they matured to pinkish-orange, pale, or grey

(Table 4.2). The morphological analysis showed that some *Colletotrichum* conidia were cylindrical with obtuse and narrowing ends, while others were fusiform with slightly rounded ends (Figure 4.1b) (Obianom and Sivakumar, 2018). During the pathogenicity assay, typical anthracnose symptoms developed on inoculated avocado fruit, which were clearly visible by 5 dpi, except in the control fruit. 80% of the isolates produced lesions characterized by brown-black spots in the exocarp and soft rot in the mesocarp (Figure 4.4a), which is similar to results obtained by Campos-Martínez *et al.*, 2016. Subsequently, symptoms of water-soaking, necrosis, and traces of mycelium were observed on the avocado fruit. After 14 days, there was extensive decay of the fruit, making it impossible to evaluate them again. *Colletotrichum* isolate Cs1 appeared to be more aggressive than Cs2, resulting in more symptoms in fruit, that would have caused them to be rejected in the market, of the fruits and contribute to production losses (Marais, 2004; Latorre *et al.*, 2019). On PDA, *Lasiodiplodia* isolates produced fast-growing cotton white to light greenish-grey mycelia and eventually became dark grey to black (Table 4.2). The conidia were initially observed as hyaline, ovoid and aseptate, and mature conidia were ovoid to ellipsoid, one-septate and slightly rod-shaped (Table 4.2) (Leong *et al.*, 2022). After 14 days of the pathogenicity test, typical stem-end rot symptoms such as soft and watery shrivelling dark spots with well-defined edges at the stem-end of the fruit developed on all inoculated avocado fruits (Hassan and Radwan, 2016). *L. theobromae* was isolated from the inoculated symptomatic fruits, confirming Koch's postulates, and isolate Ls1 appeared to be more pathogenic compared to Ls2. *Cladosporium* isolates formed olivaceous-green, velvet-like colonies on PDA medium (Figure 4.3a) with laterally branched conidiophores, ovoid to elliptical conidia, and cylindrical secondary ramoconidia (Figure 4.3b; Table 4.2) (Kim *et al.*, 2015).

After 14 dpi, the pathogens were re-isolated on a PDA medium to confirm Koch's postulate. On the few fruits with symptoms, *Cladosporium* spp. was present, as well as *Colletotrichum* spp. The artificial inoculations performed in this study were done on detached mature fruits, which may explain the presence of anthracnose on fruits inoculated with *Cladosporium* spp. (Figure 4.4c). *Colletotrichum* species' ability to cause latent infections makes it the most important postharvest pathogen. The fruits used in this study may have been infected by preharvest pathogens and symptoms only became visible during the experiment as the conditions were favourable to the pathogen. It is important to highlight that the fruits were only surface sterilized and no deep measures to kill/control latent infections on the untreated fruits were included such as hot water

treatment. The presence of *Cladosporium* spp. on the inoculated fruits was not a primary pathogen but rather a secondary invader of the existing infection, therefore confirming that fungus is not pathogenic to avocado fruits but is a saprophyte as described in a study carried out by Bara (2020). *Pseudocercospora* spp. were isolated from fruits displaying typical symptoms of Cercospora spot. The isolates were grown on PDA medium which is a rich media to help the fungus grow faster but the cultures grew very slowly regardless. However, pure cultures were placed under UV treatment which helped sporulate abundantly (Kirk *et al*., 2000). Colonies on PDA were initially greyish white at the centre and later became dull green to black. The scanning electron microscopy showed *Pseudocercospora* isolates produced conidiophores in compact fascicles bearing long conidia as described in a previous study (Figure 4.2b) (Kirk *et al.,* 2000). To comply with Koch's postulates, initial symptoms were observed at 10-14 dpi and the pathogen showed to be less virulent resulting in only 20% disease incidence. This might have been due to the inoculation technique and further investigation is needed to confirm this current result.

Previous studies showed that *Pseudocercospora* infects immature fruit at any stage of the growing period and this present study was carried out with mature avocado fruits which possibly also affected the results presented in (Table 4.3). At day 20, all fruits inoculated with *Pseudocercospora* displaced anthracnose symptoms which made it impossible for further data collection. Previous studies show that isolates sometimes fail due to secondary organisms such as *Colletotrichum* spp. that overgrow the slow-growing *Pseudocercospora* (Reina-Noreña *et al.,* 2020). The pathogen isolates associated with anthracnose in this study were also identified based on their molecular morphology. Cs1 was identified as *Colletotrichum cobbittiense* and Cs2 was identified as *Colletotrichum henanense* (Table 4.4). This strain of *C. cobbittiense* was more virulent compared to strain of *C. henanense* (Table 4.3). Isolate Ls1 was identified as *Neofusicoccum parvum*. In South Africa, members of the fungal family Botryosphaeriaceae have been reported to be the leading cause of stem-end rots, with *Lasiodiplodia* being the predominant genus (Adikaram and Karunanayake, 2020). In this current study, *Neofusicoccum parvum* was the predominant species, isolated most frequently from stem-end rot of avocado fruits in Kwa-Zulu Natal, South Africa. *N. parvum* was more virulent compared to isolate Ls2 which was identified as *Lasiodiplodia mahajangana* (Table 4.3) (Majola, 2020). These results suggest that isolate *C. cobbittiense* and *N. parvum* were the most virulent species in this study causing severe fruit rot symptoms of anthracnose and stem-end rot on avocado fruits in KwaZulu-Natal (Table 4.3, Figure 4.4).

4.6 Conclusion

Both *Colletotrichum* and *Neofusiococcum* genera were isolated from symptomatic fruits collected from different supermarkets in Pietermaritzburg (KwaZulu-Natal, South Africa). Members of these morpho-groups were found to be the most virulent in this study, indicating that both the fungal pathogens can present a problem and need effective control strategies. Future studies on *Pseudocercospora* species will be conducted using leaves as spores are transferred from leaves to fruits. This is to avoid avocado latent infections on fruits during the experiments.

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Chapter 5 In vitro and In vivo screening for antifungal and antagonistic activity of endophytic Trichoderma isolates against fungal pathogens associated with anthracnose and stem-end rot of avocado in KwaZulu-Natal, South Africa

Abstract

Several biocontrol microorganisms have been commercialized, making biocontrol agents an important component of plant protection. The objectives of the present study were to analyse the *in vitro* inhibitory effect of endophytic *Trichoderma* isolates against *Colletotrichum* and *Lasiodiplodia* species associated with avocado fruit and to evaluate the potential use of the antagonistic *Trichoderma* spp. as a biocontrol agent postharvest. Nine isolates of endophytic *Trichoderm*a were recovered from avocado fruits and screened for their *in vitro* antagonism against four groups of pathogenic isolates; *Colletotrichum cobbittiense* (Cs1), *Colletotrichum henanense* (Cs2), *Neofusicoccum parvum* (Ls1), and *Lasiodiplodia mahajangana* (Ls2) in a dual culture assay. Seven endophytic *Trichoderma* isolates with the strongest inhibitory activity, as well as a commercial biocontrol agent (Eco77®), were screened *in vivo* for efficacy on healthy avocado fruits. The most effective *Trichoderma* isolates were identified using molecular analysis of their sequences with internal transcribed spacer (ITS) regions (ITS1 and ITS2), amplified with the primer pair ITS1 and ITS4. The results showed that *Trichoderma* isolates UK1E, UK4C, and as well as Eco77® had the strongest antagonistic efficacy against all the pathogens and did not cause any harm to the fruits. Molecular studies identified the best *Trichoderma* isolates UK1E, as *Trichoderma asperellum,* and UK4C as *Trichoderma koningiopsis.* The results also revealed that *Trichoderma asperellum* (UK1E) and *Trichoderma koningiopsis* (UK4C), and Eco77®, may be effective as biocontrol agents against *N. parvum*, *C. cobbittiense*, *C. henanense*, and *L. mahajangana.* Further field studies are needed to confirm these results.

Keywords: Avocado, Colletotrichum cobbittiense, Colletotrichum henanense, Eco77®, Lasiodiplodia mahajangana, Neofusicoccum parvum, Trichoderma asperellum, Trichoderma koningiopsis.

5.1 Introduction

Fungal plant diseases pose a serious threat to the avocado (*Persea americana* Mill.) industry. Currently, they are being controlled by applying fungicides such as copper-oxychloride, bravo 720 (chlorothalonil) and copper ammonium carbonate (Duvenhage and Willis, 2003; Pérez-Jiménez, 2008). The increasing awareness of the excessive use of agrochemicals for the control of pre-and postharvest diseases caused by fungal phytopathogens has emphasized that it is not cost-efficient in agriculture and not safe for the environment (Dhananjayan *et al.*, 2020). Intensive application of fungicides results in pathogen resistance, chemical residues and pollution of crops and the environment (Chen *et al.*, 2020). These agrochemicals are now hindering the sustainable development of agriculture and food security (Aliferis *et al.*, 2021). There are currently strict regulations on pesticide use and some of these chemicals might be removed from the market (Chen *et al.*, 2020). Hence, a significant number of scientists and agricultural industries have shown increased interest in the need to develop alternative, eco-friendly, non-chemical methods to control fungal plant diseases, including bio-control agents for plant protection. Biological control measures rely on the use of antagonistic organisms (e.g., *Trichoderma* species) to control phytopathogens. Many biocontrol organisms have been commercialized, giving biocontrol agents a significant role in plant protection (Heydari and Pessarakli, 2010). *Trichoderma* species are one of the frequently isolated plant fungi and have been studied extensively for their potential to control diseases in several crops caused by plant pathogenic fungi and to replace fungicides (Das *et al.*, 2017).

Previous studies indicate that some isolates of *Trichoderma* species are endophytic microorganisms and mutualistic symbionts that live within the plant tissue for at least a period of their life cycle without causing any disease symptoms. Instead, they have beneficial effects on their host plant (Leylaie and Zafari, 2018). *Trichoderma* isolates can suppress the growth of fungal pathogens in the host using several mechanisms such antibiosis, myco-parasitism, and competition for space and nutrients (Sharma, 2011). Endophytes also play a role in promoting growth and development of plant, induce the defense response of plants by secretion of cell wall degrading enzymes such as chitinases, proteases, and gluconates, helping plant hosts to become resistant to pathogen attack (Bae *et al.*, 2008). These properties of *Trichoderma* species have resulted in many commercialized products (Sharma, 2011). In this study, endophytic *Trichoderma* species were

previously isolated and identified as potential biocontrol agents to control fruit spot and rot. *In vitro* bioassay was needed to confirm the antagonistic action of the isolates of *Trichoderma* spp. and a subsequent *in vivo* bioassay was to confirm the antagonistic action.

Therefore, the objectives of this present study were as follows:

1. Analyze the *in vitro* inhibitory effect of endophytic *Trichoderma* spp. against fungal pathogens associated with anthracnose and stem-end rot.
2. Evaluate the potential use of the endophytic *Trichoderma* spp. as biocontrol agents against the key fungal pathogens *in vivo.*

5.2 Material and Methods

5.2.1 *In vitro* screening for antagonism by dual culture method

The antagonistic effect of endophytic *Trichoderma* isolates against the fungal pathogens isolated in chapter 3; *Colletotrichum cobbittiense* (Cs1), *Colletotrichum henanense* (Cs2), *Neofusicoccum parvum* (Ls1) and *Lasiodiplodia mahajangana* (Ls2) were evaluated using a dual culture technique, as demonstrated in previous studies (Garg *et al.,* 2011). In this method, 5 mm^2 size plugs of an endophytic *Trichoderma* culture grown on Potato Dextrose Agar (PDA) and the same size plug of the target pathogens were placed on opposite sides of a Petri dish containing PDA medium, 1 cm away from the side of the dish. Petri dishes inoculated with the *Trichoderma* strains alone in the absence of the pathogens served as a control, and Petri dishes inoculated with a pathogen alone served as a second control. All pairing in this experiment was carried out in four replicates. All the inoculated plates including the controls were sealed with Parafilm and incubated at 25°C for 7 to 14 days. The growth of colonies of both *Trichoderma* and the tested pathogen were examined using a rating pattern of 1-5 to rate the degree of antagonism of each *Trichoderma* strain. The competing growth of the colonies were photographed (Ziena, 2019).

Mycelial growth inhibition was rated as follows (Ziena, 2019):

1 = Pathogen does not grow at all, strong inhibition of the pathogen by the *Trichoderma* isolate

2 = Pathogen grows only around the plug

3= Pathogen grows to the *Trichoderma* isolate but the *Trichoderma* isolate overgrows the pathogen

4 = Pathogen grows towards the *Trichoderma* isolate but there is a zone of inhibition

5 = Pathogen overgrows the *Trichoderma* isolate

5.2.2 Scanning electron microscope (SEM) studies of the interaction zone between antagonistic *Trichoderma* and the pathogen

The zone of interaction between endophytic *Trichoderma* isolates and the pathogens was studied using a scanning electron microscope (SEM) (ZEISS, EVO LS 15) (Carl Zeiss SMT Ltd., Cambridge) at the microscopy and microanalysis unit (MMU) laboratory at the University of KwaZulu-Natal. A square of 1 mm^2 of the interaction zone on PDA plates was cut for preparation and fixed in 3% buffered glutaraldehyde for 1 hour 30 minutes. The samples were then washed in 0.05M sodium cacodylate buffer twice for 5 mins, then dehydrated in ethanol (10%, 30%, 50% 70%, 90%, and 3 x 100% for 10 min each) under a fume hood. The samples were mounted on SEM stubs, transferred into critical point dryer (CPD) (Quorum Technologies Ltd., Ashford, Kent, UK) baskets under 100% ethanol, and then the ethanol was replaced with CO_2, which was heated to its critical point. The samples were coated with gold in a gold palladium sputter coater and examined with a scanning electron microscope (ZEISS, EVO LS 15) (Carl Zeiss SMT Ltd., Cambridge).

5.2.3 *In vivo* screening for antagonistic activity of selected endophytic *Trichoderma* spp.

The endophytic *Trichoderma* isolates that were able to inhibit the growth of all fungal pathogens in the *in vitro* assays were selected for the *in vivo* screening for antagonistic against target pathogens. *Trichoderma* isolates and the pathogen isolates were grown on PDA until they were sporulated, and then conidial suspensions were prepared. The pure cultures on each PDA plate were surface-washed with 10 mL of sterile distilled water and gently scraped with a glass hockey stick. The concentration of the conidial suspensions was adjusted to 1 x 10^6 conidia/ml^{-1} using a hemacytometer (Marienfeld Superior™, Germany) (Neubauer IMP kit 0.1 mm) under a light

microscope (Zeiss Axiophot, Germany). Fresh avocado fruits of the cultivar "Hass" were collected from a local farm in Richmond, KwaZulu-Natal, South Africa. The fruits were first washed using running tap water, then surface sterilized using 2% sodium hypochlorite for three minutes, rinsed three times using distilled water and air-dried before use.

The fruits were sprayed with conidial suspensions of the *Trichoderma* isolates at a concentration of 1 x 10^6 conidia/ml^{-1}, or the commercial *Trichoderma*-based product (Eco77®). Each strain was tested with six replicates (Fourie *et al.,* 2011). The fruits were then air-dried and stored in boxes with black plastic bags for relative humidity at room temperature (25°C) for 7 days. After 7 days past inoculation (dpi), the black plastic bags were removed and the fruits were inoculated with the pathogens by spraying on conidia of the isolates Cs1, Cs2, Ls1, and Ls2 with concentration 1 x 10^6 conidia ml^{-1}. The inoculation method that involved spraying the entire fruit with a conidial suspension of 1 x 10^6 conidia/ml^{-1} had been verified previously, and the pathogens had been re-isolated and identified using cultural and morphological approaches. The three controls for this investigation were fruits inoculated with the fungal pathogens only, fruits inoculated with *Trichoderma* only, and lastly, fruits sprayed with sterile distilled water (Dubey *et al.,* 2021). The fruits were then covered with black plastic for 3 weeks. This experiment was conducted twice independently to confirm the antagonistic activity of endophytic *Trichoderma* isolates. A rating scale of 1-3 was used to describe the amount of damage caused by the pathogen isolates Cs1, Cs2, Ls1, and Ls2.

The scale was:

1= perfect fruit

2 = fruit infected but largely edible

3 = fruit inedible

5.2.4 Molecular identification of endophytic *Trichoderma* strains isolated from avocado fruits.

Fresh cultures of the most antagonistic *Trichoderma* isolates were sent to Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa) for DNA extraction, Polymerase Chain Reaction (PCR) and sequencing. Genetic DNA was extracted from the fungal isolates using the Quick-DNA™

Fungal/Bacterial Miniprep kit (Zymo Research, Catalogue No. D6005). Internal transcribed spacer (ITS) target region (ITS1 and ITS2) was amplified using Primers ITS1 and ITS4 (Table 5.1)

Table 5.1: ITS Primers sequences used for the identification of endophytic *Trichoderma* biocontrol agents

Name of Primer	Target	Sequence (5' to 3')	Reference
ITS1	Internal Transcribed Spacer Region	TCCGTAGGTGAACCTGCGG	Bruns *et al.*, 1990
ITS4	Internal Transcribed Spacer Region	TCCTCCGCTTATTGATATGC	Bruns *et al.*, 1990

The PCR parameters used under this investigation were NEB OneTaq 2X MasterMix with Standard Buffer (Catalogue No. M04825), Genomic DNA (10-30ng/µl), forward primer (10 µM), reverse primer (10 µM) and nuclease-free water (catalogue No. E476). The PCR protocol followed as the initial denaturation at 94°C for 5 min, followed by 35 cycles (94°C for 30 sec, 50°C for 30 sec, 68°C for 1 min), with a final step at 68°C for 10 min and on hold at 4°C. The amplified PCR amplicons was examined in 1% agarose gel (CSL-AG500, Cleaver Scientific Ltd) stained with EZ-vision® Bluelight DNA Dye. The NEB Fast ladder was used on gels (N3238) as the size standard. The fragments were purified using the ExoSAP procedure (NEB M0293L; NEB M0371). Amplicons were purified for sequencing (Zymo Research, ZR-96 DNA Sequencing Clean-up kit□, Catalogue No. D4050), and sequenced in a forward and reverse direction (Nimagen, BrilliantDye™ Terminator Cycle Sequencing kit V3.1, BRD3- 100/1000) using an AB1 3730*x*/ Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific).

The sequences were submitted to the GenBank database, and BLASTn analysis was performed on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine if a sequence in the database matched the query sequence above a certain threshold (99% query coverage; 99% identity) (Dubey *et al.*, 2021).

5.3 Results

5.3.1 *In vitro* screening for antagonism by dual culture method

In this study, nine endophytic isolates of *Trichoderma* spp. were screened for their *in vitro* antagonism against the key fungal pathogens causing anthracnose and stem-end rot. In dual culture Petri dishes, the zone of interaction was observed between two colonies of the antagonistic endophytic *Trichoderma* isolate and the target pathogen (Figure 5.1). *Trichoderma* strains UK1E, UK1C and UK4C caused the most inhibition (≤ 2) of pathogen isolates Cs1, Cs2, Ls1 and Ls2 growth in dual culture (Table 5.2). *Trichoderma* isolate TsF3G and TsF4A showed the least inhibition of Cs1, Cs2, Ls1 and Ls2. On the other hand, UK2G kept a consistent intermediate value (≤ 3) of inhibition with all the pathogenic isolates. Hyphal interactions and antagonism were observed by the naked eye and under SEM (Table 5.2, Figure 5.1 and 5.2). Strain UK1E, UK1C and UK4C did not coil around the hyphae of pathogens as there was no contact. However, they still inhibited the pathogens Cs1, Cs2, Cs3 and Ls (Figure 5.1, Table 5.2). In contrast, isolates TsF2B, UK2G, TsF3E and UK4G only inhibited the growth of Cs1, Cs2, Ls1 and Ls2 after contact (Figure 5.1 B and C). These *Trichoderma* isolates coiled around the pathogens Cs1, Cs2, Ls1 and Ls2 (Figure 5.2), except no coiling was evident for isolate TsF3E. Finally, the strains TsF3G and TsF4A did not inhibit the pathogens Cs1, Cs2, Ls1 and Ls2 (Table 5.2).

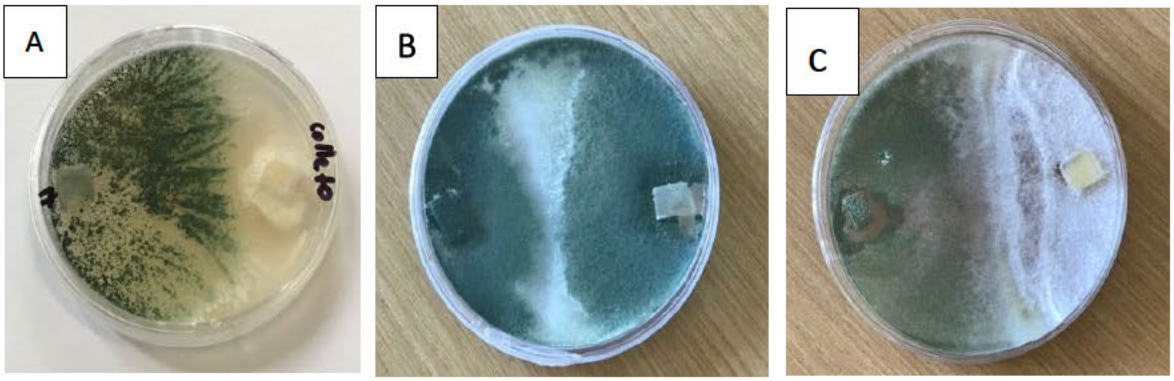

Figure 5.1: Mycoparasitism of *Trichoderma* spp. against pathogen isolate Cs2; rated 2: (Photo A) the pathogen grows only around the fungal plug; Rated 3: (Photo B) the pathogen grows towards the *Trichoderma* strain, but *Trichoderma* is able to overgrow the pathogen after contact; rated 4: (Photo C) the pathogen grows towards the *Trichoderma* spp. but there is a zone of inhibition between the pathogen and the *Trichoderma* strain on PDA, 7 days post inoculation.

Table 5.2: *In vitro* inhibitory effects of *Trichoderma* isolates against pathogen isolates causing anthracnose and stem-end rot on avocado fruits

Pathogen Strain	*Trichoderma* strain	Mean score rating	Visible interaction visible with naked eye	Microscope interaction
Cs1	UK1C	2	Inhibition before contact	No coiling
	UK1E	2	Inhibition before contact	No coiling
	TsF2B	4	Inhibition after contact	Coiling
	UK2G	3	Inhibition after contact	Coiling
	TsF3E	4	Inhibition after contact	No coiling
	TsF3G	5	No Inhibition	No coiling
	TsF4A	4	Inhibition after contact	Coiling
	UK4C	2	Inhibition before contact	No coiling
	Uk4G	3	Inhibition after contact	No coiling
Cs2	UK1C	2	Inhibition before contact	No coiling
	UK1E	1	Inhibition before contact	No coiling
	TsF2B	3	Inhibition after contact	Coiling
	UK2G	3	Inhibition after contact	Coiling
	TsF3E	3	Inhibition after contact	Coiling
	TsF3G	4	Inhibition after contact	No coiling
	TsF4A	3	Inhibition after contact	Coiling
	UK4C	1	Inhibition before contact	No coiling
	Uk4G	2	Inhibition after contact	Coiling

Ls1	UK1C	2	Inhibition before contact	No coiling
	UK1E	2	Inhibition before contact	No coiling
	TsF2B	4	Inhibition after contact	Coiling
	UK2G	3	Inhibition after contact	Coiling
	TsF3E	4	Inhibition after contact	Coiling
	TsF3G	4	Inhibition after contact	No coiling
	TsF4A	5	Inhibition after contact	No coiling
	UK4C	2	Inhibition before contact	No coiling
	Uk4G	3	Inhibition after contact	Coiling
Ls2	UK1C	2	Inhibition before contact	No coiling
	UK1E	2	Inhibition before contact	No coiling
	TsF2B	4	Inhibition after contact	Coiling
	UK2G	3	Inhibition after contact	Coiling
	TsF3E	4	Inhibition after contact	Coiling
	TsF3G	5	Inhibition after contact	No coiling
	TsF4A	5	Inhibition after contact	No coiling
	UK4C	2	Inhibition before contact	No coiling
	Uk4G	2	Inhibition after contact	No coiling

Mean score rating: 1 = Pathogen does not grow at all, strong inhibition of the pathogen by the *Trichoderma* isolate; 2 = Pathogen grows only around the plug; 3= Pathogen grows to the *Trichoderma* isolate but the *Trichoderma* isolate overgrows the pathogen; 4 = Pathogen grows towards the *Trichoderma* isolate but there is a zone of inhibition; 5 = Pathogen overgrows the *Trichoderma* isolate

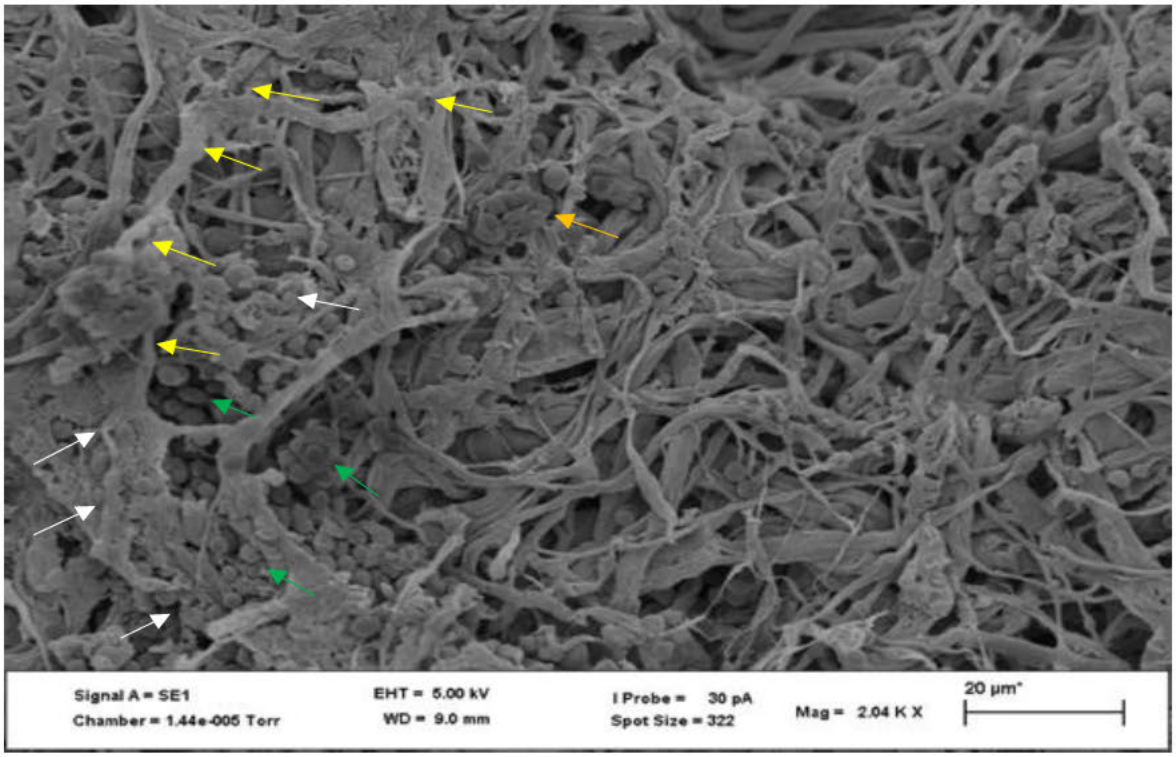

Figure 5.2: Scanning electron micrograph from an inhibition zone between an endophytic *Trichoderma* strain and a fungal pathogen strain. The white arrows indicate *Trichoderma* hyphae, the green arrows indicate the pathogen hyphae, and lastly, the yellow arrows indicate the hyphae of *Trichoderma* strain coiling around the hyphae of the pathogen.

5.3.2 *In vivo* screening for antagonistic activity of selected endophytic *Trichoderma spp*.

Complementary to the previous *in vitro* study on pathogenic isolates Cs1, Cs2, Ls1, Ls2 and endophytic *Trichoderma* isolates interactions, an *in vivo* investigation was conducted to gain better insight of the antagonistic activity of endophytic *Trichoderma* as a potential bio-control agent against anthracnose and stem-end rot. A rating scale of 1-3 was used to describe the amount of damage caused by the pathogen isolates Cs1, Cs2, Ls1, and Ls2. The scale was: 1= perfect fruit; 2 = fruit infected but largely edible; 3 = fruit inedible. The fruit were previously inoculated with endophytic *Trichoderma* strains UK1C, UK1E and UK4C that previously displayed high inhibitory activity with a mean ≤ 2 on PDA Petri dishes and *Trichoderma* strains displaying intermediate inhibitory activity with a mean ≤ 4 (TsF2B, UK2G, TsF3G, and UK4G). The avocado fruits were also inoculated with a commercial biocontrol agent, Eco77®.

Both Cs1 and Cs2 caused anthracnose symptoms, brown to black lesions on the avocado fruits at 14 dpi on the controls. Cs1 displayed more aggressiveness and pathogenicity compared to Cs2 (Figure 5.3 and 5.4). It is difficult to see external symptoms on ripe Hass avocado fruits due to the dark skin colour, but the lesions were larger, darker, and sunken on fruits inoculated with the Cs1 and Cs2 only, compared to the fruits inoculated with both the biocontrol agent and the pathogen (UK1C + Cs1) and (UK1C + Cs2) at 23 dpi (Figure 5.3 and 5.4). On the other hand, Ls1 caused black lesions, slight shriveling, and decay with a well-defined margin around the stem end at 14 dpi (Figure 5.5) in the control. This pathogenic isolate (Ls1) was very virulent in causing stem-end rot symptoms.

Strain Ls2 caused more stem-end rot symptoms when the fruit were inoculated with Ls2 compared to UK1C + Ls2 (Figure 5.6). *Trichoderma* isolates UK1E, UK4C and Eco77® displayed high antagonistic activity and disease control ratings with a mean ≤ 2 (Table 5.3). Eco77® treatment as a biocontrol agent based on *T. harzianum* reduced the disease incidence and severity of lesions caused by isolates inoculated on healthy avocado fruit (Table 5.3). The rest of the *Trichoderma* isolates displayed moderate to low antagonistic activity, and provided moderate control of Cs1, Cs2, Ls1 and Ls2 (Table 5.3).

Figure 5.3: Symptoms of anthracnose on avocado fruits artificial inoculated with *Trichoderma* strain UK1C and pathogenic strain Cs1; (Photo A) 14 dpi; (Photo B) 23 dpi. The control was represented by fruits inoculated with only the pathogen strain: (Photo C) 14 dpi, (Photo D) 23 dpi.

Figure 5.4: Symptoms of anthracnose on avocado fruits artificial inoculated with *Trichoderma* strain UK1C and pathogenic strain Cs2 (UK1C + Cs2): (Photo A) 14 dpi, (Photo B) 23 dpi. The control was represented by fruits inoculated with only the pathogen strain; (Photo C) 14 dpi; (Photo D) 23 dpi.

Figure 5.5: Symptoms of stem-end rot on avocado fruits artificial inoculated with *Trichoderma* strain UK1C and pathogenic strain Ls1 (UK1C + Ls1): (Photo A) 14 dpi, (Photo B) 23 dpi. The control was represented by fruits inoculated with only the pathogen strain: (Photo C) 14 dpi, (Photo D) 23 dpi.

Figure 5.6: Symptoms of Stem-end rot on avocado fruits artificial inoculated with *Trichoderma* strain UK1C and pathogenic strain Ls2 (UK1C + Ls2): (Photo A) 14 dpi; (Photo B) 23 dpi. The control was represented by fruits inoculated with only the pathogen strain: (Photo C) 14 dpi; (Photo D) 23 dpi.

Table 5.3: Disease incidence and severity of lesions caused by isolates inoculated healthy avocado fruit

Trichoderma Isolates	Fungal pathogen	Disease incidence						severity
		Day 8	Day 11	Day 14	Day 17	Day 20	Day 23	Non-wound Inoculation
UK1C	Cs1	No	No	No	Yes	Yes	Yes	3
	Cs2	No	No	No	No	Yes	Yes	2
	Ls1	No	No	No	Yes	Yes	Yes	3
	Ls2	No	No	No	No	Yes	Yes	2
UK1E	Cs1	No	No	No	No	Yes	Yes	2
	Cs2	No	No	No	No	No	Yes	2
	Ls1	No	No	No	No	No	Yes	2
	Ls2	No	No	No	No	No	Yes	2
T_SF2B	Cs1	No	Yes	Yes	Yes	Yes	Yes	3
	Cs2	No	No	Yes	Yes	Yes	Yes	3
	Ls1	No	No	No	Yes	Yes	Yes	2
	Ls2	No	No	No	No	Yes	Yes	2
UK2G	Cs1	No	Yes	Yes	Yes	Yes	Yes	3
	Cs2	No	No	No	Yes	Yes	Yes	3
	Ls1	No	No	Yes	Yes	Yes	Yes	3
	Ls2	No	No	Yes	Yes	Yes	Yes	2
T_SF3E	Cs1	No	Yes	Yes	Yes	Yes	Yes	3
	Cs2	No	Yes	Yes	Yes	Yes	Yes	3
	Ls1	No	Yes	Yes	Yes	Yes	Yes	3
	Ls2	No	No	No	Yes	Yes	Yes	3
UK4C	Cs1	No	No	No	No	No	Yes	2
	Cs2	No	No	No	No	No	No	1
	Ls1	No	No	No	No	No	Yes	2
	Ls2	No	No	No	No	No	Yes	2
UK4G	Cs1	No	No	No	Yes	Yes	Yes	3
	Cs2	No	No	No	No	Yes	Yes	2
	Ls1	No	No	No	Yes	Yes	Yes	3
	Ls2	No	No	No	No	Yes	Yes	2
Eco77®	Cs1	No	No	No	No	Yes	Yes	2
	Cs2	No	No	No	No	No	Yes	2
	Ls1	No	No	No	No	Yes	Yes	2
	Ls2	No	No	No	No	No	Yes	2

Scale: 1= perfect fruit; 2= fruit infected but largely edible; 3=fruit inedible

5.3.3 Molecular identification of endophytic *Trichoderma* isolates

Approximately as mentioned in chapter 4.3.4 approximately 600 bp product of the ITS region was amplified from the isolates that had the best antagonistic efficacy *in vivo,* using the primers ITS1 and ITS4, respectively. The ITS gene region is highly polymorphic with enough informative taxonomic units to separate samples up to a species level (Bruns *et al.,* 1990). The results revealed that the endophytic *Trichoderma* isolates were 100% identified as *Trichoderma asperellum* (UK1E) and *Trichoderma koningiopsis* (UK4C) (Table 5.4).

Table 5.4: The primers and GenBank accession used for the identification of *Trichoderma* biocontrol agents

Isolate name	Species name	Name of Primer	GenBank accession
UK1E	*Trichoderma asperellum*	ITS1, ITS4	MW386848.1
UK4C	*Trichoderma koningiopsis*	ITS1, ITS4	MF616361.1

5.4 Discussion

Trichoderma was introduced as a potential biocontrol agent in the early 1930s against several phytopathogenic fungi, such as *Pythium, Rhizoctonia* and *Phytophthora* (Weindling, 1934). Weindling's research inspired development and research work in many plant pathology laboratories across the world seeking a tool for the management of plant diseases and parasitic microorganisms (Ghazanfar *et al.,* 2018). In this study, endophytic *Trichoderma* isolates were screened for their *in vitro* antagonism against two fungal pathogens causing anthracnose (Cs1 and Cs2) and two causing stem-end rot (Ls1 and Ls2). In dual culture, mycoparasitism was observed and recorded, which is the direct interaction between *Trichoderma* and the pathogen (Table 5.2). Out of nine endophytic *Trichoderma* isolates, only seven isolates managed to inhibit the growth of the pathogenic isolates. Isolates UK1E, UK1C and UK4C demonstrated the strongest inhibitory effect against pathogen isolates Cs1, Cs2, Ls1 and Ls2 before contact (Table 5.2, Figure 5.1 A). Isolate TsF2B, UK2G, TsF3E and UK4G only managed to inhibit the growth of the pathogens Cs1, Cs2, Ls1 and Ls2 after contact (Table 5.2, Figure 5.1 B and C). In numerous studies, mycoparasitism has been described as a complex mechanism that involves chemotropism and

recognition (Hossain *et al.,* 2014; Chandra *et al.,* 2018). The *Trichoderma* isolates appeared to detect the pathogen colonies, grew straight towards them and produced hydrolytic cell wall degrading enzymes for penetration (Figure 5.1, Table 5.2). Mycoparasitism may involve attachment and coiling (Omann and Zeilinger, 2007). Some *Trichoderma* isolates attach themselves to the pathogen and coil their hyphae around the pathogen (Figure 5.1 C; Table 5.1; Figure 5.2). Lastly, mycoparasitism involves cell wall penetration and host cell digestion (Costa *et al.,* 2015).

The results of inoculation of pathogenic isolate Cs1, Cs2, Ls1 and Ls2 on healthy avocado fruits showed that all the pathogenic isolates were able to cause fruit rot. The fungal pathogens were able to utilize the nutrients of the avocado fruits as a substrate for development and growth. However, the fruits inoculated with both *Trichoderma* and the pathogen demonstrated different results compared to the control where the fruit was inoculated with the pathogens only. The fruit inoculated with UK1C + Cs1 showed little to no symptoms of anthracnose at 14 days post inoculation (dpi) (Figure 5.3 A). On the other hand, the fruits inoculated with Cs1 only developed a high level of disease with a mean ≥ 3, which indicated that the inoculated fruit would have been thrown away as inedible. (Figure 5.3 B; Table 5.3). At 23 dpi, both the fruit inoculated with UK1C + Cs1 and Cs1 developed anthracnose symptoms, but at different levels (Figure 5.3 C and D). All the fruits inoculated with *Trichoderma* and the pathogen demonstrated less anthracnose or stem-end rot symptoms than the fruit inoculated with the pathogen only. Whilst a certain amount of fruit rot still developed, the *Trichoderma* isolates such as UK1E, UK4C and Eco77□ were able to inhibit the growth of the pathogens.

In previous studies, *Trichoderma* strains have been found to be opportunistic, avirulent plant symbiont fungi that produce proteins such as chitinase, which offers plant host protection from phytopathogenic plant diseases (Hossain *et al.,* 2014). *Trichoderma* species are good potential biocontrol agents as they are efficient competitors of nutrition and living space, and they are prolific spore producers (Ghazanfar *et al.,* 2018). *Trichoderma* produces highly efficient siderophores that bind minerals such as iron, reducing access to iron for phytopathogens (Barka *et al*., 2022). Iron starvation results in growth inhibition for most fungal pathogens, which may explain fewer symptoms developed on fruits inoculated with *Trichoderma*, compared to fruit inoculated with the pathogens only (Figures 5.3, 5.4, 5.5, and 5.6). Previous studies show that *Trichoderma*

not only controls phytopathogens but also promotes plant growth (Abdullah *et al.,* 2021). In this present study, *Trichoderma* spp. was inoculated into fruits post-harvest, which made it difficult to observe any plant growth effects. However, the fruits inoculated with *Trichoderma spp.* took longer to ripen compared to the control fruits (Figure 5.6). The skin of "Hass" avocado turns black and soft when it has ripened, which occurred much more slowly on fruits inoculated with *Trichoderma* isolates, changing the physiology and improving the shelf-life of the fruits (Figures 5.5 and 5.6).

Out of seven endophytic *Trichoderma* isolates inoculated *in vivo*, only UK1E and UK4C were able to control all four pathogenic isolates, Cs1, Cs2, Ls1, and Ls2. The *Trichoderma* isolates were primarily identified based on morphological characteristics, but a molecular approach was used in this current study. Based on ITS1 and ITS2 gene sequence analysis, UK1E was identified as *Trichoderma asperellum* and UK4C as *Trichoderma koningiopsis* (Table 5.4). Previous studies demonstrated that a strain of *T. koningiopsis* was an effective antagonistic microorganism against postharvest anthracnose of chili pepper, using multiple mechanisms (Pitija *et al.,* 2021). Dubey *et al.,* (2021) also mentioned the application of *T. asperellum* in chilli, which induced systemic resistance against anthracnose. The pathogen isolates associated with anthracnose in this study were identified as *C. cobbittiense* (Cs1) which was more virulent compared to strain of *C. henanense* (Cs2) (Figure 5.3 and 5.4). Isolate Ls1 was identified as *Neofusicoccum parvum* and was the most virulent isolate in this study, which suggests that it is currently causing a major postharvest issue for farmers in KwaZulu-Natal (Figure 5.5, Figure 5.3). After 23 dpi, all the fruit inoculated with *N. parvum* displayed stem-end rot symptoms, including the control fruits that were only sprayed with distilled water. This was an indication that this pathogen also has a latent phase, where the infection remains latent until harvest. This is a major problem for avocado farmers exporting fruit internationally (Kimaru *et al.,* 2020). Isolate *L. mahajangana* (Ls2) was less virulent than *N. parvum*. A positive outcome was that both *T. asperellum* and *T. koningiopsis* were able to suppress both stem-end rot fungi (Table 5.2). This study is possibly the first report of *N. parvum* being a serious threat in the avocado industry in KwaZulu-Natal, South Africa, and this requires further investigation such as field trials.

5.5 Conclusion

Endophytic, antagonistic *Trichoderma* species, *T. asperellum* (UK1E) and *T. koningiopsis* (UK4C) have the potential to be commercialized as biocontrol agents against the Botryosphaeriaceae family associated with anthracnose and stem-end rot.

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Chapter 6 Conclusions and Recommendations

6.1 General discussion and conclusion

In this dissertation, we explored the biocontrol potential of endophytic Trichoderma species against postharvest pathogens infecting avocado fruits and leaves. The study aimed to develop an eco-friendly and sustainable method to mitigate the losses caused by postharvest diseases such as anthracnose, stem-end rot, Cercospora spot, and Cladosporium spot which are a threat to the avocado industry. The research covered several key areas including isolation and identification of *Trichoderma* species, isolation and identification of the key pathogens, and *in vivo* and *in vitro* efficacy tests on avocado fruits. The study further discussed the importance of using biocontrol agents such as *Trichoderma* spp. as a postharvest management practice. Future research will focus on field trials under diverse conditions to further validate the effectiveness and reliability of the isolated endophytic *Trichoderma* strains as biocontrol agents. By advancing the understanding and application of endophytic Trichoderma species, this study could contribute to the development of commercial protection to protect avocados from pre- and postharvest disease in the South African avocado industry.

6.2 Thesis Overview

Avocado (*Persea americana* Mill.) is a subtropical/tropical fruit widely produced and consumed worldwide (Belmonte-Herrera *et al.,* 2020). Several fungi infect avocado leaves and fruit pre-harvest, which cause both pre- and postharvest avocado diseases, resulting in significant crop losses (Alvarez, 2004). Anthracnose caused by *Colletotrichum* species, and stem-end rot caused by *Lasiodiplodia* species, are two of the major constraints in the avocado industry globally due to their high incidence, and their severe impact on the shelf-life and marketability of infected fruit (Obianom and Sivakumar, 2018). Avocado trees are also attacked by various fungal pathogens causing fruit and leaf spots, such as *Pseudocercospora* species that causes Cercospora spot, and *Cladosporium* species that cause Cladosporium spot (Korsten and van Eeden, 2013; Coertzen and Fourie, 2018). Historically, agrochemicals have been used to manage most avocado plant diseases. However, public sentiment has turned against agrochemicals due to residues in food, and the damage that they are perceived to cause to the environment (Majeed, 2018). Therefore, agrochemicals are being discontinued due to the minimum accepted residue levels (MRLs) being

reduced in many countries, including those of the EU, a major market for South African fruit exports (Majeed, 2018). In this respect, the use of biological control agents (BCAs), such as *Trichoderma* species, has been regarded as a promising and environmentally friendly approach to controlling plant diseases (Howell, 2003). *Trichoderma* spp. are of great economic importance, avirulent plant symbionts and are used as BCAs against plant pathogens as they produce hydrolytic enzymes, cellulase, chitinases and antibiotic that promotes plant growth (Arikit *et al*., 2020; Pandya, 2017) The purpose of this overview is to review the objectives of this dissertation and their outcomes. This overview is to also identify and recommend future research studies for the avocado industry in South Africa.

6.3 Research objectives and respective outcomes

Objectives 1: To isolate strains of *Trichoderma* spp. and to screen them for endophytic properties as potential biocontrol agents of several fruit diseases of avocado such as anthracnose, fruit rot and fruit spot.

- A total of 17 *Trichoderma* spp. isolated from avocado fruits and leaves were screened for endophytic properties *in vivo* using a non-wound inoculation technique screening.
- A total of 9 isolates out of 17 *Trichoderma* strains demonstrated high endophytic ability of 70 to 100%.
- Isolate UK1E and UK4C showed 100% endophytic ability. These endophytic *Trichoderma* strains were potential biocontrol agents to control and prevent pre-harvest infections in avocado orchards.

Objective 2: To isolate and identify pre- and post-harvest fungal pathogens causing rot and black spots on avocado fruit; to test their pathogenicity on healthy avocado fruit and to verify Koch's postulates.

- A total of 45 isolates were recovered from fruit displaying typical symptoms of anthracnose, stem-end rot, Cladosporium spot, and Cercospora spot.
- Out of 45 isolates, *Colletotrichum* species were the most frequently isolated genus, divided into two morphological groups (1 and 2).
- Stem-end rot isolates (Ls1) were the second most isolated species and isolate Ls2 was

obtained from a previous study (Majola, 2020).

- About 20% of the fungal genera such as *Pseudocercospora* and *Cladosporium* were isolated rarely.
- Pathogenicity tests of the isolates on non-symptomatic avocado fruit were performed through a non-wounding inoculation method, following surface sterilization of the fruit.
- *Cladosporium* isolates did not appear to be the primary pathogens but rather secondary invaders of existing infections.
- The fungal pathogens associated with anthracnose Cs1 and stem-end rot isolate Ls1 appeared to be more virulent than the other morpho-groups.
- Molecular studies identified Cs1 as *Colletotrichum cobbittiense* and Cs2 as *Colletotrichum henanense.*
- Ls1 was identified as *Neofusicoccum parvum* and Ls2 was identified as *Lasiodiplodia mahajangana* (Majola, 2020).

Objective 3: In vitro and in vivo screening for antifungal and antagonistic activity of endophytic *Trichoderma* isolates against fungal pathogens associated with anthracnose and stem-end rot of avocado in KwaZulu-Natal, South Africa.

- A total of 9 endophytic *Trichoderm*a isolates recovered from avocado fruits were screened for their *in vitro* antagonism against pathogenic isolates Cs1, Cs2, Ls1, and Ls2 in a dual culture assay.
- 7 endophytic *Trichoderma* isolates with the strongest inhibitory activity, as well as a commercial biocontrol agent (Eco77®) were screened *in vivo* for efficacy on healthy avocado fruits.
- The results showed that *Trichoderma* isolates UK1E, UK4C, and as well as Eco77®, had the strongest antagonistic efficacy against all the pathogens and did not cause any harm to the fruits.
- Molecular studies identified the best *Trichoderma* isolates UK1E, as *Trichoderma asperellum*, and UK4C as *Trichoderma koningiopsis.*
- This study revealed that *N. parvum* is currently a serious problem in KwaZulu-Natal as

it was the most virulent post-harvest pathogen isolated during this investigation.

- The results also revealed that *Trichoderma asperellum* (UK1E) and *Trichoderma koningiopsis* (UK4C), and Eco77®, may be effective as biocontrol agents against *N. parvum*, *C. cobbittiense*, *C. henanense*, and *L. mahajangana.*

6.4 Recommendations for Future Research

- This study revealed that endophytic, antagonistic *Trichoderma* species, *T. asperellum* (UK1E) and *T. koningiopsis* (UK4C) have the potential to be commercialized as biocontrol agents against the Botryosphaeriaceae family associated with anthracnose and stem-end rot but field trials will need to be conducted first.

- Isolating more than 100 *Trichoderma* strains would improve these results, as there are more than 104 Trichoderma species that have been recorded internationally but not all of them have been tested to control phytopathogenic fungi attacking the avocado orchards in South Africa. This could contribute to the avocado industry with a commercialised endophytic *Trichoderma* biocontrol agent.

- Several fungi infect avocado leaves and fruit pre-harvest, which cause both pre- and post-harvest avocado diseases, resulting in significant crop losses. Applying endophytic *Trichoderma* strains pre-harvest would play a big role in evaluating the ability of *Trichoderma* as a biocontrol agent on avocado fruits.

- *N. parvum* was the most virulent isolate in this study, which suggests that it is currently causing a major postharvest issue for farmers in KwaZulu-Natal and further assessment is required for this pathogen. *N. parvum* displayed stem-end rot symptoms, including in the control fruits that were only sprayed with distilled water. This was an indication that this pathogen also has a latent phase, where the infection remains latent until harvest. This is a major problem for avocado farmers exporting fruit internationally.

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Acronyms and Appendixes

BCA	Biocontrol Agent
BLASTn	Basic Local Alignment Search Tool
□C	Degree Celsius
CO_2	Carbon Dioxide
cm	Centimeter
DNA	Deoxyribonucleic Acid
dpi	Days Post Inoculation
FAO	Food and Agriculture Organizations
ITS	Internal Transcribed Spacer
LSU	Large ribosomal subunit
M	Molar
MBC	Methyl benzimidazole carbamates
Min	Minutes
mL	Milliliter
mm	Millimeter
NCBI	National Center for Biotechnology Information
PAL	Phenylalanine Ammonia Lyase
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PHI	Post-Harvest Innovation
POD	Peroxidase
PPO	Polyphenol Oxidase
QoI	Quinone Outside Inhibitors
RH	Relative Humidity
s	Seconds
SA	South Africa
SEM	Scanning Electron Microscopy
spp.	Species
TSM	Trichoderma Selective Media
UK	United Kingdom
US	United States
UV	Ultraviolet
□l	Microliter

Fungal Identification (ITS)

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Client Name:	Londeka Mkhize
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Date of sample receipt:	10/05/2023
Number of samples:	6
Type of samples:	Bacterial cultures
Deviations from Method	None
Date Analysed	15/05/2023
Date Released	15/05/2023
Quote Number	SA2023/145137

BACKGROUND:

The sequencing of the ITS gene region (internal transcribed spacer, with ITS1 and ITS2 sub-regions) is universally used as a barcode for fungal species. The ITS gene region is a useful target for taxonomic and phylogenetic studies for the following reasons:

- The ITS1 and ITS2 sub-regions are present in every cell, therefore researchers know they will always amplify their target.
- The ITS gene region is a highly polymorphic, non-coding region with enough informative taxonomic units to separate samples up to species level.
- The overall size of the ITS gene region is short (~600bp). This target is easily amplifiable, and two sequencing reads should span the whole fragment (without the need of internal primers) (White *et al.*, 1990).

MATERIAL AND METHODS:

Genomic DNA was extracted from the cultures received using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, Catalogue No. D6005). The ITS target region was amplified as presented in Table 1.

Table 1: ITS Primers sequences

Name of Primer	Target	Sequence (5' to 3')
ITS1	Internal Transcribed Spacer Region	TCCGTAGGTGAACCTGCGG
ITS4	Internal Transcribed Spacer Region	TCCTCCGCTTATTGATATGC

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PCR Parameters:

NEB OneTaq 2X MasterMix with Standard Buffer (Catalogue No. M0482S)
Genomic DNA (10-30ng/µl)
Forward primer (10µM)
Reverse primer (10µM)
Nuclease free water (Catalogue No. E476)

General PCR Protocol:

94°C for 5 min
94°C for 30 sec
50°C for 30 sec
68°C for 1 min
} 35 Cycles
68°C for 10 min
Hold at 4°C

Agarose Gel Analysis:

The integrity of the PCR amplicons was visualized on a 1% agarose gel (CSL-AG500, Cleaver Scientific Ltd) stained with EZ-vision® Bluelight DNA Dye. The NEB Fast Ladder was used on all gels (N3238) as size standard.

PCR Amplicon Purification:

Fragments are enzymatically purified using the ExoSAP procedure (NEB M0293L; NEB M0371)

Sanger Sequencing:

The amplicons are purified for sequencing (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™, Catalogue No. D4050), and sequenced in the forward and reverse direction (Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000) using the ABI 3730*xl* Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific).

Data Analysis:

FinchTV (https://finchtv.software.informer.com/1.4/) is used to view the raw chromatogram files (.abi). CLC Bio Main Workbench was used to assemble the forward and reverse sequencing reads to form a consensus sequence for each sample. BLASTn analysis (with default parameters) (Altschul *et al.*, 1997) was performed on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine if a sequence in the database matches the query sequence above a certain threshold (99% query coverage; 99% identity)..

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RESULTS:

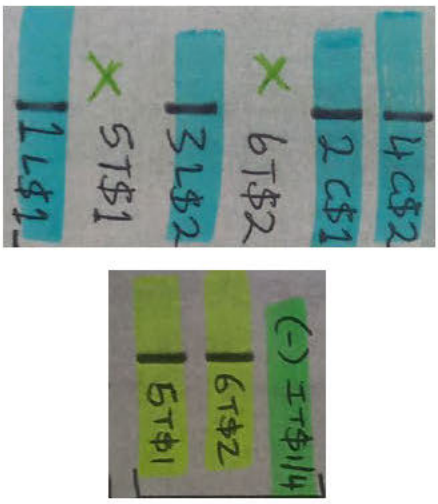

Figure 1: A photographic image of an agarose gel indicating the amplification of the ITS target region (~600bp).

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BLAST RESULTS: Similarity between the sequence queried and the biological sequences within the NCBI database.

Name of sample	Sample 1 Ls1
Request ID	5VX6S9W301N
Predicted Organism	*Neofusicoccum parvum*
GenBank Accession	KU997560.1
E-Value	0
HSP Length	582bp
% Identity	100%

Name of sample	Sample 2 Cs1
Request ID	5VX6X5PY01N
Predicted Organism	*Colletotrichum cobbittiense, Colletotrichum henanense*
GenBank Accession	MN856259.1, ON793152.1
E-Value	0, 0
HSP Length	576bp, 576bp
% Identity	100%, 100%

Name of sample	Sample 3 Ls2
Request ID	5VY5H48M013
Predicted Organism	*Neofusicoccum parvum*
GenBank Accession	KU997399.1
E-Value	0
HSP Length	582bp
% Identity	100%

Name of sample	Sample 4 Cs2
Request ID	5VY5HUV1013
Predicted Organism	*Neofusicoccum parvum*
GenBank Accession	KU997399.1
E-Value	0
HSP Length	581bp
% Identity	100%

Fungal Identification (ITS)

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Name of sample	Sample 5 Ts1
Request ID	63Y3VWVW013
Predicted Organism	*Trichoderma asperellum*
GenBank Accession	MW386848.1
E-Value	0
HSP Length	604bp
% Identity	100%

Name of sample	Sample 6 Ts2
Request ID	63Y40RCV013
Predicted Organism	*Trichoderma koningiopsis*
GenBank Accession	MF616361.1
E-Value	0
HSP Length	605bp
% Identity	100%

Fungal Identification (ITS)

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CONCLUSION:

Name of sample	Predicted Organism	Comments
Sample 1 Ls1	*Neofusicoccum parvum*	-
Sample 2 Cs1	*Colletotrichum cobbittiense, Colletotrichum henanense*	-
Sample 3 Ls2	*Neofusicoccum parvum*	-
Sample 4 Cs2	*Neofusicoccum parvum*	-
Sample 5 Ts1	*Trichoderma asperellum*	-
Sample 6 Ts2	*Trichoderma koningiopsis*	-

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