THE POTENTIAL OF COMBINED RAPID HOT WATER TREATMENT AND YEAST BIOCONTROL FOR SUPPRESSING POSTHARVEST AVOCADO ANTHRACNOSE AND STEM-END ROT DISEASES

By

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THESIS SUMMARY

Avocado (*Persea americana* Mill.) is a highly nutritious fruit, rich in vitamins, minerals, and antioxidants. In South Africa, the avocado industry experiences combined losses of about 50% due to anthracnose and stem-end rot. The fungi most commonly associated with these diseases are *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc and *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl respectively. Acceptable control levels of these diseases have been achieved by postharvest treatments with prochloraz. However, a significant reduction of the maximum residue levels by the European Union has precluded the use of this fungicide from 2020. Therefore, this study aimed to develop an alternative treatment regime to control the primary postharvest diseases of avocado. The aim of the study was to optimize a rapid hot water treatment (RHWT) and to discover an effective yeast biological control followed by the integration of these two treatments. Temperatures tested for the RHWT ranged from 20 to 80°C, combined with exposure periods ranging from 10, to 180 seconds. These were applied to “Hass”, “Fuerte”, and “Pinkerton” fruit. Levels of disease occurrence were reduced when temperatures between 52°C and 58°C were combined with exposure times of 10 to 30 seconds, which also caused no heat damage of fruit. Overall, the best treatment was a temperature and time combination of 56°C for 10 seconds. More than 100 yeast isolates were isolated and screened against the two primary pathogens. Three yeasts performed exceptionally well, including a commercial yeast variety known as B13. The combination of RHWT and all four yeasts provided a level of control comparable with that provided by the fungicide prochloraz. Excellent and consistent control was achieved from the integration of yeast strain B13 and rapid hot water treatment of 56°C for 10 seconds.

DECLARATION

I, THEMBEKA FAITH MAJOLA, declare that:

I. The research reported in this thesis, 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. This thesis does not contain other person's data, pictures, graphs or information unless acknowledged as being sourced from other persons.

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TABLE OF CONTENT

THESIS SUMMARY i

DECLARATION ii

ACKNOWLEDGEMENTS iii

INTRODUCTION 1

References 4

CHAPTER ONE: Review of literature 6

Abstract 6

1.1 Introduction 7

1.2 Avocado 8

1.3 Postharvest diseases of avocado 13

1.3.1 Anthracnose 13

1.3.2 Stem-end rot 16

1.4 Disease management strategies 20

1.4.1 Chemical control 21

1.4.2 Biological control 21

1.4.3 Physical treatment 24

1.4.4 Integrated control 26

1.5 Conclusion 27

1.6 References 29

CHAPTER TWO: Koch's postulate experiments on two isolates of avocado anthracnose and stem-end rot causal organisms in KwaZulu-Natal, Republic of South Africa 39

Abstract 39

2.1 Introduction 40

2.2 Materials and methods 41

2.2.1 Sample preparation and isolation 41
2.2.2 Cultural and morphological studies of the causal agents 41
2.2.3 Molecular identification of the causal agents 42
2.2.4 Pathogenicity studies of the causal agents 42
2.3 Results 43
2.3.1 Cultural and morphological studies of the causal agents 43
2.3.3 Pathogenicity studies of the causal agents 44
2.3.2 Molecular identification of the causal agents 46
2.4 Discussion 47
2.5 Conclusion 49
2.6 References 50
CHAPTER THREE: Assessment of a rapid hot water treatment to control postharvest anthracnose and stem-end rot of avocado fruits. 53
Abstract 53
3.1 Introduction 54
3.2 Materials and methods 55
3.2.1 Skin sensitivity test of hot water treatments to determine a safe temperature x exposure time for avocado fruit 55
3.2.2 Hot water treatment for the control of postharvest avocado rot 55
3.2.3 Testing the best rapid hot water treatment compared with prochloraz treatments 56
3.2.4 Statistical analysis 56
3.3 Results 56
3.3.1 Hot water treatments for a safe temperature and exposure time for fresh avocado fruits 56
3.3.2 Hot water treatment for the control of postharvest avocado rot 58
3.3.3 Comparison of best rapid hot water treatment with prochloraz treatments for the control of avocado rot 61

3.4 Discussion 62
3.5 Conclusion 63
3.6 References 64
CHAPTER FOUR: Biocontrol yeasts as promising control of postharvest anthracnose and stem-end rot diseases of avocado fresh fruits 66
Abstract 66
4.1 Introduction 67
4.2 Materials and methods 68
4.2.1 Pathogen isolates 68
4.2.2 Yeast antagonists sample preparation and isolation 68
4.2.3 Preventative action of selected yeasts isolates antagonistic to *Colletotrichum spp.* and *Lasiodiplodia spp.* on avocado fresh fruits 69
4.2.4 Statistical analysis 69
4.3 Results 70
4.3.1 Isolation of yeast antagonists against postharvest avocado rot 70
4.3.2 Yeast antagonists for the control postharvest avocado rot 71
4.4 Discussion 73
4.5 Conclusion 75
4.6 References 76
CHAPTER FIVE: Efficacy of rapid hot water treatment and biological yeast antagonist for the control of avocado anthracnose and stem-end rot diseases 79
Abstract 79
5.1 Introduction 80
5.2 Materials and Methods 81
5.2.1 Pathogen isolates 81
5.2.2 Hot water treatments to determine the optimum temperature and exposure time for avocado fruits 81
5.2.3 Hot water treatment for the control of postharvest avocado rot 82

5.2.4 Yeast antagonists sample preparation and isolation 82
5.2.4 Preventative action of selected yeasts isolates antagonistic to *Colletotrichum spp.* and *Lasiodiplodia spp.* on avocado fresh fruits 83
5.2.5 Integration of best rapid hot water treatment with best yeast antagonists for the control of anthracnose and stem-end rot diseases 83
5.2.5 Statistical analysis 84
5.3 Results 84
5.3.1 Integration of best rapid hot water treatment with best yeast antagonists for the control of anthracnose and stem-end rot diseases 84
5.4 Discussion 86
5.5 Conclusion 88
5.6 References 89
CHAPTER SIX: Thesis overview 91
6.1 Introduction 91
6.2 Research objectives and outcomes 91
6.3 Recommendations and future prospects 94
6.4 Relevance of the research to the avocado industry 95
6.5 References 96
APPENDICES 97

INTRODUCTION

Factors such as genetic make-up; physiology; pre- and post-harvest conditions of the crop species of interest can significantly affect shelf life (Singh and Singh, 2011). This is an important factor in avocado production. The avocado *(Persea americana* Mill.) is a spherical to pear-shaped fruit with over 400 varieties that change in flavour and aroma during their ripening process. They are affected by postharvest anthracnose and stem-end rot diseases (Bill *et al*., 2014). Anthracnose causes irregular brown to black lesions which expand as the fruit ripens, leading to body rot of the fruit (Tchatchou, 2012). Stem-end rot disease appears as small dark brown to black spot at the stem-end of the fruit leading to fruit rot (Alemu, 2014). Collectively these diseases can cause between 50 – 90% of the losses of postharvest avocado due to spoilage (Joseph *et al*., 2015; Tesfay *et al*., 2017). Acceptable control levels of these diseases are achieved by the application of the fungicide prochloraz (Lundqvist *et al*., 2016).

Prochloraz (CAS no. 67747-09-5; N-propyl-N-[2-(2,4,6 trichlorophenoxy) ethyl]-1H-imidazole-1-carboxamide) has been intensively and successfully applied in agriculture for the pre- and postharvest control of fungal plant pathogens (Lundqvist *et al*., 2016). It is registered in South Africa as a non-systemic imidazole fungicide with effective control against plant fungal, bacterial, and viral diseases (Obianom, 2018). Prochloraz is widely applied in the avocado industry for the control of avocado diseases as both pre- and postharvest treatments (Obianom, 2018). The registered emulsifiable concentrate (EC) formulation for “Chronos” (active ingredient: prochloraz) is 1100 mL per 100 L of water, i.e., 11,000 parts per million (ppm). However, for export, the permitted maximum residue level (MRL) for prochloraz is 2 ppm or lower, depending on the importer (Daneel *et al*., 2016). This can negatively impact fruit exports (Obianom, 2018). Strict rules and regulations on the use and application of new and existing fungicides will prohibit the application of prochloraz by the year 2020 (Quinn *et al*., 2011; Wisniewski *et al*., 2016). For this reason, there is great urgency in the search for alternatives to prochloraz in postharvest avocado protection.

There has been growing interest in the application of hot water treatment and antagonistic yeasts for postharvest disease control in fruit, as alternatives to fungicides

(Abraham, 2010). Hot water treatment involves treating fresh produce with hot water at optimal temperature and time combinations for the control of infections (Palou, 2009). Biological control using antagonistic yeasts involves the application of naturally occurring yeasts to control pest organisms through competition for nutrients (Liu *et al.,* 2018). Both these methods are harmless to human health, environmentally friendly, and easy to apply (Asio and Cuaresma, 2016). However, when applied as stand-alone methods, both hot water treatment and biological control yeast do not always provide acceptable disease control levels (Zhang *et al*., 2008). On the other hand, during attempts to optimize the efficacy of individual treatments (Terao *et al*., 2017), the integrated application of hot water treatment and antagonistic yeasts has been found to significantly reduce the levels of postharvest fruit rot relative to conventional treatments (Abraham, 2010).

The aim of this study was to develop an integrated pest management approach for the control of postharvest anthracnose and stem-end rot diseases of avocado *(Persea americana* Mill.) fruit, caused by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc, and *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. respectively.

The objectives of this study included the following:

- To identify the causal organisms of postharvest avocado anthracnose and stem-end rot.
- To assess the pathogenicity of isolated anthracnose and stem-end rot pathogens on avocado fresh fruits.
- To determine the optimum temperature and time combinations for hot water treatment for the control of postharvest avocado anthracnose and stem-end rot.
- To screen and determine the best antagonistic yeasts for the biological control of postharvest avocado anthracnose and stem-end rot.
- To determine the best-performing integrated control methods using rapid hot water treatment and antagonistic yeasts for the control of anthracnose and stem-end rot diseases of avocado.

This dissertation is made up of five chapters, one being a review of literature, followed by four research chapters with a specific focus on postharvest avocado anthracnose

and stem-end rot. Each chapter is compiled in the form of a discrete, scientific paper, with all references formatted in accordance with the Journal of Biological Control.

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CHAPTER ONE

Review of literature

Abstract

Avocado *(Persea americana* Mill.) is a popular crop due to its palatability, nutritional content, and its processed products. However, the fruit is highly susceptible to anthracnose, typically caused by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., and stem-end rot, often caused by *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. These pathogens occur globally, and cause crop losses of 40 - 90%. Major avocado producing countries, including South Africa, apply a systemic postharvest fungicide, prochloraz, to control these pathogens. The continued use of this one fungicide may lead to the development of resistance by the pathogens. Furthermore, restrictions on the maximum residual level of pesticides are becoming increasingly strict on exports to the Organisation for Economic Cooperation and Development (OECD) countries. Therefore, alternative approaches to controlling postharvest diseases of avocado is a priority for the avocado industry. Alternative treatments can include the individual or combined use of biological and physical control approaches, e.g., the use of surface applications of a biocontrol yeast after a rapid hot water treatment of the whole fruit. As well as inhibiting postharvest avocado diseases, some methods can also improve the nutritional and taste properties of the fruit.

Keywords: *Avocado, anthracnose, stem-end rot, hot water treatment, biocontrol, yeast antagonists.*

1.1 Introduction

Avocado, *Persea americana* Mill., belongs to the family *Lauraceae* (Boadu *et al*., 2019; Colombo and Papetti, 2019). The species originated in the West Indies, Central America, and South America, but is currently grown worldwide in tropical and subtropical regions (Tchatchou, 2012). In 2017, Mexico was the global leader in avocado production, producing 2,029,886 tons per year (Campos-Martínez *et al*., 2016; FAOSTAT, 2019). At that time, South Africa was the 19th largest avocado producer, with 62,840 tons per year (FAOSTAT, 2019). A major challenge to the global avocado industry is postharvest diseases (Everett, 2002), especially anthracnose and stem-end rot (Tesfay *et al*., 2017). These diseases reduce the marketability of the fruit due to latent infections that manifest themselves as the fruit ripen (Obianom *et al*., 2019). Local and global reports suggest that the incidence of anthracnose is usually greater (36 - 80%) than stem-end rot (13 – 38%) (Marais, 2004; Demoz, 2005; Djeugap *et al*., 2015; Tesfay *et al*., 2017; Xoca-Orozco *et al*., 2017; Fischer *et al*., 2018; Obianom and Sivakumar, 2018). General approaches to postharvest disease control include pre-harvest disease management practices such as orchard sanitation and the application of copper-based chemicals to combat key pathogens at the pre-harvest stage (Lundqvist *et al*., 2016; De Silva *et al*., 2017). However, the major postharvest pathogens still infect fruit after these pre-harvest activities, appearing during ripening prior to consumption (Obianom and Sivakumar, 2018).

In typical commercial packhouses, fungicides are applied to control the postharvest diseases of avocados during the storage period (Campos-Martínez *et al*., 2016; Fischer *et al*., 2018). However, export regulations are making it difficult for avocado farmers to continue to depend upon fungicides (Madhupani and Adikaram, 2017; Lastochkina *et al*., 2019). As a result, biological control agents and physical control treatments, including the exposure of avocados to hot water for defined time intervals, are being explored as alternatives to such synthetic chemicals (Sharma *et al*., 2009). Several examples of such alternatives exist: in South Africa, the product Yield plus®, with the active ingredient *Cryptococcus albidus* (Saito) C.E. Skinner., is commercially available for the control of postharvest diseases of pome fruits caused by *Botrytis, Penicillium*, and *Mucor* species (Spadaro and Droby, 2016). Complete control of *Monilinia fructicola (*Winter) Honey in nectarines, peaches, and plums has also been achieved using a hot water treatment (Usall *et al*., 2016). The combination of heat

treatment and the biocontrol yeast *Metschnikowia pulcherrima* on stored apples has been shown to provide a high level of control of anthracnose, caused by *Colletotrichum acutatum* Simmonds, than either treatment alone (Sui *et al*., 2016). Hence, there is potential for this approach to be used to protect harvested avocado fruit.

This review describes avocado cultivation, two of the major postharvest diseases of avocado, i.e., anthracnose and stem-end rot, and their impact on the industry. It further describes and discusses the various options available for their control, including chemical treatments, biocontrol agents, and physical treatments. Integrated management strategies for controlling major postharvest diseases are described, revealing the potential of integrated, non-chemical strategies for the control of postharvest diseases of avocado.

1.2 Avocado

The avocado fruit is round to pear-shaped with a medium to large seed and palatable flesh (Bill *et al*., 2014). The skin may vary in texture and colour, from smooth to rough, and from green to purple (Nelson, 2008; Abraham *et al*., 2018). The fruit flesh changes in flavour and aroma over time during the ripening process (Marais, 2004). Different shapes, sizes, flavours, and aromas are characteristic of the different varieties of avocado (Abraham *et al*., 2018).

There are approximately 400 recognized avocado varieties, with fruits of different shapes and sizes (Álvarez *et al*., 2015). These varieties were developed from avocados that were first domesticated in Mesoamerica, approximately 10,000 years ago (Oduro, 2009; Barbosa-Martín *et al*., 2016; Campos-Martínez *et al*., 2016). The cultivation of avocados subsequently spread to many tropical and subtropical parts of the world (Tchatchou, 2012). In South Africa, avocado orchards are situated in the provinces of Limpopo, Mpumalanga, KwaZulu-Natal, Eastern Cape, and Western Cape (Demoz, 2005; DAFF, 2012; DAFF, 2017; Donkin, 2019).

The spread of avocado to various parts of the world resulted in different varieties being developed with characteristics that were influenced by the environmental conditions specific to the geographical regions in which they were cultivated (Marais, 2004). These avocado varieties originated from three different geographically isolated races, i.e., the Mexican (*Persea americana* Miller var. *drymifolia)*, West Indian/Antillean

(*Persea americana* Miller var. *americana)*, and Guatemalan (*Persea nubigena* Miller var. *guatemalensis)* races (Dabas *et al*., 2013; Duarte *et al*., 2016). The climatic conditions for cultivation and the variation in the season of maturity for these races, described by Hurtado- Fernández (2018), is shown in Table 1, along with several fruit characteristics that distinguish them.

Table 1 Comparison of avocado race characteristics (Hurtado- Fernández *et al*., 2018)

Trait(s)	**Race(s)**		
	Guatemalan (G)	**Mexican (M)**	**West Indian (WI)**
Optimal climatic growth conditions	Semi-tropical	Sub-tropical	Tropical
Maturity season	September to January	June to October	May to September
Fruit size	Small to large	Very small to medium	Medium to very large
Seed size	Small	Large	Variable
Fruit weight (average)	309.8g	98.8g	312.5g
Skin thickness	Thick	Very thin	Medium
Skin surface	Rough	Waxy bloom	Shiny
Seed cavity	Tight	Loose	Variable
Pulp flavour	Often nutty	Spicy	Mild
Oil content	High	Very high	Low

The hybridization of these races has given rise to many commercial cultivars (Griesbach, 2005). The majority of commercial cultivars have been derived from the hybridization of the Guatemalan and Mexican races. Selection criteria for commercial cultivar development include the length of the season, fruit skin quality, fruit size, and flavour (Tchatchou, 2012). In Table 2, examples are provided of some of the hybrid

cultivars produced from the Guatemalan and Mexican races, with the associated fruit characteristics that they were selected for.

Table 2 Examples of avocado cultivars developed from crosses between the Guatemalan, West Indian and Mexican races (Arpaia *et al*., 2012; Price, n.d.)

Race(s)	Cultivar	Characteristics of the fruit
Guatemalan (G) X Mexican (M)	Hass	Taste excellent with a good storage shelf life
	Fuerte	Taste excellent with a fair storage shelf life
	Gwen	Taste very good with an average storage shelf life
	Bacon	Taste good with a fair storage shelf life
	Lamb Hass	Taste very good with an average storage shelf life
	Pinkerton	Taste very good with an excellent storage shelf life
Mexican (M) X West Indian (WI)	Brogdon	Rich in flavour with high quality green flesh
Guatemalan (G) X West Indian (WI)	Choquette	Smooth tasting flesh with excellent quality oil and mild nutty flavour
	Day	Excellent rich and nutty taste
	Lula	Almost smooth with dark and glossy green skin

The increasing demand for avocado fruit worldwide is based on their nutritional value and perceived dietary health benefits because avocado fruit is rich in essential minerals, fatty acids, and vitamins (Obianom and Sivakumar, 2018), as shown in Table 3, which lists the nutrients typically found in avocado fruit

Table 3. Typical avocado fruit nutritional information per 100g (Dreher and Davenport, 2013; Hurtado-Fernandez *et al*., 2018)

	Nutrients	**Value per 100g**
Nutritional composition	Energy	160 kcal
	Protein	2.00 g
	Water	73.23 g
	Total lipids	14.66 g
	Carbohydrates	8.53 g
	Sugars	0.66 g
	Total dietary fibre	6.70 g
	Starch	0.11 g
Lipids	Monounsaturated	9.80 g
	Saturated	2.13 g
	Unsaturated	1.82 g
	Cholesterol	0 mg
	Campesterol	5.0 mg
	Beta-sitosterol	76.0 mg
	Stigmasterol	2.0 mg
Vitamins	Folate (DFE)	89 µg
	Pyridoxine (B6)	0.26 mg
	Vitamin A (RAE)	7 µg
	Thiamine (B1)	0.07 mg
	Niacin (B3)	1.74 mg
	Vitamin E (α-tocopherol)	2.07 mg
	Vitamin C (ascorbic acid)	10.00 mg
	Riboflavin (B2)	0.13 mg
	Vitamin K (phylloquinone)	21 µg
	Pantothenic acid	1.46 mg
Minerals	Calcium (Ca)	12 mg
	Iron (Fe)	0.55 mg
	Phosphorus (P)	52 mg
Sodium (Na)		7 mg

Zinc (Zn)	0.64 mg
Potassium (K)	485 mg
Magnesium (Mg)	29 mg
Manganese (Mn)	0.15 mg
Copper (Cu)	0.17 mg
Selenium (Se)	0.40 µg

In addition to the nutritional and dietary value of the fresh fruit, avocado fruit pulp can be processed into cosmetics, soaps, shampoo, oil, and processed foods (guacamole, frozen products, and avocado paste) (Dorantes *et al*., 2004; DAFF, 2012; Duarte *et al*., 2016).

Avocado fruit production in South Africa is aimed primarily for export to the European market (DAFF, 2017; Prabhu *et al*., 2017; Obianom and Sivakumar, 2018). During 2016, the international market share of South African avocado production was at 1.7%, equating to the export of approximately 57,867 tons of avocado fruits at a value of approximately one billion rand (DAFF, 2017). These export volumes are affected by the quality and food safety standards for avocado fruit in the importing countries (Obianom and Sivakumar, 2018). In particular, the residue levels for synthetic pesticides are one of the crucial issues for avocado fruit exports (Daneel *et al*., 2016). The European Union (EU) has set maximum residue levels (MRLs) for food products to ensure safety for consumers and the environment (Obianom *et al*., 2019). Commodities containing more pesticides residues than the allowed levels will be removed from the EU market (Nigro *et al*., 2018).

The application of pesticides is aimed at reducing crop losses (Quinn *et al.*, 2011). However, in addition to fungal rots, avocado production may be affected by post-harvest losses due to poor fruit quality that can be caused by chilling injury during storage, as well as from mechanical damage during handling (Dorantes *et al*, 2004; Marais, 2004). A lack of adequate fertilization of avocado trees in the orchard pre-harvest can also result in internal fruit damage, characterized by staining or vascularization of the fruit flesh (Dorantes *et al*, 2004). However, prevention of fungal damage is considered to be the most significant cause of crop losses because this type of damage causes the greatest losses by reducing avocado fruit shelf life, and

quality during transportation, storage, and marketing (Dorantes *et al*, 2004; Twizeyimana *et al*., 2013). The South African avocado industry now faces the challenge of delivering high-quality fruit to a very demanding consumer without the use of postharvest synthetic pesticides.

1.3 Postharvest diseases of avocado

1.3.1 Anthracnose

Postharvest anthracnose is a term used to describe a plant disease resulting in a body rot of fruit or vegetables. The pathogens involved are mostly members of the genus *Colletotrichum* (Tchatchou, 2012). This genus has a wide host range, as shown in Table 4, which lists some of the crops that suffer from postharvest anthracnose, as well as the causal agent (Fagundes *et al*., 2018).

Table 4. Hosts affected by anthracnose, caused by various *Colletotrichum* species (De Silva and Michereff, 2013)

Host	**Pathogen**
Avocado (*Persea americana* Mill.); Papaya (*Carica papaya* L.); Citrus (*Citrus sinensis* (L.) Osbeck)	*C. acutatum* Simmonds
Banana (*Musa X paradisiaca* L.)	*C. musae* (Berk. & Curtis) Arx
Papaya (*Carica papaya* L.)	*C. magna* (Jenkins & Winstead) Bhairi, Buckley & Staples
Acerola (*Malpighia emarginata* Sessé & Moc ex DC.)	*C. dematium* (Persoon) Grove
Mango (*Mangifera indica* L.)	*C. asianum* Prihastuti, Cai & Hyde
Avocado (*Persea americana* Mill.); Acerola (*Malpighia emarginata* Sessé & Moc ex DC.); Mango (*Mangifera indica* L.);	*C. gloeosporioides* (Penz.) Penz. & Sacc

Avocado anthracnose is often associated with *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. (Sarkhosh *et al*., 2017). This pathogen belongs to the family *Glomerellaceae,* in the phylum Ascomycota (Weir *et al*., 2012). It is the anamorphic, imperfect, or asexual state of a fungal teleomorph, *Glomerella cingulata* (Stoneman) Spauld. & H. Schrenk (Gautam, 2014). In other words, the fungus has two life cycles, and the asexual and sexual stages have been given different names.

Microscopic characteristics of C. *gloeosporioides* include conidia that can be symmetrical, cylindrical, broad or oblong, with rounded ends, which are occasionally oval in shape (Kimaru *et al*., 2018). Sizes can vary from 11-16 x 4-6 µm, with a mean size of 13.8 x 4.8 µm, and when cultured on potato dextrose agar (PDA) (Sharma and Kulshrestha, 2015), C. *gloeosporioides* produces cottony colonies that are a pale brown to greyish colour. The optimal environmental conditions required for infection are a temperature between 25-28°C, a pH of 5.8-6.5, and a relative humidity of 95 % or higher (Sharma and Kulshrestha, 2015). If these conditions are not met, then the pathogen will remain dormant until conditions become favourable (Gautam, 2014). *C. gloeosporioides* follows a hemibiotrophic mode of infection, whereby biotrophic and necrotrophic phases occur sequentially (De Silva *et al*., 2017). The infection process is initiated when a fungal conidium adhere to a fruit surface, and germinates to produce an appressorium, and this develops an infection peg that penetrates the epidermal cells of the skin (Gautam, 2014). However, the fungus then goes dormant, and remains a latent infection until fruit ripening changes the biochemical profile of the fruit (Sharma and Kulshrestha, 2015). The initial, latent stage of infection is called the biotrophic phase (De Silva *et al*., 2017). When the fruit begins to ripen, antifungal compounds found in unripe fruit diminish, allowing the fungus to grow aggressively, spreading throughout the fruit, killing the host cells (Jabeen, 2016). This is called the necrotrophic phase. In the advanced stages of the disease, lesions develop as visible symptoms, typically a dark brown patch, in the middle of which develops a structure called an acervulus, from which salmon pink spores emerge (De Silva *et al*., 2017). The two stages of the process are illustrated in Figure 1, which shows the typical infection cycle of *C. gloeosporioides* with tomato as the host model.

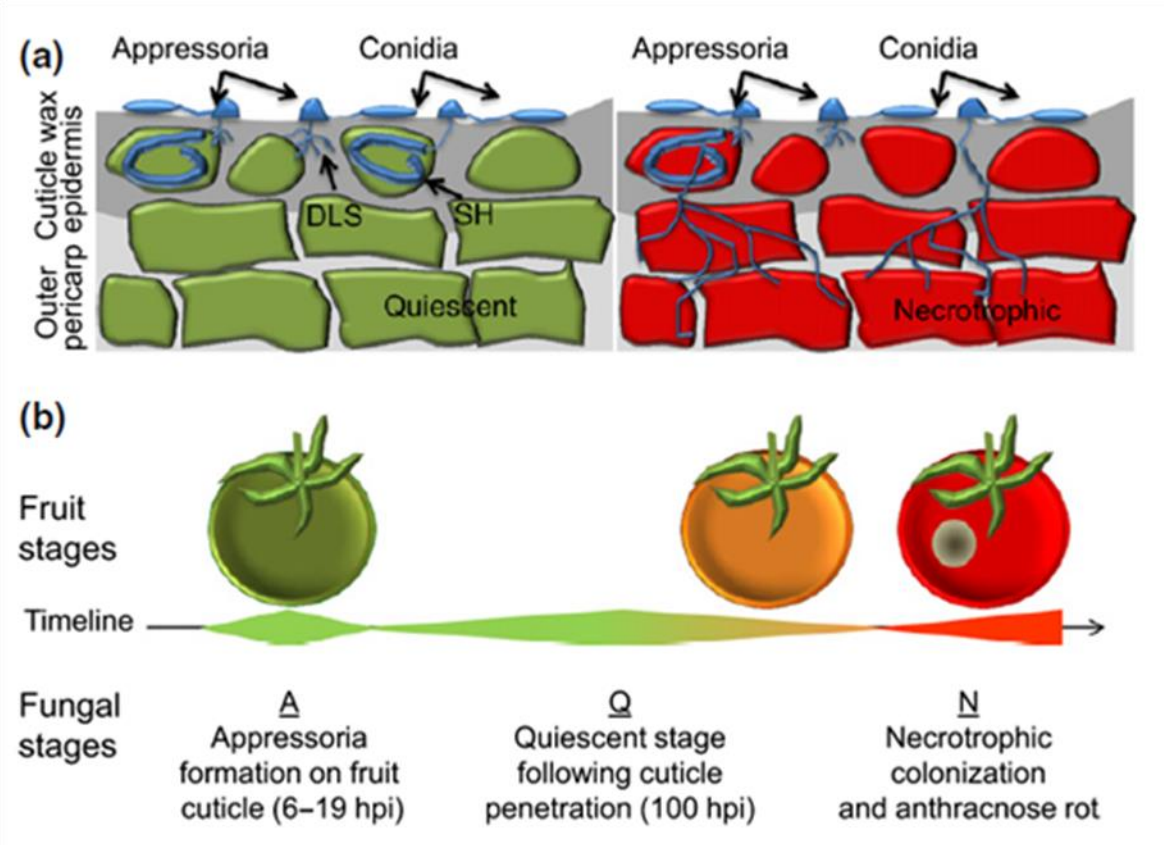

Figure 1 General infection process of anthracnose on a typical tomato model (Alkan *et al.,* 2014).

Postharvest symptoms of anthracnose caused by *C. gloeosporioides* on avocado fruits are irregular brown to black lesions which expand as the fruit ripens (Sharma and Kulshrestha, 2015). In the advanced stages of development, the salmon pink conidial masses can be observed in the center of the lesions (Demoz, 2005), generally under conditions of high humidity. These disease characteristics can be seen in the photo shown in Figure 2.

Figure 2 Symptoms of anthracnose caused by *C. gloeosporioides* on avocado fruits (Image taken by T.F., Majola).

1.3.2 Stem-end rot

The disease is named “stem-end rot” because lesion start at the pedicel end of the fruit during ripening (Madhupani and Adikaram, 2017). Stem-end rot may be caused by numerous fungal pathogens, usually by members of the family *Botryosphaeriaceae,* but sometimes by species from other genera, including *Colletotrichum*, *Phomopsis*, *Thyronectria*, *Rhizopus* and *Botryodiplodia* (Alves *et al*., 2008; Twizeyimana *et al*., 2013). In Table 5, examples of species responsible for stem-end rot are shown. These species can all cause severe rot at the stem-end of avocado fruits, and their importance often varies by geographic regions (Twumasi *et al*., 2014).

Table 5. Examples of pathogens causing stem-end rot disease (Twizeyimana *et al*., 2013; Valencia *et al*., 2019)

Family	**Specie(s)**
Nectriaceae	*Thyronectria pseudotrichia* (Schw.) Seeler
	Fusarium sambucinum Fuckel
	Fusarium decemcellulare Brick
	Nectria pseudotrichia (Schwein.) Berk. & M.A. Curtis
Botryosphaeriaceae	*Lasiodiplodia theobromae* (Pat.) Griffon & Maubl
	Neofusicoccum luteum (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips
	Neofusicoccum ribis (Slippers, Crous & M.J. Wingfield) Crous, Slippers & A.J.L. Phillips
Glomerellaceae	*Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc.
Diaporthaceae	*Phomopsis perseae* Zerova
Sporocadaceae	*Pestalotiopsis versicolor* (Speg.) Steyart
Pleosporaceae	*Bipolaris setariae* (Sawada) Shoemaker
Mucoraceae	*Rhizopus stolonifer* (Ehrenb.:Fr.) Vuill

The most common causal agent of stem-end rot in avocado fruits is *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl., an adaptable fungus known to cause disease in at least 500 different hosts, including humans (Xie *et al*., 2016; Felix *et al*., 2018). Some of the hosts that may be infected by *L. theobromae* are highlighted in Table 6.

Table 6. Major hosts of *Lasiodiplodia theobromae*

Host(s)	Disease(s)	Reference(s)
Mango (*Mangifera indica* L.)	Dieback	(Ismail *et al*., 2012)
Ricinodendron heudelotii (Baill.) Pierre ex Hecke	Shoot blight	(Djeugap *et al*., 2016)
Tea (*Camellia sinensis* (L.) Kuntze (Theaceae)	Leaf necrosis	(Li *et al*., 2019)
Papaya (*Carica papaya* L.)	Stem-end rot	(Netto, 2014)
Avocado (*Persea americana* Mill.)	Stem-end rot	(Twizeyimana *et al*., 2013)
Humans (*Homo sapiens)*	Keratomycosis and Phaeohyphomycosis	(Summerbell *et al*., 2004)
Physic nut (*Jatropha curcas* L.)	Collar rot disease	(Latha. *et al*., 2009)

L. theobromae, formerly known as *Botryodiplodia theobromae* Pat., belongs to the family *Botryosphaeriaceae,* Phylum Ascomycota (Manawasinghe, 2016). The asexual stage of *L. theobromae* is *Botryosphaeria rhodina* (Berk and Curtis) Arx. The sexual stage of this family is rarely seen in nature (Alves *et al*., 2008).

Under the microscope, immature fungal conidia are unicellular, hyaline, ellipsoid to subovoid, and the mature conidia are dark brown, bi-cellular, thick-walled, and ellipsoid (Latha. *et al*., 2009). In culture, *L. theobromae* initially produces white fluffy colonies with aerial mycelia, when grown on potato dextrose agar (PDA) (Djeugap *et al*., 2016). After one to two weeks, the colonies become black in colour, which is more visible on the underside of colonies grown in transparent Petri dishes (Alemu, 2014; Xie *et al*., 2016).

Optimal conditions for infection of fruit are temperatures of 27 - 33°C and relative humidities of more than 80% (Demoz, 2005). *L. theobromae* enters the fruit via wounds, or via natural openings, such as the pedicels and stem-end scars of fruit, penetrating the host tissue of the fruit during the harvesting and storage process (Alemu, 2014). Infection can result from the dispersal of the spores by rainwater onto the surface of unripe fruits (Jabeen, 2016). Once the conidia germinate on the unripe host fruit surface, they remain dormant until the ripening processes commence. As the fruit ripens, disease symptoms become visible (Alemu, 2014). A summary of the infection process and disease cycle of *L. theobromae* is shown in Figure 3.

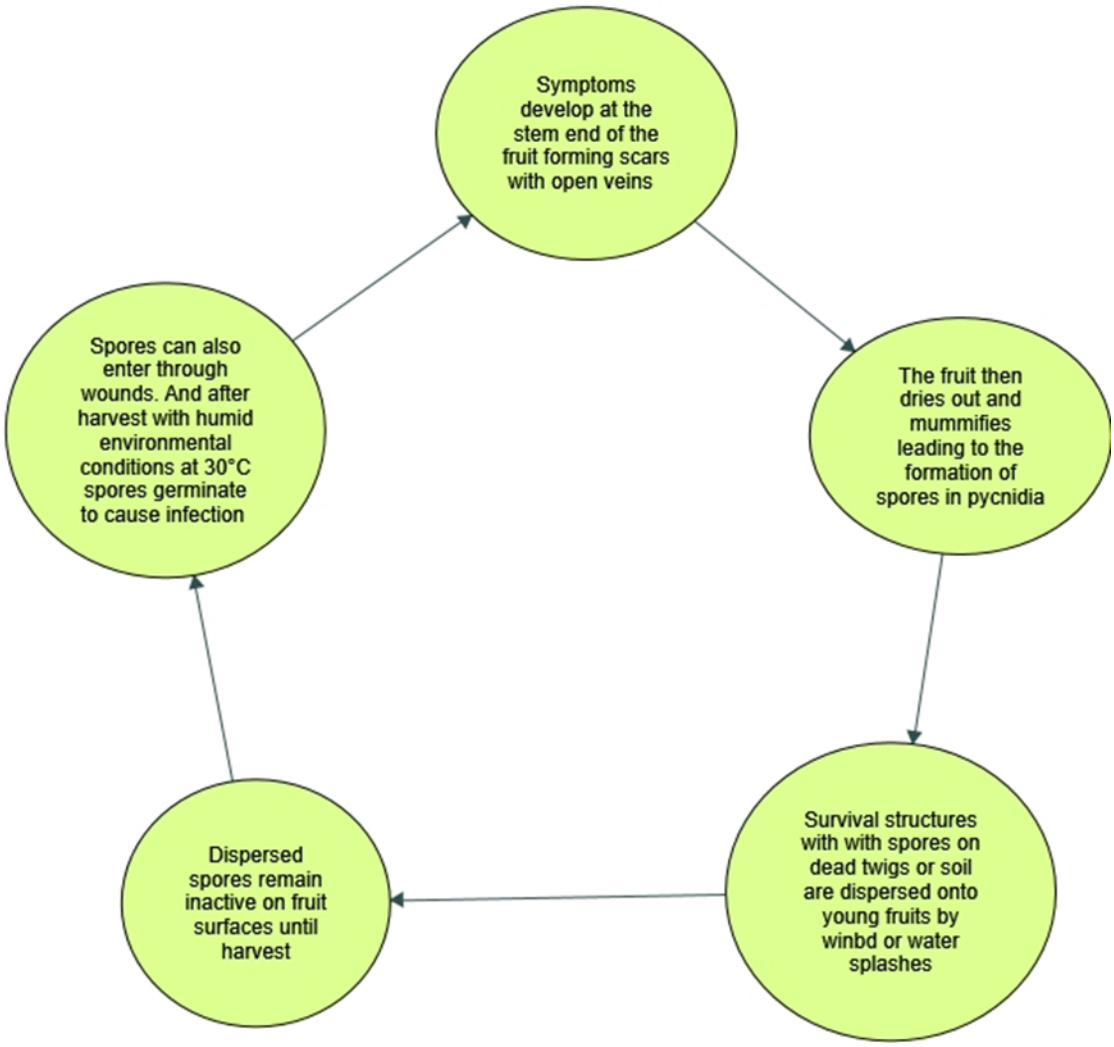

Figure 3 Typical disease cycle of *L. theobromae* on avocado fruits (Alemu, 2014).

At the early ripening stage, stem-end rot symptoms appear as small dark brown to black spots at the fruit stem-end (Alemu, 2014). In advanced stages of ripening, stem-end rot causes fruit decay, visible as fruit discolouration, brown flesh, and the softening of tissue until it becomes a dark brown-coloured liquid (Manawasinghe, 2016). This necrotic process eventually spreads throughout the fruit (Jabeen, 2016), as highlighted in Figure 4.

Figure 4 Stem-end rot symptoms on an infected avocado fruit (Madhupani and Adikaram, 2017).

1.4 Disease management strategies

There are several strategies for maintaining post-harvest fruit quality (Usall *et al*., 2015). While biological and physical control methods are being investigated and explored, historically chemical control has been the most common approach (Palou, 2013). However, each approach has its own benefits and challenges (Palou *et al*., 2008).

1.4.1 Chemical control

Chemical control commonly refers to the application of synthetic fungicides to fruit to increase the shelf life of the fresh produce (Palou *et al*., 2016). It is implemented to reduce postharvest losses of avocado fruits due to anthracnose and stem-end rot diseases (Obianom and Sivakumar, 2018). This is usually achieved by the application of prochloraz (CAS no. 67747-09-5; N-propyl-N-[2-(2,4,6 trichlorophenoxy) ethyl]-1H-imidazole-1-carboxamide), which is applied immediately after harvest in packhouses at a rate of 1100 mL prochloraz per 100 L of water (Demoz, 2005; Obianom and Sivakumar, 2018). It is a registered post-harvest fungicide in South Africa, Australia, and New Zealand (Campos-Martínez *et al*., 2016; Obianom and Sivakumar, 2018). However, fungicide residue on the surfaces and inside the fruit has raised concerns about potential adverse impacts on the health of terrestrial and aquatic ecosystems, as well as negative effects on consumer health (Campos-Martínez *et al*., 2016). Therefore, the post-harvest application of chemical fungicides on avocado fruits is now facing restrictions (Quinn *et al*., 2011).

1.4.2 Biological control

Biological control involves the use of naturally occurring microorganisms to control a population of pathogenic microorganisms, insects or weeds (McEvoy, 2017). Such agents were first applied to plant pathogens in 1914 by a pioneer in biological control, Carl Freiherr von Tubeuf (Hajek, 2004). To date, biocontrol agents (BCAs) have been widely investigated and have demonstrated success (Sharma *et al*., 2009; Abraham, 2010; Singh and Sharma, 2018). For a BCA to be successful and to be applied commercially, it should ideally possess the following properties: (1) non-pathogenic to humans and environmentally friendly; (2) non-pathogenic to the host plant; (3) genetic stability; (4) compatibility with commercial manufacturing procedures with simple nutrient requirements; (5) efficacy at low concentrations; (6) tolerance of a wide range of environmental conditions; (7) a stable shelf-life of at least 12 months; (8) efficacy against a wide range of target pathogens (Mbili, 2012; Asio and Cuaresma, 2016).

Some research has been undertaken to develop pre-harvest applications of microbial BCAs for the control of post-harvest diseases, including those of avocados (Korsten *et al*., 1988). Dukare *et al* (2019) recently reviewed the use of BCAs against postharvest pathogens of commercial fruit crops. This is summarized in Table 7.

Table 7. Successful bio-control agents used for the control of postharvest diseases (Dukare *et al*., 2019)

Antagonist(s)	Pathogen(s)	Crop(s)
Bacillus pumilus and *Bacillus thuringiensis*	*C. gloeosporioides*	Mango
Pseudomonas fluorescens and *Bacillus subtilis*	*Lasiodiplodia theobromae*	Mango
Paenibacillus polymyxa and *Bacillus subtilis*	*Colletotrichum gloeosporioides* *Colletotrichum acutatum, Botryosphaeria dothidea*	Apple
Bacillus amyloliquefaciens	*Alternaria citri, Colletotrichum gloeosporioides, Penicillium crustosum*	Citrus

Of particular interest, beneficial yeasts been developed for application on various fruits (Sharma *et al*., 2009). Yeasts are single-celled fungi (Mbili, 2012). They are potentially good BCAs because they can grow rapidly, colonize the entire fruit surface and outcompete pathogens through competition for nutrients, and can tolerate most agrochemicals (Dukare *et al*., 2019). Yeasts are also easily produced and applied (Sharma *et al*., 2009). They live naturally on healthy fruit surfaces, and are generally regarded as safe when applied to food crops (Dukare *et al*., 2019). Many yeasts have demonstrated their efficacy as biocontrol agents of post-harvest diseases in a variety of commercial fruit crops, as shown in Table 8.

Table 8. Yeasts used as bio-control agents against postharvest diseases (Wisniewski *et al*., 2016; Dukare *et al*., 2019)

Antagonist(s)	Pathogen(s)	Crop(s)
Candida oleophila	*Colletotrichum musae*	Banana
	Penicillium expansum, Botrytis cinerea	Apple
Cryptococcus albidus	*P. expansum, B. cinerea*	Apple
Metschnikowia fructicola	*P. expansum*	Apple
	Penicillium digitatum	Citrus
Pichia guilliermondii	*Colletotrichum capsici*	Chilies
	Rhizopus nigricans	Tomato
	B. cinerea	Apple, Kiwifruit
Candida sake	*P. expansum*	Apple
	B. cinerea, P. expansum	Grape
Rhodotorula mucilaginosa	*P. expansum*	Pear
Debaryomyces hansenii	*Rhizopus stolonifer*	Peach
	P. digitatum	Citrus
Pichia anomala	*Colletotrichum musae, Fusarium moniliforme*	Banana
Cryptococcus laurentii	*B. cinerea*	Strawberry

The investigation into yeasts as potential BCAs of post-harvest avocado diseases is validated to the extent that research, such as that listed in Table 9, has led to the development and release of a variety of commercial products that are available for this purpose. Several of these commercial products and their target pathogens and hosts are listed in Table 9. Biological control options are becoming mainstream methods of dealing with post-harvest fruit disease management because they are effective, non-toxic, and biologically and commercially viable (Junaid *et al*., 2013).

Table 9. Commercial yeast-based biological control products (Romanazzi *et al*., 2016; Dukare *et al*., 2019)

Product name	Producing country	Target pathogen	Host fruit
Candifruit	Spain	*Penicillium, Botrytis, Rhizopus*	Pome
Shemer	Netherlands	*Botrytis, Penicillium, Rhizopus, Aspergillus*	Table grape, pome, strawberry, stone fruit, sweet potato
Aspire	USA	*Botrytis, Penicillium, Monilinia*	Pome, citrus, stone fruit, strawberry
Nexy	Belgium	*Botrytis, Penicillium*	Pome
Yield Plus	South Africa	*Botrytis, Penicillium, Mucor*	Pome, citrus
Boni Protect	Austria	*Penicillium, Botrytis, Monilinia*	Pome

1.4.3 Physical treatment

Physical treatments of crops are non-chemical alternatives to chemical pesticides or BCAs to reduce diseases, without leaving a residues (Usall *et al*., 2016; Ayon-Reyna *et al*., 2017). They are inexpensive, hazard-free, simple, effective and easy to implement (Palou *et al*., 2008). Examples of physical treatments are cold storage; heat treatments such as hot water treatment (HWT), rapid hot water treatment (RHWT), hot air treatment, radiofrequency and microwave treatment, hypobaric and hyperbaric pressure treatments, and light treatments such as ultraviolet-C (Terao *et al*., 2017; Singh and Sharma, 2018). Of these physical treatments, heat treatments as disease control methods are of particular interest in the postharvest industry, including HWT applied as a hot water dip, sometimes including rinsing, brushing, or both (Abraham, 2010; Li *et al*., 2013).

The first reported evaluation of an HWT to control a postharvest disease was in 1960 in California citrus packinghouses to control brown rot (Palou, 2009). A subsequent

version of the HWT system was developed in Israel during the 1990s (Palou, 2013). The machinery system consisted of a packing line that cleans and disinfects fresh produce by the application of hot water over rotating brushes at a relatively high temperature (54-62^{0}C) for a very short period of time (8-60 seconds) (Palou, 2013). The main factors to consider when developing an efficient HWT system are balancing the two treatment factors, hot water temperature and period of exposure, versus the need to avoid damage to the fruit (Singh and Sharma, 2018). Research in the last 15 years has shown that HWT only needs to be applied for a relatively short period of time (under 20 seconds). This is linked to our understanding that HWT works via the plant's own immune system. The previous concept of how HWT worked was that the heat applied directly killed the fungal pathogens, which is incorrect. A brief exposure (say 10 seconds) to hot water at a critical temperature (say 56^{0}C) triggers the host immune system without damaging the plant tissues (Li *et al*., 2013). Previous studies have indicated that heat shock proteins 70 and 90 (HSP 70 and HSP90) possess the ability to directly interact with heat shock factors to produce host immunity (Sable *et al*., 2018). As a bonus, the shelf life of fruit may be enhanced by a delay of the normal ripening process. HWT induces systemically acquired resistance (SAR) in the host, via a complex cascade of biochemical reactions. The SAR results in the release of natural fungicides called phytoalexins that kill fungi (Usall *et al*., 2016). Responses often depend on the type of fruit being treated; the treatment temperature and duration; seasonal and climate conditions; fruit size; and the fruit condition (Palou, 2009).

Physical postharvest disease control using various HWT conditions has been implemented against several fruit pathogens, as shown in Table 10.

Table 10. Successful hot water treatments against postharvest disease (Usall *et al*., 2016)

Crop	Disease	Hot water dip treatment
Papaya (*Carica papaya* L.)	Anthracnose	54°C for 3-4 min
Potato (*Solanum tuberosum* L.)	Bacterial soft rot Dry rot	57.5°C for 20–30 min
Strawberry (*Fragaria* × *ananassa* Duchesne)	Gray mould, Soft rot	55–60°C for 30 s
Pear (*Pyrus communis* L.)	Blue mould	46°C for 15 min
Mango (*Mangifera indica* L.)	Anthracnose,	53°C for 20 min;
	Stem-end rot	55°C for 5 min;
Apple (*Malus domestica*)	Bull's eye rot; Blue mould	45°C for 10 min
Grapefruit (*Citrus* × *paradisi* Macfad)	Green mould	53°C for 3 min

1.4.4 Integrated control

Integrated control strategies are combinations of several control treatment methods. These are often adopted when stand-alone biological or physical postharvest disease control treatments do not provide adequate and consistent levels of disease control (Zhang *et al*., 2008). Postharvest disease management strategies that utilize combinations of eco-friendly approaches, rather than a single approach, may be able to provide viable alternatives to synthetic fungicides (Terao *et al*., 2017). Korsten *et al*. (1997) demonstrated that integrated chemical and biological control was able to reduce postharvest disease in avocado fruits, warranting further exploration of novel integrated control combinations, such as BCAs and physical treatments.

The application of a heat treatment combined with a microbial antagonist provides a synergistic effect against the postharvest diseases being controlled (Palou *et al*.,

2008). HWT provides a curative action against existing pathogenic infections but does not provide protection from future infections (Usall *et al*., 2015). Yeasts as BCAs provide preventative protection by colonising the surface of fruit, including wounds and infection sites, thereby preventing future infections by pathogens (Obagwu and Korsten, 2003). Examples of integrated control strategies against postharvest diseases are shown in Table 11.

Table 11. Heat and bio-control integrated control strategies against the postharvest disease

Crop	Disease	Treatment	Reference
Banana	Anthracnose	Hot water treatment with *Burkholderia cepacia*	(De Costa and Erabadupitiya, 2005)
Orange	Green mould	Hot water dip with yeast isolate B13	(Abraham, 2010)
Peach	Grey mould and blue mould	Hot water dip, yeast antagonist and modified atmosphere packaging	(Karabulut and Baykal, 2004)
Orange	Green and blue mould	*Bacillus subtilis* with hot water treatment	(Obagwu and Korsten, 2003)
Pear	Blue mould	Hot water treatment with *Rhodotorula glutinis*	(Zhang *et al*., 2008)
Orange	Green mould	Hot water brushing with yeast isolate, *C. membranifaciens* CMAA-1112	(Terao *et al*., 2017)

1.5 Conclusion

For the effective control of postharvest diseases of avocado, an integrated suite of disease control strategies should be implemented, both before and after harvest, to control the plant pathogens responsible for postharvest diseases (Everett, 2002). It has been demonstrated that effective levels of control can be achieved using such strategies. However, future studies should also include the testing of fruit nutritional

content, flavour, aroma and texture after treatment, and shelf life, in comparison with chemical treatments, to see if further benefits arise from the alternative treatment methods. These benefits could encourage the use of alternative postharvest disease control methods, which would increase the market value of South African avocados in a globally competitive market.

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CHAPTER TWO

Koch's postulate experiments on two isolates of avocado anthracnose and stem-end rot causal organisms in KwaZulu-Natal, Republic of South Africa.

Abstract

The avocado industry experiences great losses due to postharvest avocado spoilage caused by fungal rot. Identifying the causal organisms involved in postharvest avocado fungal rot is imperative for developing new approaches to control the two major diseases of postharvest avocado, i.e., anthracnose and stem-end rot. To confirm the pathogenicity and identity of fungi isolated from diseased avocado fruit, Koch's postulate experiments were performed. It was found that the fungus isolated from fruit showing stem-end rot was caused by a member of the genus *Lasiodiplodia*. The isolate from the fruit showing anthracnose symptoms was shown to be a member of the genus *Colletotrichum.* In both instances both the Internal Transcribed Spacer (ITS) regions, i.e., ITS1 and ITS2 regions sequence data were inadequate for the determination of the taxonomic assignment of either of these isolates to species level. However, both isolates were able to produce the original symptoms in inoculated fruits and showed the epidemiological and morphological characteristics of the species typically associated with these avocado diseases. These isolates were sufficiently pathogenic and adequately characterized for use in subsequent studies on the control of the two major postharvest diseases of avocado.

Keywords: *Isolation, identification, phylogeny, avocado rot, and postharvest.*

2.1 Introduction

Avocado (*Persea americana* Mill) is a climacteric fruit that experiences postharvest damage caused by chilling, mechanical damage, and fungal damage (Dorantes *et al*., 2004). The most significant losses are due to fungal damage and the rotting process of the fruit is often directly correlated with the ripening process when the conditions that are favourable for infections to occur (Terander, 2002). The fruit ripening process takes place after harvesting as fruit begin to senesce. Once ripening begins, the dormant fungal pathogens begin to grow and lead to the development of fungal symptoms (Marais, 2004). The two major postharvest avocado fruit rot diseases are anthracnose and stem-end rot. These are associated with severe symptoms that reduce fruit quality for consumption (Obianom *et al*., 2019). Anthracnose causes body rot of the fruit with irregular brown to black lesions which expands as the fruit ripens, later forming pink conidial masses on the body of the fruit (Demoz, 2005; Sonavane *et al*., 2017). Stem-end rot of avocado appears as small brown to black lesions at the stem-end of the fruit (Alemu, 2014), which later advances to the rest of the fruit flesh during the ripening stages (Twizeyimana *et al*., 2013).

Globally, species of the genera *Colletotrichum* and *Lasiodiplodia* are the most prevalent causal agents of anthracnose and stem-end rot diseases of avocado fruits respectively. They can infect avocado fruit alone or simultaneously (Demoz, 2005; Twizeyimana *et al*., 2013; Valencia *et al*., 2019). Approximately 36% and 13% of South African avocado postharvest losses are due to stem-end rot and anthracnose diseases respectively (Korsten *et al*., 1991). With increasing health concerns regarding the application of chemical fungicides and growing restrictions on their use, there is an urgent need to identify and understand the nature of the causal organisms, to limit and manage the presence of these diseases on fresh avocado fruits using alternative approaches (Everett, 2002; Campos-Martínez *et al*., 2016).

To confirm that the causal agents of anthracnose and stem-end rot diseases belong to the genera *Colletotrichum* and *Lasiodiplodia* respectively, Koch's postulate studies are employed to isolate the pathogens confirm their taxonomic assignments. Koch's postulate involves the process whereby suspected causal organisms consistently associated with the disease are isolated from infected plant material and grown in pure

culture. Following inoculation of a healthy susceptible host with the potential pathogen from pure culture, the host is observed for the development of symptoms of the original disease. Finally, the same microbial organism must be re-isolated from plants infected under these experimental conditions (Thangamani *et al*., 2011; Valencia *et al*., 2019). Koch's postulate is a valuable tool to establish a causative relationship between a fungal microbe and a disease (Thangamani *et al*., 2011) and is often employed in studies that investigate new methods for the control of plant diseases.

For the purpose of investigating the control of the two major postharvest fungal avocado diseases, the objectives of this study were:
1-to isolate the causal agents of postharvest avocado anthracnose and stem-end rot;
2-to characterise the isolated causal organisms of anthracnose and stem-end rot using morphological, microscopic, and molecular methods; and
3- to conduct pathogenicity trials using the isolated pathogens.

2.2 Materials and methods

2.2.1 Sample preparation and isolation

Diseased avocado fruit showing anthracnose and stem-end rot symptoms were collected from different fruit markets in Pietermaritzburg, South Africa (29.6006°S, 30.3794°E). Pieces of fruit tissues taken from the advancing margin of disease lesions were surface disinfected with 70% ethanol for 3 minutes, washed in sterile distilled water and allowed to dry. They were transferred to Potato Dextrose Agar (PDA) (Merck, Germany) plates and incubated for seven days at 28°C. For long term storage, cultures were cultured on quarter strength PDA agar slants.

2.2.2 Cultural and morphological studies of the causal agents

After seven days of incubation at 28°C the colony characteristics were observed and wet mounts were observed at various magnifications under a light microscope.

2.2.3 Molecular identification of the causal agents

Pure culture isolates were sent to Inqaba biotechnical Industries (Pty) Ltd (South Africa) for DNA-based identification, sequencing the ITS1 and ITS2 regions in forward and reverse directions. The consensus sequence for each isolate was submitted to Genbank, selecting only for similar sequences from type cultures. Select aligned sequence fragments with greater than 97% similarity and 100 % coverage were downloaded. Along with the isolate sequences, these sequences were aligned for the construction of a neighbour joining (NJ) bootstrap consensus tree to infer the probable identity of the isolates. All sequence processing and phylogenetic tree construction was performed using MegaX software (version 10.0.5).

2.2.4 Pathogenicity studies of the causal agents

Koch's postulate experiments were performed by inoculating asymptomatic fruit with the pathogen isolates and then re-isolating and identifying the pathogens from the fruit after the development of disease symptoms. The procedure took place as follows: asymptomatic avocado fruit were surface sterilized using 70% ethanol and wounded by creating a 3 x 3 mm circular hole in the fruit surface using a sterile scalpel. Wounded fruit were inoculated with pure culture mycelial plugs of the pathogens of interest and the control fruit were inoculated with sterile PDA plugs. There were ten fruit per replicate and three replicates per treatment, as per the method used by Abd-Elsalam *et al.* (2010). The fruit were incubated at 25°C and a relative humidity of 95% for 10 days. After fruit ripening, the wounds were examined to confirm the presence of infections by the isolated causal agents. Morphological examinations were performed to compare the cultures from the initial isolation with the secondary isolation from the inoculated fruit (Thangamani *et al*., 2011).

2.3 Results

2.3.1 Cultural and morphological studies of the causal agents

After the incubation period at 28°C, cultures on plates grew as per images in figure 2.1.

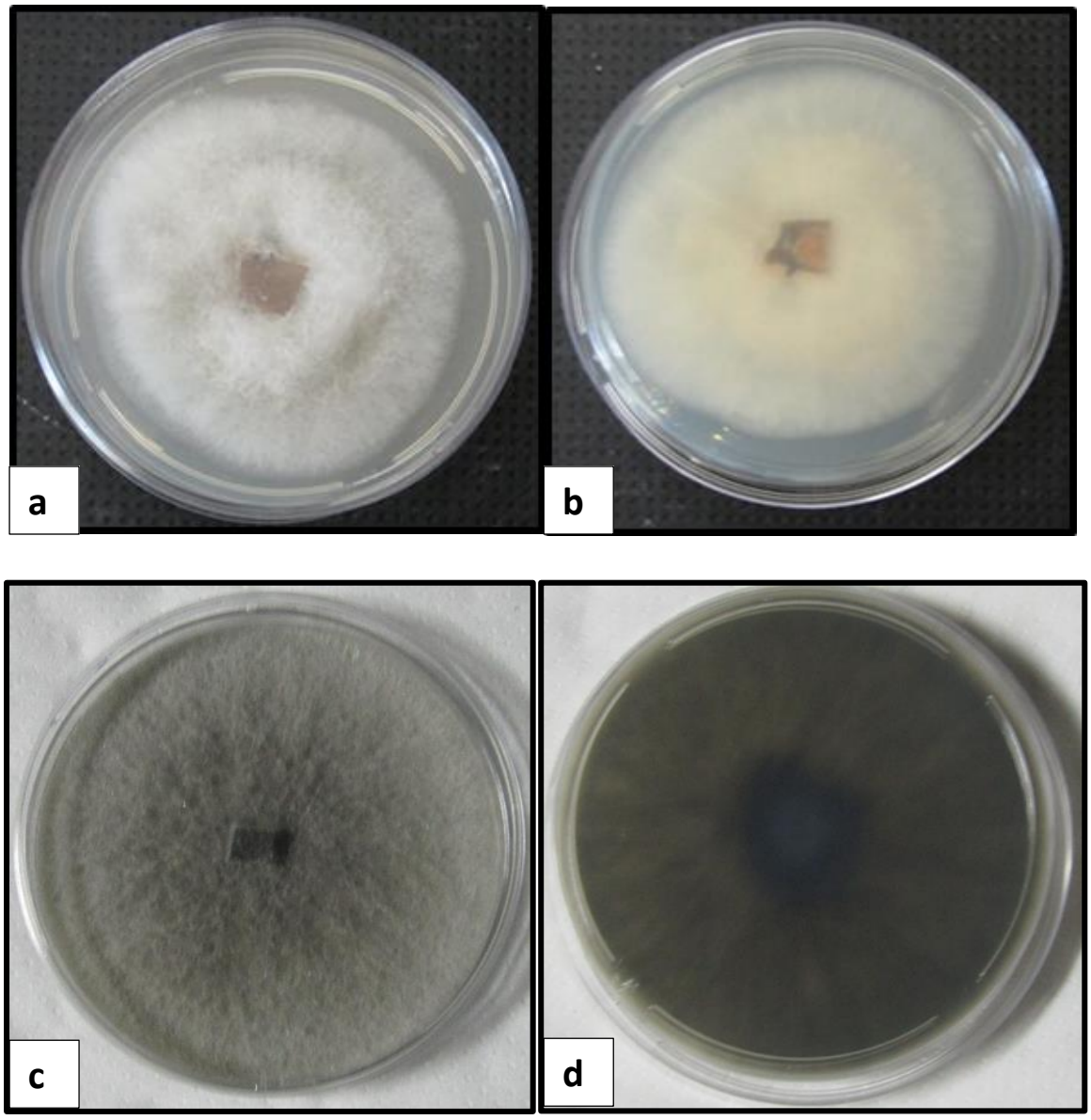

Figure 2.1 *Colletotrichum* and *Lasiodiplodia* species isolates on PDA with surface views shown in **(a)** and **(c),** and the undersides of the plates in (**b)** and **(d)**.

Images (a) and (b) show cottony colonies that are of cream-white greyish to orange in colour belong to cultures isolated from anthracnose diseased avocado fruits, (c) shows

colonies that are grey to black in colour, (d) shows reverse plate with black colonies obtained from stem-end rot diseased avocado fruits

Microscopic images from wet mounts of each of the culture isolates are shown in figure 2.2.

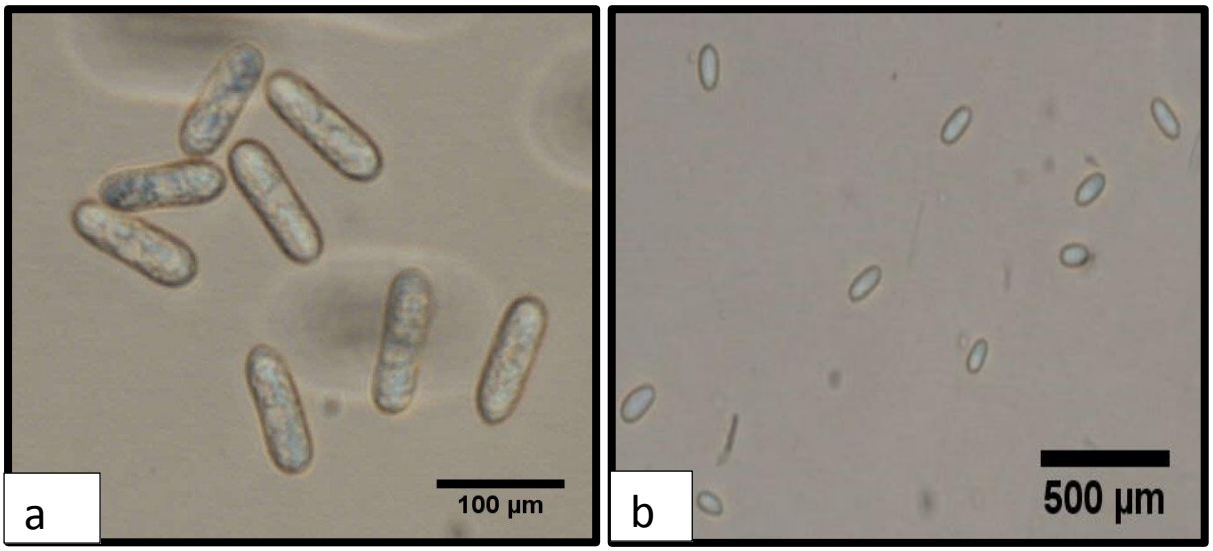

Figure 2.2 Conidial spores at (100x) magnification of (a) *Colletotrichum* and (b) *Lasiodiplodia* species isolates.

Figure 2.2 (a) shows that the *Colletotrichum* conidia were slightly oval to rod-shaped with rounded ends, symmetrical, and approximately 100 µm in length. The *Lasiodiplodia* conidia isolates were ellipsoid to slightly rod-shaped and around 500 µm in length as seen in Figure 2.2 (b).

2.3.3 Pathogenicity studies of the causal agents

The requirements for the fulfilment of Koch's postulates were met for the fungal cultures isolated from avocados that showed anthracnose and stem-end rot diseases in this work. Symptoms from the inoculation of the healthy avocado fruits for experimental purposes are shown on the images in figure 2.3.

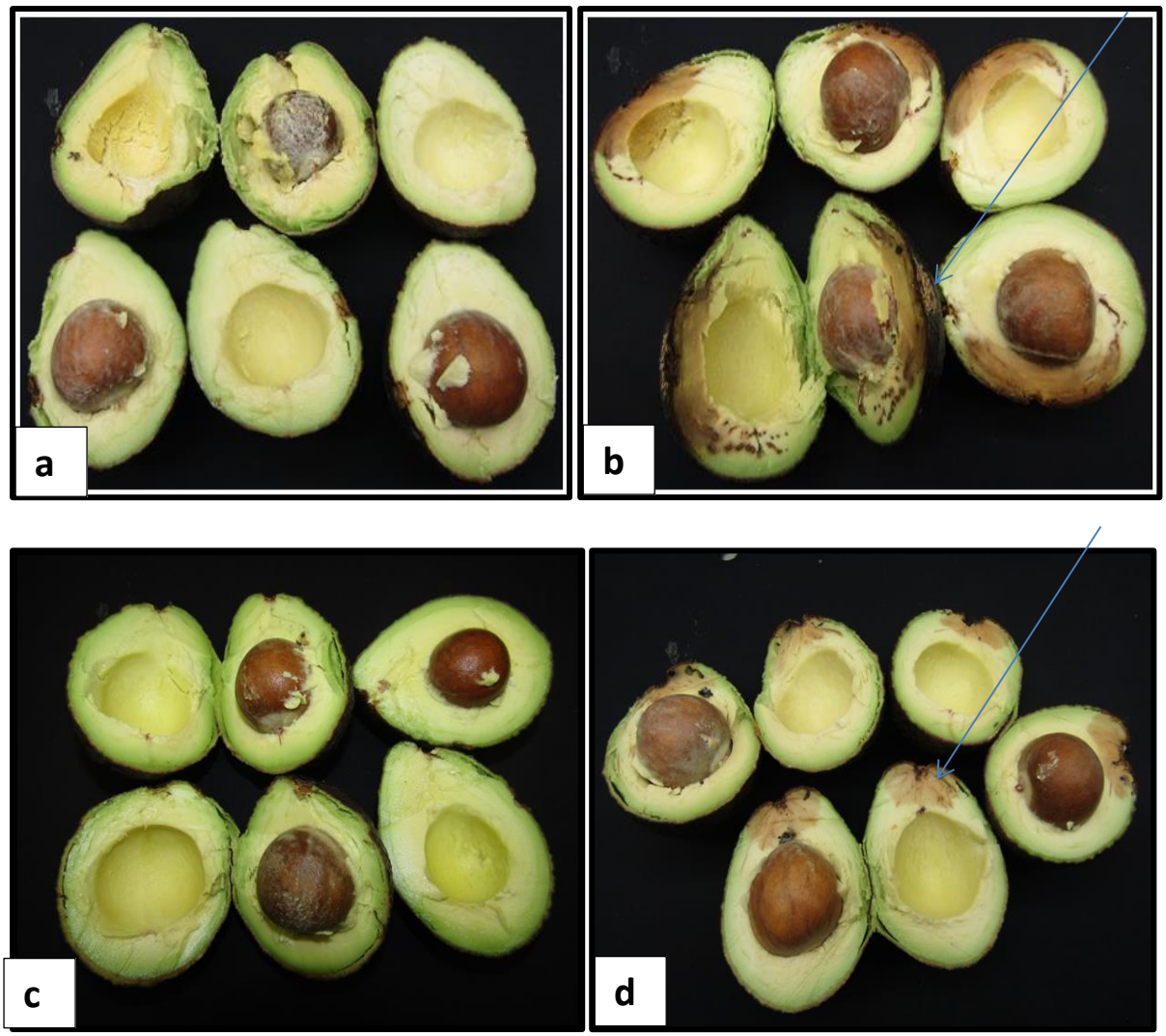

Figure 2.3 Pathogenicity avocado images of (a) anthracnose uninfected controls and (b) anthracnose infected fruit; and (c) stem-end rot uninfected controls and (d) stem-end rot infected fruit.

For both anthracnose and stem-end rot treated avocado fruits, respective characteristic symptoms were observed in fruit inoculated with both pathogen isolates. Figure 2.3 (b) shows the pink conidial masses that are characteristic of anthracnose, while figure 2.3 (d) shows the start of rot at the stem-end of the fruit, characteristic of stem-end rot. These symptoms of anthracnose and stem-end rot diseases were the same as those observed in the original diseased fruit from which the pure cultures were isolated.

2.3.2 Molecular identification of the causal agents

The NJ bootstrap consensus tree developed from the sequence alignment of the isolate that caused anthracnose is shown in figure 2.4.

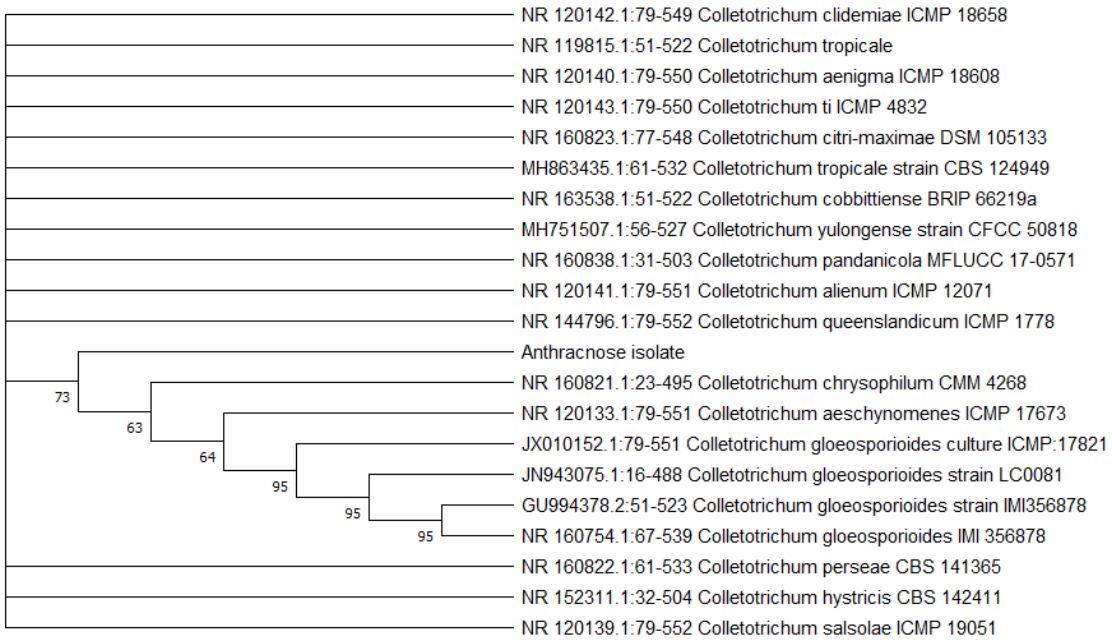

Figure 2:4 A NJ bootstrap consensus tree displaying the phylogenetic tree from the ITS nucleotide sequence of the *Colletotrichum sp*. isolated from avocado fruit showing anthracnose symptoms, and type culture sequences that had high similarity to the query sequence.

The phylogenetic tree suggests that the isolated causal agent of anthracnose of avocado fruits isolated in this work is potentially related to *C. gloeosporioides* or *C. aeschynomenes* species.

The NJ bootstrap consensus tree developed from the sequence alignment of the isolate that caused stem-end rot is shown in figure 2.5.

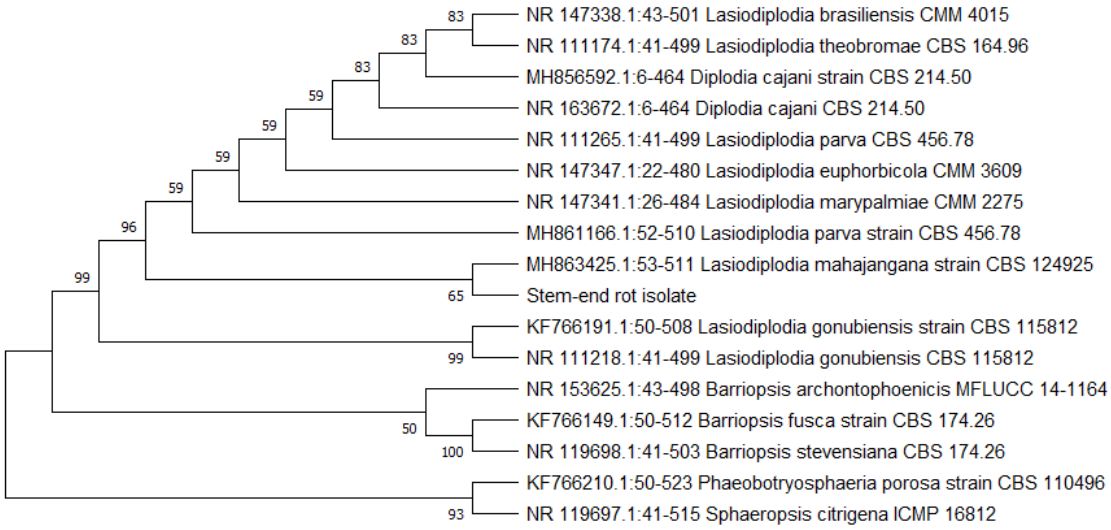

Figure 2.5 An NJ bootstrap consensus tree displaying the phylogenetic tree from the ITS nucleotide sequence of the *Lasiodiplodia sp.* isolated from avocado fruit showing stem-end rot symptoms, and type culture sequences that had high similarity to the query sequence.

The tree indicates that the isolated causal agent of stem-end rot of avocado fruit in this study was possibly related to *Lasiodiplodia mahajangana*.

2.4 Discussion

Members of the genera *Colletotrichum* and *Lasiodiplodia* are known to cause anthracnose and stem-end rot respectively in avocado fruit. As fruit ripen under postharvest conditions the levels of infectious diseases that lead to fruit rot increase (Marais, 2004). Such postharvest avocado fruit spoilage is of great concern to farmers. Therefore, the identity and properties of the microorganisms associated with fruit rot need to be elucidated to develop methods to prolong fruit freshness. This is critical to the avocado industry (Wogu, 2014).

To confirm that the two isolates from this study were the causal agents of the two major postharvest avocado diseases of interest, the Koch's postulate experiment was performed and it confirmed that the isolates obtained from the infected parts of symptomatic fruit were able to cause the disease in asymptomatic fruit, because the

symptoms in artificially inoculated fruit were identical to those observed in the naturally infected avocado fruit from which they were isolated (Ukeh and Chiejina, 2012).

The morphological characteristics of the mycelia and conidia of the two isolates were positively correlated to those characteristics of anthracnose and stem-end rot respectively. In this study the mycelia of the isolated causal agent of anthracnose were fluffy-cottony cream-white greyish to orange colonies as described by Kimaru *et al*.(2018) and Honger *et al*. (2016). The conidia of the isolated causal agent of anthracnose were rod-shaped with rounded ends that occasionally appeared oval in shape, which supported the conclusion that this was a *Colletotrichum* sp as described by Honger *et al*. (2016) who identified *Colletotrichum* species that were isolated from avocado, citrus, and pawpaw in Ghana. Furthermore, according to Sharma and Kulshrestha (2015), *C. gloeosporioides* has been reported to cause anthracnose in many different fruits and vegetables.

The mycelia of the isolated causal agent of stem-end rot appeared grey to black in colour, as described in the work by Djeugap *et al*. (2016) in the first report of *Lasiodiplodia theobromae* causing shoot blight of *Ricinodendron heudelotti* seedlings in Cameroon. Alemu (2014) also reported this morphological appearance in a study of the dynamics and management of major postharvest fungal diseases of mango fruits. The conidia of the avocado stem-end rot isolate in this work were ellipsoid to rod-like in shape, as described by Latha *et al*. (2009) in the first report of *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl causing root rot and collar rot disease of physic nut (*Jatropha curcas* L.) in India.

To narrow down the identification of the isolated causal agents of anthracnose and stem-end rot to species level, phylogenetic trees were generated to infer the probable identity of the isolates due to the similarity of their ITS region sequences to other sequences that were publicly available in Genbank. Several *Colletotrichum* species shared high ITS sequence similarity with the avocado anthracnose isolate, making it difficult to provide a definite taxonomic assignment to the isolate based on the ITS DNA sequence. However, it has been shown that sequencing of ITS regions are inadequate for identifying *Colletotrichum* to species level (Khodadadi *et al*., 2020). However, the dendrogram (Fig. 2.4) did show that the isolate clustered with *C.*

gloeosporioides. Therefore, when considering the epidemiological and morphological characteristics, it is possible that this isolate is a strain of *C. gloeosporioides.*

The NJ bootstrap consensus tree for stem-end rot (Fig. 2.5) inferred that the isolated avocado stem-end rot pathogen was a member of the genus *Lasiodiplodia*. Members of this genus can cause a variety of infections which have been reported in up to 500 different host plants found in tropical and subtropical regions (Netto *et al*., 2014). The isolate had a high sequence similarity to be *Lasiodiplodia mahajangana.* This species is commonly recognised in Madagascar as infecting *Terminalia catappa* (Begoude *et al.*, 2009; Van der Linde *et al.*, 2011). This study is possibly a first report of this species infecting avocado fruit in South Africa and requires further investigation and confirmation.

This work confirms that the anthracnose isolate was a member of the genus *Colletotrichum*, which is associated with anthracnose in other types of fruit; and that the avocado stem-end rot isolate was a member of the genus *Lasiodiplodia.*

2.5 Conclusion

The avocado anthracnose and stem-end rot isolates from South African avocados have been shown to belong to the genera typically associated with these diseases. These isolates have been confirmed to be the causal agents of the two main postharvest diseases and can be studied further to develop effective alternatives to synthetic chemical fungicides that pose a threat to human health.

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CHAPTER THREE

Assessment of a rapid hot water treatment to control postharvest anthracnose and stem-end rot of avocado fruits.

Abstract

Hot water treatment has been in practice for decades and has gained interest for the control of postharvest diseases of fresh produce. It is an affordable non-chemical control strategy to reduce levels of postharvest diseases. The aim of the study was to assess the efficacy of hot water treatment for the control of postharvest anthracnose and stem-end rot diseases of avocado fruits. Hot water treatment tests were performed on different avocado fruit cultivars in a range of temperature and time combinations. Various temperatures at varying durations were tested to determine the level of skin damage from heat treatment. Further tests were then conducted to determine the optimum temperature and time combination effective in controlling anthracnose and stem-end rot of avocado fruits and comparing this to the commercial fungicide prochloraz. The temperature range of 52°C to 58°C and exposure times of 10 to 30 seconds caused no skin damage. Rapid hot water treatment at 56°C for 10 seconds was more effective than the fungicide prochloraz at a dosage level of 550 mL per 100 L water, but less effective than 1100 mL prochloraz per 100L water. Therefore, rapid hot water treatment shows great potential as part of an integrated pest management programme against postharvest avocado fruit rot.

Keywords: *Disease control, fruit rot, physical treatment, chemical control, and avocado*.

3.1 Introduction

Systemic acquired resistance (SAR) is a valuable tool for disease control in plants. SAR occurs when host immune system biochemical reactions are triggered to naturally protect the host plant (Usall *et al*., 2016). This resistance in plants can be induced using high temperature stress to promote wound healing and the synthesis of proteins and antifungal compounds (Li *et al*., 2013; Di Francesco *et al*., 2018). High temperature stress by means of hot water treatment (HWT) is a non-chemical control strategy that can reduce levels of postharvest diseases (Mirshekari *et al*., 2012; Li *et al*., 2013). The process involves dipping fruit (or other fresh produce) in water heated to specific temperatures for certain lengths of time that have been determined to provide optimal disease control (Usall *et al*., 2016). Hot water treatments do not directly inhibit pathogens, i.e., the heat does not kill the pathogen. Instead, it has been shown that the primary effect is the induction of plant host resistance mechanisms (Palou, 2009). Another form of HWT known as rapid hot water treatment (RHWT) involves dipping fruit in water heated to the temperatures ranging between 50 - 59°C for comparatively short periods of time ranging from 10-30 seconds (Usall *et al*., 2016). This RHWT is fast, inexpensive, efficient, and easy to implement (Palou, 2009). As with HWT the heat shock induces the host immune resistance for protection against the infection (Palou, 2013) and has be shown to be effective against postharvest green mould on citrus (Abraham, 2010).

The fungicide Chronus EC45 with the active ingredient prochloraz (CAS no. 67747-09-5; N-propyl-N-[2-(2, 4,6 trichlorophenoxy) ethyl]-1H-imidazole-1-carboxamide) has been widely used to control postharvest anthracnose and stem-end rot diseases of various fruit crops including avocado (Obianom and Sivakumar, 2018). However, the use and application of prochloraz will be discontinued in 2020 due to human safety and environmental concerns (Campos-Martínez *et al*., 2016).

This work aims to show that RHWT at an optimal time and temperature combination can control anthracnose and stem-end rot diseases of avocado fruits to an economically significant level. To test for the successful disease control using RHWT, other fruit properties such as cultivar, seasonal and climate conditions, and fruit size need to be considered as they can influence levels of control (Usall et al., 2016). Therefore, seasonally available cultivars of avocado fruit obtained from different farms

across Southern Africa treated with rapid hot water at varying temperatures and durations are needed to determine the optimum temperature and time combination for disease control compared with the performance of the prochloraz-containing fungicide.

To achieve the aim of this work, the following objectives were necessary:

1-To determine the temperature and time combinations of RHWT that did not cause skin damage on avocado fresh fruits;

2- To determine the optimum temperature and time combination best for the control of anthracnose and stem-end rot diseases compared with the fungicide prochloraz.

3.2 Materials and methods

3.2.1 Skin sensitivity test of hot water treatments to determine a safe temperature x exposure time for avocado fruit

Healthy mature avocado fruit from three cultivars, i.e., 'Hass', 'Fuerte', and 'Pinkerton' were treated in a hot water bath in a series of temperature x time combinations, as follows: 20, 45, 50, 55, 60, 65, 70, 75 and 80°C (± 0.1°C) x 20, 30, 45, 60, 75, 90, 105, 120 and 180 seconds. Each treatment combination was applied to 10 avocado fruit. The treated fruits were then air dried, placed in cardboard boxes on a bench in a complete randomised design (CRD) and maintained at 25°C for seven days until they were ripe. Once ripe, the fruits were classified according to the following skin colours: (1) green, (2) green-black, (3) brown-black, and (4) black. The experiment was performed twice per treatment.

3.2.2 Hot water treatment for the control of postharvest avocado rot

Healthy (un-inoculated) mature avocado fruits (depending on the cultivars available in a season) were submitted to the following temperatures: 25 (control), 54, 56, and 58°C (± 0.1°C). For each temperature, the avocado fruit were exposed for the following period of time: 0 (control), 10, 15, 20, and 30 seconds. Each treatment was applied to 25 avocado fruits and replicated four times. The treated fruit were then air dried, placed in cardboard boxes on a bench in a CRD at 25°C until they were ripe. After storage to ripeness stage, the fruits were classified as follows: A = healthy avocado

fruits (0% infection); B = approximately 90% of the fruit flesh is edible, with no bitter smell (10% infection); and C= the fruit is rotten, smells and is not edible (100% infection). The experiment was conducted twice.

3.2.3 Testing the best rapid hot water treatment compared with prochloraz treatments

The best performing temperature and time combination treatment for RHWT (56°C X 10 seconds) was compared with full strength prochloraz (1100 mL per 100 L water), half strength prochloraz (550 mL per 100 L water), and a control (no RHWT or fungicide) treatments were applied to 25 avocado fruit, repeated four times. The treated fruit were then air dried, placed in cardboard boxes on a bench in a CRD design at 25°C until they were ripe. After storage to ripeness stage, the fruits were classified as follows: A = healthy avocado fruits (0% infection); B= approximately 70% of the fruit flesh is edible, with no bitter smell (30% infection); and C= the fruit is rotten, smells and is not edible (100% infection). The experiment was conducted twice.

3.2.4 Statistical analysis

The data were subjected to analysis of variance (ANOVA) using the agricolae package in the R Statistical Analysis Software (version 3.6.1) (de Mendiburu, and de Mendiburu, 2020; R Core Team, 2013) to determine differences between treatments. Fisher's Least Significant Difference Test was used for treatment means separations ($P < 0.05$).

3.3 Results

3.3.1 Hot water treatments for a safe temperature and exposure time for fresh avocado fruits

Hot water treated fruit showed skin colour changes depending on temperature and time combinations. Fruit that remained green showed no skin damage and were deemed to be suitable for consumption. Fruit that were green-black showed slight skin damage and were deemed unacceptable for commercial sale. Brown-black fruits were skin-damaged, as were those that were black in colour, and were considered unsuitable for sale or consumption, because at the stage of ripeness for the majority of the fruit, all the brown-black and black fruit were both skin-damaged and rotten. The

results for the skin sensitivity test for HWT of various avocado fruit cultivars at different temperature and time combinations are summarized in Figure 3.1.

	20s	30s	45s	60s	75s	90s	105s	120s	180s
45c	Green	Green	Green	Green	Green	Green	Green	Green	Green
50c	Green	Green	Green	Green	Green	Green	Green	Green	Green
55c	Green	Green	Green	Green	Green	Green	Green	Green	Green
60c	Green	Green	Green-Black	Green-Black	Green-Black	Green-Black	Green-Black	Black	Black
65c	Black	Black	Black	Black	Black	Black	Black	Black	Black
70c	Black	Black	Black	Black	Black	Black	Black	Black	Black
75c	Black	Black	Black	Black	Black	Black	Black	Black	Black
80c	Black	Black	Black	Black	Black	Black	Black	Black	Black
Control	Green	Green	Green	Green	Green	Green	Green	Green	Green
(a)									

	20s	30s	45s	60s	75s	90s	105s	120s	180s
45c	Green	Green	Green	Green	Green	Green	Green	Green	Green
50c	Green	Green	Green	Green	Green	Green	Green	Green	Green
55c	Green	Green	Green	Green	Green	Green-Black	Green-Black	Green-Black	Green-Black
60c	Green	Green	Green	Green	Green	Green-Black	Green-Black	Green-Black	Green-Black
65c	Green-Black	Green-Black	Brown-Black	Brown-Black	Brown-Black	Brown-Black	Brown-Black	Brown-Black	Brown-Black
70c	Brown-Black	Brown-Black	Brown-Black	Brown-Black	Brown-Black	Brown-Black	Brown-Black	Brown-Black	Brown-Black
75c	Brown-Black	Brown-Black	Brown-Black	Brown-Black	Brown-Black	Brown-Black	Brown-Black	Brown-Black	Brown-Black
80c	Brown-Black	Brown-Black	Brown-Black	Brown-Black	Brown-Black	Brown-Black	Brown-Black	Brown-Black	Brown-Black
Control	Green	Green	Green	Green	Green	Green	Green	Green	Green
(b)									

	20s	30s	45s	60s	75s	90s	105s	120s	180s
45c	Green	Green	Green	Green	Green	Green	Green	Green	Green
50c	Green	Green	Green	Green	Green	Green	Green	Green	Green
55c	Green	Green	Green	Green	Green	Green	Green	Green	Green
60c	Green	Green	Green	Green	Green	Green	Green	Green-Black	Brown-Black
65c	Green	Green	Green-Black	Green-Black	Green-Black	Green-Black	Brown-Black	Brown-Black	Brown-Black
70c	Brown-Black	Brown-Black	Brown-Black	Brown-Black	Brown-Black	Brown-Black	Brown-Black	Brown-Black	Brown-Black
75c	Brown-Black	Brown-Black	Brown-Black	Brown-Black	Brown-Black	Brown-Black	Brown-Black	Brown-Black	Brown-Black
80c	Brown-Black	Brown-Black	Brown-Black	Brown-Black	Brown-Black	Brown-Black	Brown-Black	Brown-Black	Brown-Black
Control	Green	Green	Green	Green	Green	Green	Green	Green	Green
(C)									

Figure 3.1 Skin sensitivity test results for the HWT at different temperature and time combination of avocado cultivars: (a) ‘Hass’, (b) ‘Fuerte’, and (c) ‘Pinkerton’.

For optimum disease control, temperature and time combination of between 52°C to 58°C, combined with exposure time of 10 to 30 seconds were selected due to their ability to cause less or no skin damage from hot water treatment heat. Fruit treated under these parameters and conditions were successful in inducing disease control equivalent to RHWT in addition to most avocado cultivars.

3.3.2 Hot water treatment for the control of postharvest avocado rot

The efficacy of RHWT treatments at 52, 54, 56 and 58°C, combined with exposure time of 10, 15, 20 and 30 seconds were tested to determine the optimal temperature and time combination for the control of postharvest anthracnose and stem-end rot of different healthy avocado fruit cultivars. The data for the Hass cultivar is summarised in Table 3.1, which shows the mean percentage of healthy (Class A) fruit for each of the treatment combination replicates.

Table 3.1 Percentage of healthy Hass fruit, free from avocado rot, after rapid hot water treatment. Values with the same letters were not significantly different.

Treatments	Mean percentage of healthy fruits
T56 x t10	67 a
T52 x t20	66 a
T58 x t10	63 a
T54 x t15	54 ab
T56 x t15	50 abc
T54 x t20	48 abc
T52 x t30	38 bc
control	34 c
P-value	0.01174
F-value	3.3849
LSD-value	19.74235
CV%- value	25.76714

T = (temperature °C)

t = time (seconds)

Rapid hot water treatment at 56°C for 10 seconds was the best treatment providing control against avocado rot in Hass avocado fruits. This was followed by treatment at

52°C for 20 seconds and 58°C for 10 seconds. The untreated control had the lowest levels of healthy and edible fruits.

The results for the Gem cultivar avocado fruit is summarized in Table 3.2 below.

Table 3.2 Percentage of healthy Gem fruit, free from avocado rot, after rapid hot water treatment. Values with the same letters are not significantly different.

Treatments	Mean percentage of healthy fruits
T56 x t10	67 a
T54 x t20	58 a
T56 x t15	58 a
T58 x t10	54 a
T54 x t15	49 a
T52 x t20	46 a
T52 x t30	42 a
Control	41 a
P-value	0.4701
F-value	0.9772
LSD-value	26.57144
CV%- value	35.0981

T = (temperature °C)

t = time (seconds)

While there were no significant differences among the treatments applied to Gem avocado fruit, a greater number of healthy fruit were also obtained after fruit were treated at 56°C for 10 seconds. The lowest number of healthy fruit were seen in the untreated control.

The data for the Fuerte avocado cultivar is summarised in Table 3.3.

Table 3.3 Mean percentage of healthy Fuerte fruit, free from avocado rot, after rapid hot water treatment. Values with the same letters are not significantly different.

Treatments	Mean percentage of healthy fruits
T54 x t15	71 a
T52 x t10	64 a
T52 x t20	61 a
T54 x t10	61 a
T54 x t20	59 a
T50 x t10	58 a
T50 x t20	55 ab
T50 x t15	52 ab
T52 x t15	52 ab
Control	36 b
P-value	0.07942
F-value	1.9693
LSD-value	19.12189
CV%- value	23.27126

T = (temperature °C)

t = time (seconds)

The highest level of disease control was achieved from exposing fruits to 54°C for 15 seconds of RHWT. The second best treatment was 54°C for 15 seconds, while the least disease control was seen in the untreated control.

Because it was the most common best-performing treatment combination, the 56°C for 10 seconds was decided upon as the optimal combination for further experiments with RHWT.

3.3.3 Comparison of best rapid hot water treatment with prochloraz treatments for the control of avocado rot

The data from the experiment comparing the efficacy of RHWT with the fungicide prochloraz at 1100 mL per 100 L water and at 550 mL per 100 L and an untreated control for the Hass avocado cultivar is summarized in Table 3.4.

Table 3.4 Efficacy of best temperature and time combination compared to prochloraz treatments for the Hass cultivar. Mean values with the same letters are not significantly different.

Treatments	Percentage of healthy fruits
Full strength prochloraz	91 a
T56 x t10	89 a
Half strength prochloraz	75 b
Control	71 b
P-value	4.303e-06
F-value	33.222
LSD-value	5.33698
CV%- value	4.250431

T = (temperature °C)

t = time (seconds)

Full strength prochloraz at 1100 mL per 100 L water

Hall strength prochloraz at 550 mL per 100 L water

The fungicide prochloraz at 1100ml per 100L water provided the best control, followed by the rapid hot water treatment at 56°C x 10 seconds. However, there was no significant difference between the performance of these two treatments. Both treatments performed significantly better than the half strength prochloraz and untreated control. There was no significant difference between the latter.

3.4 Discussion

Rapid hot water treatment can be an environmentally friendly alternate control strategy towards postharvest anthracnose and stem-end rot of avocado fresh fruits (Li, *et al*. 2013). This is evident from the results obtained in this work.

Avocado fruits are often cooled to 16°C after harvest (Bill, *et al.* 2014) to delay the ripening rate of the fruits because ripening at higher temperatures can lead to darkening of the fruit skin and subsequent spoilage (Terander, 2002). Therefore it was necessary to check the effect of high temperatures on the fruit skin to limit heat phytotoxicity of hot water treatment (Palou, *et al.* 2008). However, it was possible to conclude that, as demonstrated by Abraham (2010), exposing fruits to higher hot water treatment temperatures and time periods was detrimental to fruit quality, there was a range of temperatures and durations, i.e. 52 to 58 °C, that did not damage the fruit and which induced a host immune reaction from the fruit.

Rapid hot water treatment optimisation against anthracnose and stem-end rot of climacteric avocado fruit cultivars (Bill, *et al*. 2014), were effective at 56°C for 10 seconds for most avocado fruit cultivars. These findings were in agreement with the findings of Sui *et al*. (2016) and Usall, *et al*. (2016). Similarly, treatment of fresh produce in a packing line machinery at 55–65ºC for 10–30 seconds with rotating brushes has shown good results in controlling postharvest spoilage (Palou, *et al.*, 2008). This work also shows that treating fruit at certain temperatures for short time periods is fast, safe, cheap, and effective in controlling postharvest disease.

Avocado fruit treatment at 56°C for 10 seconds provided a level of control that was slightly lower compared with the full strength prochloraz treatment commonly used to treat postharvest avocado effectively. However, both treatments were significantly better than the half strength treatment, which means that the reduction of fungicide strength to meet export regulation rules will reduce the levels of fruit quality, as explained by Daneel *et al*. (2016). Therefore, to meet the demand in the avocado industry for fungicide free fruit, alternate control measures should be implemented as suggested by Li *et al*. (2013) and Usall *et al*. (2016). The results of this work show that this is possible using rapid hot water treatment.

Further work is required to develop automated machinery that can be used at pack houses for the commercial implementation of the rHWT technology, adopting a route that has already been taken by Israelis farmers (Palou, 2013).

Future studies will involve elucidating on the biochemical reactions that occurs when heat induces systemically acquired resistance in a host. However, in the meantime, rapid hot water treatment can be implemented to conform to the rules, regulations, and restrictions pertaining towards the application of synthetic fungicides for the control of postharvest avocado rot.

3.5 Conclusion

Rapid hot water treatment is a viable alternative to fungicide application for the control of fungal disease in postharvest avocado fruit. This can be implemented as part of an integrated pest management strategy along with other safe measures such as improved cultivation practices as well as biological control of diseases of pre-harvest and postharvest avocado fruit.

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CHAPTER FOUR

Biocontrol yeasts as promising control of postharvest anthracnose and stem-end rot diseases of avocado fresh fruits

Abstract

Since the first application of biological control agents (BCAs) extensive studies have been conducted on yeasts as potential antagonists against plant pathogens. Having demonstrated success in disease control due to their various physiological properties, yeast antagonists are considered to have potential as environmentally friendly alternatives to chemical fungicides in the control of avocado postharvest diseases. This study investigated the efficacy of yeast antagonists in the control of anthracnose and stem-end rot diseases of postharvest avocado fruit. Yeasts were isolated from fruit surfaces and then screened for antagonism towards anthracnose and stem-end rot disease of avocado. Selected isolates were further assessed for their control efficacy compared with a commercial yeast BCA and the fungicide prochloraz. The commercial yeast BCA provided the best control against both anthracnose and stem-end rot compared to the other yeasts isolates. However, prochloraz (1100ml per 100L of water) provided superior control of both diseases compared to all other experimental treatments. For effective control comparable of anthracnose and stem-end rot of avocado fruit an integrated pest management strategy combining both BCAs and other non-fungicidal treatments needs to be considered.

Keywords: *yeast antagonist, prochloraz, avocado rot, and postharvest diseases.*

4.1 Introduction

Avocado *(Persea americana* Mill.) fruit has a very short shelf-life. Ripe fruit can on average be stored for 3-6 days before spoilage leads to considerable losses. This is a challenge for the export market that requires prolonged periods of storage (Wogu and Ighile, 2014). When avocado fruit quality deteriorates, it is almost always due to fungal fruit rots (Everett, 2002). Avocado postharvest diseases occur as latent infections and secondary infections associated with stem-end rot disease. These often enter the fruit through wounds and natural openings to cause fungal rot symptoms (Alemu, 2014). Due to the shift away from the application of synthetic fungicide due to human health concerns and resistance build-up, there is an increasing interest in the application of yeast antagonists as biological control agents against postharvest avocado diseases (Zhang *et al*., 2018).

Biological control occurs when the presence and activity of a pathogen is prevented or inhibited by other naturally occurring or introduced organisms (Irtwange, 2006). It has been applied extensively and successfully for decades with promising potential for fruit disease control, especially in the form of yeast antagonists of disease organisms (Abraham, 2010; Singh and Sharma, 2018). Prior research in the development of a commercial yeast BCA, known as “B13” for the control of postharvest green mould of citrus and litchi has been successful (Abraham, 2010).

Yeasts are safe to use, low cost and easy to formulate. In addition, they can tolerate most agrochemical applications (Dukare *et al*., 2019). Yeast antagonist mechanisms can be direct antagonism (hyperparasitism) and indirect antagonism (competition and or induction of host resistance) as preventative modes of action against future fungal pathogen infections (Pal and Gardener, 2006). Hypothetically, yeast BCAs can effectively control postharvest diseases of avocado fruits anthracnose and stem-end rot diseases. Such yeast antagonists would be of significant commercial value in the postharvest industry (Asio and Cuaresma, 2016).

The aim of this study was to investigate the ability of yeast isolates to inhibit the causal agents of anthracnose and stem-end rot disease in avocado and compare their efficacy with a commercial yeast BCA and a commonly applied chemical fungicide, i.e., prochloraz.

The aims were to be achieved by the following objectives:

1- To isolate and screen yeasts antagonists against the causal agents of anthracnose and stem-end rot in postharvest avocado fruits;

2- To conduct *in vivo* trials to compare the efficacy of selected yeasts isolates with a commercial yeast BCA and the fungicide prochloraz for the control of anthracnose and stem-end rot diseases of postharvest avocado fruit.

4.2 Materials and methods

4.2.1 Pathogen isolates

Previously isolated and identified pathogenic strains the causal agents of anthracnose and stem-end rot of avocados were used for the trials in this work.

4.2.2 Yeast antagonists sample preparation and isolation

Undamaged avocado fruits harvested from commercial orchards and home gardens in KwaZulu-Natal and Limpopo, South Africa, were rinsed with distilled water in order to wash off any potential yeasts antagonists on fruit surfaces. The rinsate was collected and serially diluted (10 fold dilution series prepared up to 10^{-4}) and plated onto (PDA) Merck, Germany) supplemented with 5 mg L^{-1} of chloramphenicol Merck, Germany) to discourage possible bacterial growth, and then incubated for three days at 28°C. Yeasts colonies were visually identified and selected based on their typical colony morphology, and further confirmed microscopically by cell size, shape, and bud formation. Over 100 yeast culture isolates were screened for their ability control of *Colletotrichum spp.* and *Lasiodiplodia spp.* This was conducted *in vitro* on malt extract agar (Merck, Germany) petri dish plates with malt extract agar (Merck, Germany) supplemented with 5mg/L of chloramphenicol (Merck, Germany) to test the growth inhibition of the pathogens by the yeast isolates.

4.2.3 Preventative action of selected yeasts isolates antagonistic to *Colletotrichum spp.* and *Lasiodiplodia spp.* on avocado fresh fruits

The following was done to compare the preventative action of the isolated yeasts with yeast B13, full strength prochloraz (1100ml per 100L of water), and half strength prochloraz (550ml per 100L of water):

For each treatment, four replicates of 25 avocado fruit each were surface sterilized with 70% alcohol and allowed to dry. For stem-end rot experiments, wounds were created at the stem-end of the fruit with a sterile scalpel, and for anthracnose experiments, wounds were created equatorially on the fruit, also with a sterile scalpel. Wounded fruit were sprayed with a standardized suspension of yeast cells (1×10^8 cells mL^{-1}) to cover the entire fruit, allowed to dry for three hours and then sprayed separately with a standardized conidial suspension for anthracnose and stem-end rot at 1×10^4 conidia mL^{-1}. Suspensions had been quantified using a haemocytometer and adjusted to the final concentrations by dilution. Control fruits were treated with sterile distilled water. Treatments were placed in trays arranged in a complete randomized design (CRD) at 25°C until they were ripened. After storage to the ripening stage, the fruits were evaluated using subjective scale: A= healthy avocado fruits (0% infection); B= approximately 90% of the fruit flesh is edible, with no bitter smell (10% infection); and C= the fruit is rotten, smells bitter, and is not edible (100% infection). The experiment was conducted twice.

4.2.4 Statistical analysis

The data were subjected to analysis of variance (ANOVA) using the agricolae package in the R Statistical Analysis Software (version 3.6.1) (R Core Team, 2013; de Mendiburu, and de Mendiburu, 2020) to determine differences between treatments. Fisher's Least Significant Difference Test was used for treatment means separations ($P < 0.05$).

4.3 Results

4.3.1 Isolation of yeast antagonists against postharvest avocado rot

Isolated yeasts that showed antagonism towards the isolated species of the genus *Colletotrichum* and *Lasiodiplodia* respectively from postharvest avocado, as well as yeast B13 are shown in figure 4.1 below.

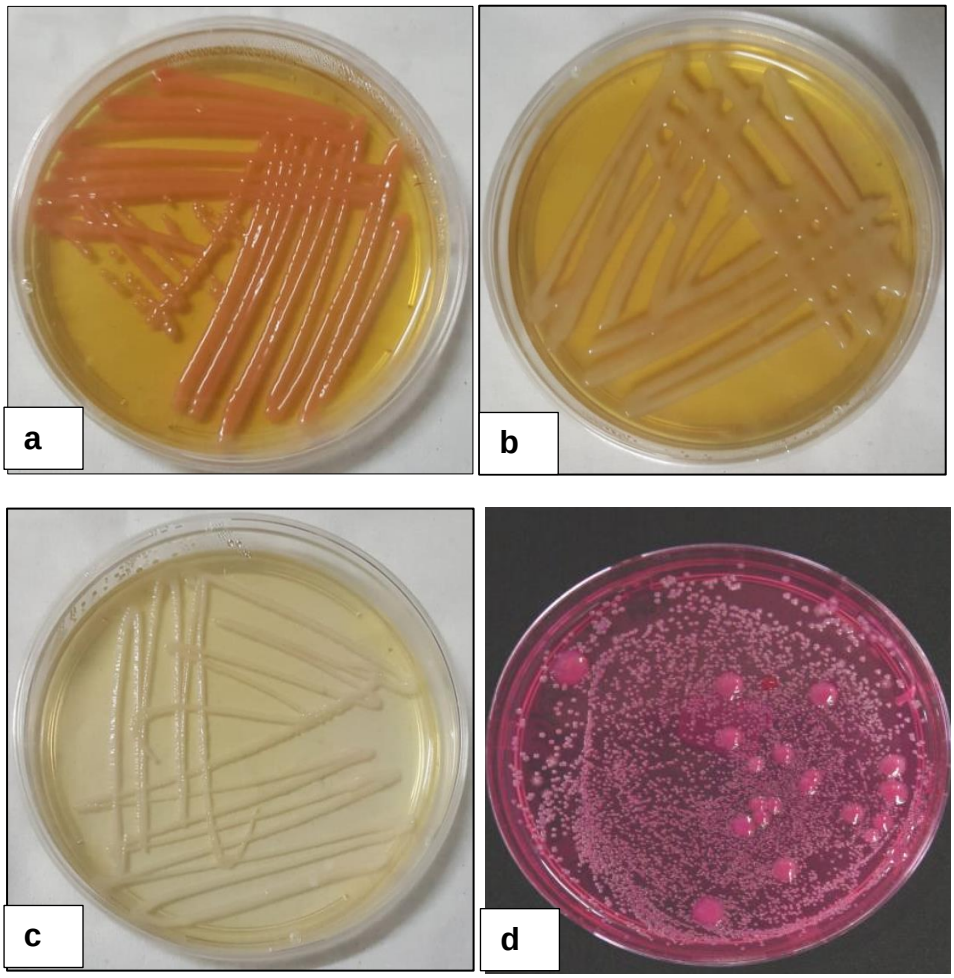

Figure 4.1 Plate cultures of yeasts isolates (a) Yeast B; (b) Yeast I; (c) Yeast J, isolated from avocado fruit surfaces for their antagonistic effect against the isolated fungal pathogens of the genus *Colletotrichum* and *Lasiodiplodia* of avocado (images taken by T.F., Majola); and (d) B13, yeast isolated on PDA media amended with Rose Bengal after three days of incubation at 25°C (image by Abraham, 2010).

4.3.2 Yeast antagonists for the control postharvest avocado rot

The antagonistic action performance of the biological control yeast antagonists against postharvest avocado rot caused by anthracnose and stem-end rot tested on Hass cultivars is summarised in Table 4.1.

Table 4.1 The mean percentage of healthy fruit free from anthracnose after biological yeast treatment compared with B13 and prochloraz treatments. Means with the same letters are not significantly different.

Treatments	Percentage of healthy fruits
Full strength prochloraz	74 a
Yeast I	72 a
Half strength prochloraz	68 ab
Yeast B	66 ab
Yeast B13	62 ab
Yeast J	59 ab
Control	49 b
P- value	0.184
F- value	1.6462
LSD- value	19.57174
CV%- value	20.70367

Full strength prochloraz at 1100 mL per 100 L water

Hall strength prochloraz at 550 mL per 100 L water

All treatments were significantly different from the control treatment.

The performance of the yeast isolates compared with yeast B13 and the full and half strength prochloraz treatments are shown in table 4.2.

Table 4.2 The mean percentage of healthy fruit free from stem-end rot after biological yeast treatment compared with B13 and prochloraz treatments. Means with the same letters are not significantly different.

Treatments	Percentage of healthy fruits
Full strength prochloraz	68 a
Half strength prochloraz	64 ab
Yeast B13	59 ab
Yeast B	55 ab
Yeast I	54 ab
Yeast J	50 ab
Control	47 b
P- value	0.2624
F- value	1.396
LSD- value	18.6117
CV%- value	22.31649

Full strength prochloraz at 1100 mL per 100 L water

Hall strength prochloraz at 550 mL per 100 L water

The full strength prochloraz treatment provided the best control against stem-end rot. Yeast B13 was the most effective BCA against stem-end rot, all of the BCA treatments were significantly more effective than the control.

4.4 Discussion

Biological control yeast antagonists can provide control against the major postharvest diseases of avocado fresh fruits, i.e. anthracnose and stem-end rot (Janisiewicz and Korsten, 2002; Sharma *et al.,* 2009). However, for a biological control to be approved for commercial application it must be as effective, if not better than the existing fungicide (Korsten *et al.,* 1991). In our study, the full-strength prochloraz fungicide provided the best control for both anthracnose and stem-end rot diseases of avocado fresh fruits as compared with the BCAs, which was expected, because it is still commonly used to protect postharvest avocados against these diseases.

While the yeast antagonists did not provide better control than the fungicide prochloraz, they were effective when compared with the control. When this is weighed against the health concerns of prochloraz, it is evident that these BCAs are the next best alternate control to fungicides since they provide least to none harm to the environment and humans (Parafati *et al*. 2015). This study showed that these BCAs have great potential, because they have the ability to grow rapidly and colonize surfaces with simple nutritional requirements. This is a requirement for a yeast antagonist BCA to be considered useful for postharvest application (Dukare *et al*., 2019). Other examples of successful yeast antagonists in the postharvest industry such as *Cryptococcus albidus* (Saito) C.E. Skinner., for the control of postharvest diseases caused by *Botrytis, Penicillium*, and *Mucor* on pome fruits (Spadaro and Droby, 2016); and yeast isolate B13 for the control of postharvest green mould of citrus and litchi (Abraham, 2010) can be tested more extensively and in combination with other non-chemical treatments to try and replace prochloraz as the primary strategy for protecting avocados from postharvest diseases.

While the yeasts isolated in this study were not able to perform better than prochloraz, the Yeast I isolate provided significant and useful control against anthracnose. While this demonstrates the great potential of such easily isolated yeasts, the effectiveness against stem-end rot was inadequate. Despite the fact that isolated yeast antagonists provided more than 50% of healthy/edible fruits from individual yeast isolates treatments, an ideal yeast BCA needs to be effective against a wide spectrum of pathogens (Mbili, 2012; Asio and Cuaresma, 2016).

Interestingly, the commercial yeast isolate B13 successfully controlled both diseases at a similar level demonstrated for citrus and litchis by Abraham (2010). This yeast isolate B13 is effective at low concentrations, it is compatible with commercial manufacturing procedures, thus currently manufactured by the plant protection company (Plant Health Protection) in Howick, Pietermaritzburg-South Africa (Abraham, 2010). It is an example of a BCA against which new isolates can be benchmarked in future studies.

In this work, the results showed that stem-end rot was more damaging compared with anthracnose. However, according to the literature, anthracnose is responsible for more crop and fruit losses than stem-end rot (Marais, 2004; Demoz, 2005; Djeugap *et al*., 2015; Tesfay *et al*., 2017; Xoca-Orozco *et al*., 2017; Fischer *et al*., 2018; Obianom and Sivakumar, 2018). The discrepancy between the literature and data obtained in this work may be due to a) the pathogenicity of the strains that were isolated, especially since stem-end rot species can differ geographically; b) the presence or amount of disease inoculum already on the avocados prior to experimental inoculation; and c) bias in the experimental rating scale, since fruits with a bitter smell but only traces of spoilage were classified as inedible, and stem-end rot is often associated with a bad smell.

This work shows that BCAs, and yeast antagonists, in particular, can inhibit postharvest anthracnose and stem-end rot in avocados. These findings support efforts to advance the field of biological control, particularly in the avocado industry, by suggesting yeast antagonists as alternative BCAs to bacteria and mycelial fungi. In addition to testing new yeast isolates, future studies can involve testing yeast B13 against more avocado postharvest disease pathogen strains to determine its ability to control a wider range of pathogenic isolates in order to fulfil the stipulated requirements regarding the safety and efficacy of yeasts as bio-control agents for commercial application.

4.5 Conclusion

While yeast antagonists may not currently demonstrate the level of control provided by synthetic fungicides, they show great potential as alternative and reliable measures for the control of postharvest anthracnose and stem-end rot diseases of avocado. These yeasts antagonists can be applied as part of an integrated pest management strategy in addition to other control strategies for the safe and effective control of postharvest diseases of avocado.

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CHAPTER FIVE

Efficacy of rapid hot water treatment and biological yeast antagonist for the control of avocado anthracnose and stem-end rot diseases

Abstract

The replacement of chemical fungicides by integrated disease control strategies is an attractive option for crop production. This approach has been developing over many decades and has been refined for specific crops, types of diseases, and for specific environmental factors. This study aimed to develop an integrated control strategy that provided acceptable levels of disease control of anthracnose and stem-end rot in postharvest avocado, in comparison with the fungicide prochloraz. Rapid hot water treatment of 56°C for 10 seconds has a significant effect when integrated with different biological control yeast antagonists (I; J; B; and B13). The integration of rapid hot water treatment at 56°C for 10 seconds combined with yeast isolate B13 provided the same or better control of avocado anthracnose and stem-end rot diseases when compared with full strength prochloraz. A protocol that considers the factors that affect the crop and pathogen of interest, combined with an integrated disease control method as was developed in this work, has great potential for future agricultural disease management.

Keywords: *integrated disease management; avocado rot, prochloraz, bio-control; hot water treatment.*

5.1 Introduction

According to the Food and Agriculture Organisation of the United Nations, integrated pest management control is the application of pest control techniques to discourage pest population development through economically and ecologically justifiable interventions (FAO, 2017). Due to the rise in the global human population and the increasing need for improved health through healthy eating, this means that farmers should increase yields on existing land while maintaining, protecting biodiversity, and limiting the use of toxic pesticides. Integrated disease management strategy helps farmers in maximizing production while minimizing yield losses, while reducing or eliminating the need for dangerous chemicals. The benefits of integrated pest managements strategies includes the production of consistent crops and yields, improved consumer confidence in terms of the safety and quality of food products, and an increased market share (Wassermann *et al*., 2019) for farmers.

Integrated disease management strategies have been practised by commercial farmers for several decades. However, these strategies have recently gained more attention due to the emergence of precision farming as well as an increasing demand for the reduction or discontinuation of synthetic pesticides applications (Wardlow. and O'Neill, 1992). Understanding the interactions between crops, pests, and the environment is crucial for developing an integrated disease management strategy, because disease cycles often have different life stages which require the responsive modification of the integrated disease management strategies (Ehi-Eromosele *et al*., 2013). Promising disease control levels have been achieved through applications of physical and biological control treatments in several crops (Asio and Cuaresma, 2016).

A viable integrated control strategy for avocado postharvest industry can involve the application of physical treatment combined biological control treatments. Due to the latent nature of anthracnose and stem-end rot diseases of avocado, rapid hot water treatment (RHWT) is a physical treatment that can be effective because it triggers the host immune system (Palou, 2009) and biological control yeast antagonist can protect the fruit against future infections (Zhang *et al*., 2018). Often physical and biological control treatments individually do not provide acceptable control levels when compared to the application of synthetic fungicides (Zhang *et al*., 2008). Biological control often poses limitations since their mode of action depends on environmental

factors, thereby failing to provide persistent control (Usall *et al*., 2015). Several studies have been conducted to improve the efficacy of bio-control antagonists by combining them with hot water treatments.

The application of RHWT followed by the biological control yeast antagonists can provide levels of control acceptable for commercial disease management. The aim of this work was to determine the optimum temperature and time combinations for treating avocados, combined with the application of yeast isolates, to provide effective control of the postharvest diseases of avocado, anthracnose and stem-end rot, as an alternative control to the fungicide prochloraz.

5.2 Materials and Methods

5.2.1 Pathogen isolates

Previously isolated and identified pathogenic strains the causal agents of anthracnose and stem-end rot of avocados were used for the trials in this work.

5.2.2 Hot water treatments to determine the optimum temperature and exposure time for avocado fruits

This experiment was performed to determine what the optimal temperatures and duration of exposure were that would not result in damage to the fruit caused by the treatment itself. Healthy mature avocado fruits of three cultivars, i.e., 'Hass', 'Fuerte', and 'Pinkerton' were treated in a hot water bath with in a series of temperature x time combinations, i.e., 20, 45, 50, 55, 60, 65, 70, 75 and 80°C (±0.1°C) for 20, 30, 45, 60, 75, 90, 105, 120 and 180 seconds. Each treatment was performed on 10 avocado fruits per replicate. The treated fruits were then air-dried, placed in carboard boxes on a bench in a complete randomised design (CRD) maintained at 25°C for seven days until they ripened. At the ripening stage, the fruits were assessed using a classification system based on the skin colour change: (1) green, (2) green-black, (3) brown-black, and (4) black.

5.2.3 Hot water treatment for the control of postharvest avocado rot

After determining the safest time and temperature combinations that would not damage the fruit (results not shown), an experiment was performed to determine the effect of selected time and temperature combinations of the development of anthracnose and stem-end rot in avocados Healthy mature avocado fruits were tested at the following temperatures: a room temperature control at ± 25, and controlled temperatures of 25, 54, 56, and 58°C ±0.1°C. For each temperature, the avocado fruits were exposed for a period of 0 (control), 10, 15, 20, and 30 seconds. Each treatment was applied to 25 avocado fruits per replicate with four replicates per treatment. After treatment, the fruits were air-dried, and then placed in cardboard boxes on a bench in a CRD at 25°C until they were ripe. After storage to the ripening stage, the fruits were rated according to the following scale: A= healthy avocado fruits (0% infection); B= approximately 90% of the fruit flesh is edible with no bitter smell (10% infection); and C= the fruit is rotten, smells bad, and is not edible (100% infection). The experiment was conducted twice.

5.2.4 Yeast antagonists sample preparation and isolation

Undamaged avocado fruits harvested from commercial orchards and home gardens in KwaZulu-Natal and Limpopo, South Africa, were rinsed with distilled water in order to wash off any potential yeasts antagonists on fruit surfaces. The rinsate was collected and serially diluted (10 fold dilution series prepared up to 10^{-4}) and plated onto (PDA) Merck, Germany) supplemented with 5 mg L^{-1} of chloramphenicol Merck, Germany) to discourage possible bacterial growth, and then incubated for three days at 28°C. Yeasts colonies were visually identified and selected based on their typical colony morphology, and further confirmed microscopically by cell size, shape, and bud formation. Over 100 yeast culture isolates were screened for their ability control of *Colletotrichum spp.* and *Lasiodiplodia spp.* This was conducted *in vitro* on malt extract agar (Merck, Germany) petri dish plates with malt extract agar (Merck, Germany) supplemented with 5mg/L of chloramphenicol (Merck, Germany) to test the growth inhibition of the pathogens by the yeast isolates.

5.2.4 Preventative action of selected yeasts isolates antagonistic to *Colletotrichum spp.* and *Lasiodiplodia spp.* on avocado fresh fruits

For each treatment, four replicates of 25 avocado fruit each were surface sterilized with 70% alcohol and allowed to dry. For stem-end rot experiments, wounds were created at the stem-end of the fruit with a sterile scalpel, and for anthracnose experiments, wounds were created equatorially on the fruit, also with a sterile scalpel. Wounded fruit were sprayed with a standardized suspension of yeast cells (1×10^8 cells mL^{-1}) to cover the entire fruit, allowed to dry for three hours and then sprayed separately with a standardized conidial suspension for anthracnose and stem-end rot at 1×10^4 conidia mL^{-1}. Suspensions had been quantified using a haemocytometer and adjusted to the final concentrations by dilution. Control fruits were treated with sterile distilled water. Treatments were placed in trays arranged in a complete randomized design (CRD) at 25°C until they were ripened. After storage to the ripening stage, the fruits were classified using subjective scale: A= healthy avocado fruits (0% infection); B= approximately 90% of the fruit flesh is edible, with no bitter smell (10% infection); and C= the fruit is rotten, smells bitter, and is not edible (100% infection). The experiment was conducted twice.

5.2.5 Integration of best rapid hot water treatment with best yeast antagonists for the control of anthracnose and stem-end rot diseases

The following was done to compare the preventative action of the isolated yeasts with yeast B13, full strength prochloraz (1100ml per 100L of water), and half strength prochloraz (550ml per 100L of water):

A set 25 healthy mature avocado fruits with four replicates were hot water treated for 10 seconds at 56°C and air-dried at room temperature for one hour. For stem-end rot, hot water treated fruits were wounded at stem-end of the fruit with a sterile scalpel, and for anthracnose, hot water treated fruits were wounded equatorially on the fruit. Wounded fruits were sprayed with a suspension of yeast cells (1×10^8 cells mL^{-1}) and allowed to dry for three hours and later sprayed with a suspension of anthracnose and or stem-end rot at a concentration of 1×10^4 conidia mL^{-1}. Treated fruit were placed in a compete randomized design (CRD) at 25°C until they were ripe. After storage to ripeness, the fruits were classified using the subjective scale: A = healthy avocado

fruits (no infection); B = approximately 90% of the fruit flesh is edible with no bitter smell (10% infection); and C = the fruit is rotten, smells and is not edible. The experiment was conducted twice.

5.2.5 Statistical analysis

The data were subjected to analysis of variance (ANOVA) using the agricolae package in the R Statistical Analysis Software (version 3.6.1) (R Core Team, 2013; de Mendiburu, and de Mendiburu, 2020) to determine differences between treatments. Fisher's Least Significant Difference Test was used for treatment means separations ($P < 0.05$).

5.3 Results

5.3.1 Integration of best rapid hot water treatment with best yeast antagonists for the control of anthracnose and stem-end rot diseases

Results of the control levels provided by the integrated treatments: rapid hot water and yeast antagonists compared to the fungicide prochloraz for the control of postharvest anthracnose of avocado fresh fruits are summarised in Table 5.1 below.

Table 5.1 Mean percentage of healthy fruit after the integrated treatment against anthracnose disease. The RHWT was at 56°C for 10 seconds. Mean values with the same letters are not significantly different.

Treatments	Percentage of healthy fruits
Yeast I+RHWT	81 [a]
Yeast B+RHWT	78 [ab]
Yeast B13+RHWT	76 [ab]
Yeast J+RHWT	74 [ab]
Full-strength prochloraz	73 [ab]
Half-strength prochloraz	70 [b]
Control	60 [c]
P- value	0.006
F- value	4.29
LSD- value	9.65
CV %	8.97

Full strength prochloraz at 1100 mL per 100 L water

Hall strength prochloraz at 550 mL per 100 L water

The best control of avocado anthracnose disease was obtained from a combination of Yeast I and RWHT. The least effective treatment was the half-strength prochloraz fungicide.

Results of the control action provided by the integrated treatments: rapid hot water and yeast antagonists compared to the fungicide prochloraz for the control of postharvest stem-end rot of avocado fresh fruits are summarised in Table 5.2 below.

Table 5.2 Mean percentage of healthy fruits from integrated control against stem-end rot of avocado. The RHWT was at 56°C for 10 seconds. Means with the same letters do not differ significantly.

Treatments	Percentage of healthy fruits
B13+RHWT	74 [a]
B+RHWT	72 [ab]
I+RHWT	72 [ab]
Full strength	70 [ab]
J+RHWT	70 [ab]
Half strength	68 [ab]
Control	59 [b]
P- value	0.332
F- value	1.23
LSD- value	13.07
CV %	12.83

Full strength prochloraz at 1100 mL per 100 L water

Hall strength prochloraz at 550 mL per 100 L water

The best control of stem-end rot was by the integrated method using RHWT and yeast B13. Again, the least control was provided by treating fruits with half-strength prochloraz.

5.4 Discussion

Research has shown that integrated physical and biological control management strategies for the control of plant diseases can provide control comparable to that of certain fungicides (Ehi-Eromosele *et al*., 2013). Several successful studies have been conducted which advocate the use of such integrated control strategies. These include research on the hot water treatment of bananas applied with *Burkholderia cepacia* against anthracnose (De Costa and Erabadupitiya, 2005); hot water treatment with *Rhodotorula glutinis* against blue mould of pears (Zhang *et al*., 2008); and hot water

brushing with the yeast isolate. C. *membranifaciens* CMAA-1112 for the prevention of green mould on oranges (Terao *et al*., 2017). These studies support the finding of this work that shows that successful control against anthracnose and stem-end rot of avocado can be achieved using hot water treatment and a yeast biological control agent.

It was shown that the best control against anthracnose was provided by the integration of (RHWT = 56°C for 10 seconds) with yeast isolate I, while best control against stem-end rot was provided by the integration of (RHWT = 56°C for 10 seconds) with yeast isolate B13. Both diseases were successfully controlled by the integration of yeast antagonists with rapid hot water treatment providing better control than the full-strength prochloraz fungicide. These findings are supported by the work conducted by Abraham (2010) which involved the integrated use of yeast, hot water, and potassium silicate treatments for the control of postharvest green mould of citrus and litchi. In that work it was concluded that the integration of hot water treatment and yeast antagonist treatments provided better control against disease infections in comparison with the chemical fungicide. On the other hand, studies conducted by Obagwu and Korsten (2003) looking at integrated control of citrus green and blue moulds using *Bacillus subtilis* in combination with sodium bicarbonate or hot water concluded that fungicide provides better control when compared to physical and biological treatments combined. This suggests that yeasts are more effective than bacteria when used in combination with RHWT. Yeast biological control agents provide superior efficacy by tolerating numerous agrochemicals and by direct competition for nutrients with pathogens through rapid growth (Dukare *et al.,* 2019). While both studies were performed on the same crops, the differences between the findings of Abraham (2010) and Obagwu and Korsten (2003) are also possibly due to differences in the temperature and time combinations applied (Palou *et al.,* 2008).

It must also be noted that for effective control of postharvest avocado rot, effective pre-harvest management strategies are also necessary to minimise the level of inoculum present at postharvest (Everett, 2002). Therefore, the integrated approach can incorporate wider interventions beyond the scope of this work. The use of postharvest adoption of the combination of RHWT and a yeast application was

effective. Considering that the intention of using integrated control strategies is to minimise negative impacts on the fruit, and to promote cost-effective short and long-term control, (Ehi-Eromosele *et al*., 2013), this work shows that this approach can be successfully implemented for the control of major postharvest diseases of avocado.

5.5 Conclusion

Integrated control strategies using physical and biological control agents are viable approaches for replacing synthetic fungicides used to control postharvest diseases of crops. Working together crop scientists and plant pathologists can identify the best strategies, which is of great value to agriculture.

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CHAPTER SIX

Thesis overview

6.1 Introduction

The growing trend of consuming organic meat and vegetable produce has caused a change in attitudes towards the application of fungicides to maintain high crop yields in agriculture (Sharma *et al*., 2009). For several decades, there has been an impetus for finding safe, effective, and commercially viable alternatives to fungicides. This has extended to become a primary focus in reducing postharvest losses of harvested crops (Wisniewski *et al*., 2016). Aside from preharvest disease management, the integration of physical and biological treatment has shown to be effective in reducing postharvest diseases and losses of fresh produce, thereby optimising agricultural productivity while minimizing the use of dangerous pesticides (Terao *et al*., 2017).

6.2 Research objectives and outcomes

This study aimed to contribute to the transition to safer pesticide alternatives by focusing on the major causes of postharvest avocado losses in South Africa. The study objectives were:

- **Objective 1:** To identify the causal organisms of postharvest avocado anthracnose and stem-end rot diseases.

    a) Pure cultures of the causal organism for diseases anthracnose and stem-end rot were cream white to orange cottony colonies and grey to black fluffy colonies respectively. Similar characteristics to those found in literature.

    b) Conidial spores of the organism casing anthracnose were slightly oval to rod-shaped with rounded ends, and ellipsoid to slightly rod-shaped for the organism causing stem-end rot of avocado fruits. Comparable to those found in literature.

c) ITS DNA sequences for molecular identification for the causal organism of anthracnose were inadequate for species level identification. However, it was able to identify the organism to genus level, *Colletotrichum.*

Molecular identification for the causal organism of stem-end rot based on ITS DNA sequence was highly similar to *Lasiodiplodia mahajangana,* a first report of this species infecting avocado fruit in South Africa.

- **Objective 2:** To assess the pathogenicity of isolated anthracnose and stem-end rot pathogens on avocado fruit.
-

a) Koch's postulates requirements for the fungal cultures isolated from avocados fruits showing anthracnose and stem-end rot diseases in this study was successfully fulfilled.

- **Objective 3:** To determine the optimum temperature and time combinations for rapid hot water treatment for the control of postharvest avocado anthracnose and stem-end rot.
-
- a) Temperature and time combination of between 52°C to 58°C, combined with exposure time of 10 to 30 successfully cause less or no skin damage from hot water treatment heat and were effective in inducing disease control.
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- b) Rapid hot water treatment at 56°C for 10 seconds was the best treatment providing control against avocado rot in most avocado fruit cultivars.
-
- c) There was no significant difference between the performance of the fungicide (prochloraz at 1100 mL per 100 L water) and rapid hot water treatment (56°C x 10 seconds). However, the fungicide prochloraz at 1100ml per 100L water provided the best control, followed by the rapid hot water treatment at 56°C x 10 seconds.

- **Objective 4:** To screen and determine the best antagonistic yeasts for the biological control of postharvest avocado anthracnose and stem-end rot.
-
- a) Antagonistic biological control yeasts were successfully isolated against the isolated causal organisms of anthracnose and stem-end rot avocado fruits.
-
- b) Prochloraz (1100 mL per 100 L water) provided excellent control against anthracnose followed by the yeast isolate I.
-
- c) Prochloraz (1100 mL per 100 L water) was the best at controlling stem-end rot followed by prochloraz (550 mL per 100 L water), with yeast isolate B13 being the third best control of all treatments and the number one best among the biological control treatments.
-

- **Objective 5:** To determine the best-performing integrated control methods using rapid hot water treatment and antagonistic yeasts for the control of anthracnose and stem-end rot diseases of avocado fresh fruits.
-
- a) The best control of avocado anthracnose disease was obtained from a combination of Yeast isolate I and rapid hot water treatment (56°C for 10 seconds).
- b) The best control of stem-end rot disease was obtained by integrating rapid hot water treatment (56°C for 10 seconds).with yeast isolate B13.
-
- c) With a mean percentage of 76 against anthracnose and 74 against stem-end rot. Integration of rapid hot water treatment (56°C for 10 seconds).and yeast isolate B13 can consistently control postharvest diseases of avocado fresh fruits and has a wide control spectrum. Indeed biological control yeast

antagonists integrated with rapid hot water treatment can provide control comparable to that of accepted commercial levels.

-

6.3 Recommendations and future prospects

The following recommendations and research studies would complement this frame of research study:

a) Isolating numerous pathogenic species of the genus *Colletotrichum* and *Lasiodiplodia* affecting avocado in South Africa in order to understand their diversity in the environment and determine the most prevalent and pathogenic species for effective control against them.

b) Developing automated machinery that can be used at pack houses for the commercial implementation of the RHWT technology, adopting a route that has already been taken by Israelis farmers (Palou, 2013).

c) Investigating biochemical reactions that occurs when heat induces systemically acquired resistance in a host for the control of postharvest avocado rot.

d) Continued screening of yeast isolates for biological control against postharvest avocado anthracnose and stem-end rot.

e) Screening of yeast isolate B13 against other postharvest diseases of avocado fresh fruits.

f) Conduct molecular characterisation studies for yeast isolate B13 along with toxicological studies to qualify for routine postharvest treatments for the industry.

g) Implementing an integrated management programme for postharvest anthracnose and stem-end rot of avocado in large scale for commercial application.

6.4 Relevance of the research to the avocado industry

As of the year 2020, applications of the fungicide prochloraz are facing restrictions (Quinn *et al*., 2011). The emulsifiable concentrate (EC) formulation for “Chronos” (active ingredient: prochloraz), has raised concerned regarding residues on the surfaces and inside the fruit for consumer health (Campos-Martínez *et al*., 2016). With severe impact on the export market for the avocado industry in failing to meet the standard for the permitted maximum residue level (MRL) for prochloraz (Daneel *et al*., 2016). The need to save the avocado industry with consumer and environmentally friendly technology effective in controlling avocado rot is imperative hence this study.

6.5 References

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APPENDICES

Appendix 2A

Location of the fruit sources for pathogen isolations.

Avocado fruits showing symptoms similar to those of anthracnose and stem-end rot were obtained from:

a) Mkhondeni fruit and vegetable market (Pietermaritzburg, Republic of South Africa).
b) City central fruit and vegetable market (Pietermaritzburg, Republic of South Africa).
c) Laager centre fruit and vegetable market (Pietermaritzburg, Republic of South Africa).
d) East street fruit and vegetable market (Pietermaritzburg, Republic of South Africa).

Appendix 4A

Location of the effective isolated yeast antagonists screened against anthracnose and stem-end rot diseases of avocado fresh fruits.

Yeast antagonists were isolated from undamaged avocado fruits harvested from commercial orchards and home gardens in KwaZulu-Natal and Limpopo, South Africa. However, the ones that provided effective control against anthracnose and stem-end rot of avocado were:

a) B , Isolated from fruit from Waterford (Richmond, KwaZulu-Natal)
b) J, Isolated from fruit from Waterford (Richmond, KwaZulu-Natal)
c) I, Isolated from fruit from Westfalia (Tzaneen, Limpopo)