Integration of Rapid Hot Water Treatments and Biocontrol Agents to Control Postharvest Pathogens of Tomato

By

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Dissertation Summary

Anthracnose and sour rot caused by *Colletotrichum* and *Galactomyces* (syn. *Geotrichum)* species, respectively, are major fungal postharvest pathogens causing significant losses of tomato fruit. The growing public concern over human health and environmental risks posed by pesticides, the accumulation of chemical residues in fruit, and the production of secondary effects on fruit, as well as the development of resistant strains has reduced the available options of synthetic fungicides to control these pathogens. Finding alternatives or integrated approaches to provide disease control comparable to the use of synthetic fungicides is therefore needed, especially for the control of postharvest diseases, while maintaining a high quality of fruit during storage and marketing. The overall objective of this study was to develop an integrated treatment that combined rapid hot water treatments with biological control agents to control two postharvest pathogens of tomato, *Colletotrichum* and *Galactomyces* spp., and to track their impact on the postharvest quality of tomato fruit. The mechanisms of rHWTs and antagonist yeasts involved in decay control were also investigated, in passing.

Isolation and Identity of the Pathogens

Isolation and identification of fungal pathogens associated with tomato fruits were carried out to determine the most common fungi associated with tomato spoilage in South Africa. A total of 55 isolates were recovered from symptomatic tomato fruits with typical symptoms of anthracnose and sour rot. The cultural and morphological characteristics of all isolates were observed and compared with standard descriptions to establish their identity. Pathogenicity tests were performed. The effects of wound and non-wound inoculation methods on the infection process and disease development were studied by scanning electron microscopy (SEM). The identities of one of the most pathogenic isolates of each pathogen were then determined using the consensus sequences and the nucleotide Basic Local Alignment Search Tool (BLASTn) on The National Center for Biotechnology Information (NCBI) website. Out of the 55 isolates, 33 were *Colletotrichum* spp., and the other 22 isolates were *Galactomyces* speciesp. *Colletotrichum* isolates were further classified into *Colletotrichum gloeosporioides* and *Colletotrichum acutatum*, based on cultural and morphological analyses. All the *Galactomyces* isolates were similar and were identified as strains of *Galactomyces candidum*. Among the isolated strains, C24 and C37A from the *Colletotrichum*

isolates, and G18, G23 and G29 from the *Galactomyces* isolates, were extremely pathogenic. SEM results showed that all wound and non-wound *Colletotrichum* inoculated fruits developed anthracnose, whereas non-wound *Galactomyces* inoculated fruits failed to develop sour rot, indicating that *Galactomyces* requires a wound for infection to occur. Molecular analyses confirmed the identities of the pathogens as *Colletotrichum gloeosporioides* (Penz.) and *Galactomyces candidum* Butler & Petersen (anamorph: *Geotrichum candidum* Link). The detection of these predominant fungal pathogens in this study indicated that both fungal pathogens are widely distributed on tomato fruit in KwaZulu-Natal. There is therefore a need to roll out effective and sustainable control strategies.

Isolation, screening and identification of yeast strains

A total of 148 yeast isolates were recovered from the surface of tomato fruits and were screened for antifungal activity *in vitro* using a dual culture assay. Only 25 isolates had strong antifungal activity against *C. gloeosporioides* and *G. candidum*. These isolates were then screened for phytotoxicity on healthy tomato fruits. Subsequently ten yeast isolates, which were non-phytotoxic to tomato fruits and which inhibited both pathogens, were selected for *in vivo* testing of their antifungal activity and their effects on tomato quality. The effects of delays between pathogen inoculation after yeast treatment, as well as the mechanism of decay control, were studied using SEM. Out of these 25 isolates, 4 were excluded for showing phytotoxic effect on the fruits. Isolates Y108, Y121 and Y124 showed strong antagonistic effects against both pathogens with no detrimental effect on the fruit. However, the application of the best 10 antagonist yeasts had no effect on the general quality parameters of the tomato fruits. The identity of the best three antagonist yeast isolates was then determined using molecular analysis of their sequences of the internal transcribed spacer (ITS) regions, which identified the best three isolates as strains of *Meyerozyma guilliermondii* (Wick) Kurtzman. The biocontrol efficacy of the yeast isolates was affected by the timing of their application. The yeast cells needed time to multiply, and thereby provide preventative protection. The sooner the application of the yeast treatments, the better was the biocontrol efficacy of the antagonist yeasts. Competition for nutrients, attachment to fungal hyphae and production of an extracellular matrix were among the probable modes of action of the antagonist yeasts in this study. The best isolates of *M. guilliermondii*, especially isolate Y108, were

effective as biocontrol agents against *C. gloeosporioides* and *G. candidum* and could provide a sustainable alternative to the use of chemical pesticides.

Hot water treatments with temperatures of 20, 44, 47, 50, 53, 56, 59, 62, 65, 68, 71 and 80°C were applied to tomato fruit for periods of 10, 20 and 30s on non-inoculated and inoculated fruit, in order to determine the optimal temperature x time combinations on pathogen control and postharvest quality traits of fruits. The effect of shorter times at the best working temperatures were also tested. The mechanism of heat treatments on decay control was then studied using the SEM. The temperature regimes at which no heat damage occurred on the skin of tomato fruits were 20°C, and from 44°C to 59°C, at all exposure times, and at 62°C for 20s. With increased temperature x time combinations above these levels, all treatments caused heat damage, which appeared as peeling, scalding, cracking and ageing either at the same time of treatment, or after 10 days of storage at 25°C. The best combinations of the rHWTs significantly reduced disease incidence, while maintaining fruit quality. These were: 56°C x 20s, 59°C x 10s and 62°C x 10s. Moreover, the combinations of 56°C x 15s, and 62°C x 8s were even more effective. Heat treatments caused the melting of the wax platelets of the fruit, sealing cracks in the wax cover of fruit, which remained highly visible on control fruits. Induction of host defence, and inhibition of sporulation and mycelial growth were among the possible modes of action of HWTs in this study. The results have demonstrated the high potential of rHWTs to control *C. gloeosporioides* and *G. candidum*, while maintaining postharvest quality during storage, thus prolonging the shelf-life of tomato fruit. Therefore, rHWTs should be considered as a viable technology for the control of postharvest diseases of tomato fruits on a commercial level. rHWT, equivalent to pasteurization, is a rapid process, and avoids introducing a delay in the processing time of large volumes of fruit going through a commercial packhouse.

The application of rHWTs and antagonist yeasts each provided significant control of both *C. gloeosporioides* and *G. candidum*. The combination of these two treatments enhanced the efficacy of both individual treatments. The integration of rHWTs at 62 x 8s with the yeast *M. guilliermondii* isolate Y108 resulted in the best disease control against both *C. gloeosporioides* and *G. candidum*, and delivered enhanced tomato fruit quality postharvest. This enhanced effect of rHWTs in combination with antagonistic yeasts could be the result of various interactions between the heat treatments, antagonist yeasts and the fruit.

The results presented in this thesis highlight the potential to use biological and physical disease control management strategies, as stand-alone treatments or in combination, as alternative control measures against postharvest tomato anthracnose and sour rot. Although both rHWTs and antagonist yeasts reduced both *C. gloeosporioides* and *G. candidum* incidence, the combined treatment provided the best disease control with the best fruit quality. Heat treatments partially disinfect fruit, allowing for the successful colonization of the fruit surfaces and wound sites with antagonist yeasts, which then provide a residual disease control effect for the fruits. Integration of these treatments enhanced persistence and stability of each single treatments, which would be valuable in the tomato industry as part of an effective disease management strategy, which would be economically viable, readily implemented and environmentally sound. Further research is required to implement the technology at an industrial scale.

Declarati

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Contents

Chapter 1: General Introduction

1.1 Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most widely grown and extensively consumed vegetable worldwide (Sandoval *et al*., 2015). According to the recently available data from the Food and Agriculture Organizations (FAOSTAT, 2019), world tomato production is about 182 million tons from around 5 million hectares. However, the climacteric ripening and high perishability of the fruit is affected by various pre-harvest practices. In particular, the treatment of the fruit during harvest and postharvest processes may expose tomato fruit to various pathogenic microorganisms, leading to the fruit's rapid deterioration and loss after harvest (Arah *et al*., 2015). Worldwide postharvest loss of tomato crops is estimated to be around 40% (Pinheiro *et al*., 2013; Sibomana *et al*., 2016). South Africa is reported as one of few countries that produce tomatoes throughout the year, with annual production reported to be 600,000 tonnes (PHI, 2017). Although there is little information, postharvest losses of tomato have been estimated to exceed 10.2% (worth R336 million) in South Africa (Sibomana *et al*., 2016). An estimated 50% of the losses of harvested tomato fruit are believed to be caused by microbial pathogens (Abd-Alla *et al*., 2009; Pinheiro *et al*., 2013; Sibomana *et al*., 2016). Postharvest diseases, especially those caused by fungal pathogens, cause significant economic losses (Klein and Kupper, 2018) because of their abundance, spore formation and resistance to several drying and environmental stress factors (Etebu *et al*., 2013). Major fungal species associated with tomato loss are *Colletotrichum* and *Galactomyces* (Wolf-Hall, 2010). Control measures are essential to reduce postharvest losses and ensure food security. Postharvest losses are traditionally controlled by the application of synthetic fungicides pre-harvest, and the rinsing of tomato fruit with chlorinated water during postharvest processing of the fruit. However, these applications are associated with the accumulation of chemical residues as well as secondary effects on fruit qualities. In addition, the loss of the effectiveness of conventional fungicides due to the appearance of resistant strains has increased the search for low cost, non-chemical approaches for the control of postharvest diseases (Liu *et al*., 2013; Sibomana *et al*., 2016). Integration of rapid hot water treatments with biocontrol agents may provide effective disease management for tomato fruit, postharvest.

1.2 Aim of the study

The main objective of the study was to investigate an integrated/ combined effect of rapid hot water treatments with biological control agents to control primary postharvest pathogens of tomato, *Colletotrichum* and *Galactomyces* spp. and for their effect on the postharvest quality of tomato fruit.

The specific objectives of this study were: -

1. To review the available literature on the use of rapid hot water treatments and biological control agents to control postharvest pathogens of tomato with special reference to *Colletotrichum* and *Galactomyces* spp.
2. To isolate and identify primary pathogens of tomato causing anthracnose and sour rot after harvest, to test their pathogenicity and study the effect of a wound and non-wound inoculation methods on infection process and disease development using scanning electron microscopy (SEM).
3. To isolate and screen yeast cells for the control *Galactomyces* and *Colletotrichum in vitro* and *in vivo*; to investigate their effect on the postharvest quality of tomato fruit and investigate the mechanism of biocontrol using scanning electron microscopy (SEM).
4. To identify the optimal temperature x exposure time combinations of rapid hot water treatments and evaluate their effect on the control of anthracnose and sour rot and improvement of postharvest quality and to investigate possible mechanisms of decay control using scanning electron microscopy (SEM).
5. To investigate the efficacy of combinations of rapid hot water treatments with biocontrol agents to control *Colletotrichum* and *Galactomyces* infection on tomato fruit.

1.3 Thesis layout

The dissertation consists of 7 chapters. The Harvard system of referencing was used in the chapters and unless indicated otherwise, the current study follows the style used in the journal Florida Entomologist (Florida Entomological Society). The chapters are focused on the key concepts from literature from the last 10 years. Following the dominant thesis format adopted by the University of KwaZulu-Natal, the dissertation is in the form of research papers, except for the Introduction

and Conclusion. Each chapter forms a stand-alone research paper, hence, there is some repetition of references and some introductory information between chapters. The last chapter portrays the overall conclusions and recommendations of the research.

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Chapter 2: Literature Review

2.1 Origin, history and cultivation of tomato

Tomato (*Solanum lycopersicum* L.) originated in the Andean region, South America, which now encompasses Peru, Bolivia, Chile and Ecuador (Veronique, 2004; Heuvelink, 2005). Its early history, domestication and classification have been the subject of controversy through much of history (Razdan and Mattoo, 2006). Its predomestication history has been traced back to a Mexican origin, although a Peruvian origin has also been proposed (Paran and Van Der Knaap, 2007; Bergougnoux, 2014). According to Sims (1979), Mexico is favoured as the most probable region where the tomato was first domesticated. It was consumed by the ancient Mexican tribes called the Aztec starting from the 700 AD. Tomato was imported to Europe early in the 16th century through the Spanish conquistador Cortes, who introduced its yellow fruits to Spain (Veronique, 2004; Heuvelink, 2005). From Spain, tomatoes reached Italy through the Naples (Bergougnoux, 2014), where they were known as "Pomid'oro", or Golden Apple in English, (Sims, 1979; Paran and Van Der Knaap, 2007), as reported in the first written record of early cultivation found in the Herbal of Matthiolus in 1544. At first, tomatoes were cultivated merely for ornamental purposes because the fruit was considered poisonous because of its close resemblance to *Solanum dulcamara* L. of the nightshade families (Heuvelink, 2005; Morris and Taylor, 2017). It took almost two centuries before tomatoes started being incorporated in the local cuisines and used for human consumption (Tan *et al*., 2010; Bergougnoux, 2014). Thereafter, tomato consumption was expanded to the North and became very common in England by the mid-18th century. From England, tomatoes were exported to the Middle East/Asia by a British diplomat, John Barker. Finally, tomatoes migrated back to North America through English colonization (Bergougnoux, 2014). According to Arah *et al*. (2015) tomatoes were accepted as an edible fruit in the 1840s. By the beginning of the 20th century, tomatoes became popular and have been produced throughout the world (Morris and Taylor, 2017).

The 20th century was marked by profound technological advancements that have benefited the mechanized processing of tomatoes due to the rapid increase in its production and the demand on the markets (Bergougnoux, 2014). Consequently, tomato fruit has gained considerable importance, being ranked as one of the most economically important horticultural crops in the world. The

differences in size, shape, maturity, colour, disease resistance and plant type among others, are the results of adaptation to varying growing conditions (Heuvelink, 2005; Gillett, 2006). Breeding, combined with the application of recent technologies such as genomics, has simplified the development of new cultivars by a spontaneous mutation, natural outcrossing and recombination of pre-existing genetic variation, which in turn, have induced drastic physiological and morphological changes and reduced the genetic diversity of cultivated tomatoes (Bergougnoux, 2014). Desirable features are selected for varietal improvements such as appearance, size, quality, plant architecture, enhanced disease resistance and ease of commercial harvest as well as enhanced human consumption (Paran and van der Knaap, 2007; Tan *et al*., 2010; Morris and Taylor, 2017).

2.2. Botanical description and classification of tomato

The classification of tomato as a fruit or vegetable was the subject of a fierce debate in the 19th century, with a special case of Nix vs. Hedden – 149 U.S. 304 (1893), following a 10% tariff increase on vegetables imported to the US. In spring 1886, the Nix family imported tomatoes from the western Indies. Edward L. Hedden, the tax collector of New York port assessed the tomatoes as vegetables. Nix refused to accept the decision made by the tax collector to recover taxes on the imported tomatoes and took the case to the court. The US Supreme Court ruled unanimously that the imported tomatoes should be taxed as vegetables rather than the less taxed fruits. The court reasoned that tomatoes are not like fruits which are eaten after a meal like a dessert but as the main course of a meal. However, the court has also acknowledged that tomatoes are fruits from a botanical point of view (Bergougnoux, 2014).

Linnaeus, in 1753, placed the tomato in the genus *Solanum* as *Solanum lycopersicum* (Heuvelink, 2005). *Solanum* is the largest genus in the Family *Solanaceae* which contains more than 3000 species including potatoes, eggplants, petunias, tobacco, peppers and Physalis. Approximately 1250 to 1700 species are present on all continents and are remarkable for their morphological and ecological diversity. The genus *Solanum* is also one of the most economically important genus for containing crops and many other species known to produce compounds that are either poisonous or with medicinal properties (Weese and Bohs, 2007).

In 1768, Philip Miller disagreed with the Linnaean classification and placed the tomato in its own separate genus. He named it *Lycopersicon esculentum* meaning "edible wolf's peach''. The debate

over the proper tomato botanical classification continued into the 20th century when taxonomists agreed with the Miller classification. However, genetic studies offered evidence suggesting tomatoes are part of the *Solanum* genus. *Solanum* sec. *Lycopersicon* was then adopted as the scientific name of tomato (Peralta and Spooner, 2001).

Taxonomic classification
Common name: Tomato
Latin name: *Solanum lycopersicon*
Genus: *Solanum*
Family: Solanaceae
Order: Solanales
Kingdom: Plantae
Chromosome number: Diploid; 2n = 24
Plant group: Dicotyledon

Source: (Heuvelink, 2005)

2.3. National and Global Production of Tomato Fruit

Tomato is one of the most grown and extensively consumed vegetable following potato worldwide (Veronique, 2004; Mujtaba and Masud, 2014). According to the recently available data from the FAO (FAOSTAT, 2019), the world tomato production accounts for about 182 million tons from around 5 million-hectare under cultivation. In 2017 China was the largest producer, followed by India, USA, Turkey and Egypt (Figure 1) (FAOSTAT, 2019). Asia dominates tomato production with 57.9% of the total world's production, followed by America, Europe and Africa from the years 2007 to 2017 (FAOSTAT, 2019).

Tomatoes are believed to have been introduced into Africa in the 16th century (OECD, 2017). Africa accounted for 12% of the total production share of tomato by region, with Egypt being one of the top five tomato producer countries in the world (FAOSTAT, 2019). South Africa is the major tomato producing country in sub-Saharan Africa (Heuvelink, 2018), producing 608 000 tons from 8006 ha in 2017 (FAOSTAT (2019). Its production increased by 3.5% from 587 772 tons in 2016 to 608 306 tons in 2017 (FAOSTAT, 2019).

The morphological diversity of tomato enables production in all nine provinces of South Africa (SA) (Directorate Marketing, 2015; Malherbe and Marais, 2015; PHIP, 2017; Heuvelink, 2018). Limpopo is the best-suited province for production due to its warm climate, and accounts for more than 75% of the total area planted with tomatoes in SA, with a production area of 3590 ha (2700 ha in Northern Lowveld and 890 ha in far northern areas of Limpopo). Bertie Van Zyl (EDMS) BPK (ZZ2®) is the largest tomato producer, and is based in Limpopo province (Sibomana *et al*., 2016). Other main producing areas of SA are Mpumalanga province (770 ha) and Eastern Cape Province (450 ha) (Directorate Marketing, 2015; Heuvelink, 2018). Production is dominated by commercial and small-scale farmers which contribute to 95% and 5% of the total tomato production in SA, respectively (Directorate Marketing, 2015). In winter, production is very limited, so they are grown only in frost-free areas or under protection (Directorate Marketing, 2015; PHI, 2017).

Tomato is produced throughout the year in SA (DAFF, 2018). SA tomato export represents only 0.1% of world tomato export because most production is sold to the domestic market (DAFF, 2018; Heuvelink, 2018). According to DAFF (2018), the number of tomatoes exported has increased by 0.4% from 16 663 tons to 16 737 tons in 2016/17. Approximately 75.7% of the tomato fruit were exported to Mozambique, 7.4% to Zambia and 5.8% to Angola, while a small percentage of processed tomatoes are exported to Belgium, Germany, Italy, The Netherlands, Sweden and UK, with a preferential tariff of 0% due to EU-SA Free Trade Agreement (DAFF, 2018; Heuvelink, 2018). Trading in fresh tomato produce and processed products is a major global business (Heuvelink,2018). Trade in fresh tomato fruit occurs mainly between neighbouring countries due to the perishability of the fruit. Freight and tariff benefits between neighbouring countries are also other reasons (Heuvelink, 2018). Mexico is leading in export volumes worldwide, exporting more than 7,745,243 tons of tomatoes, which accounted for 25% of the world export market for tomatoes in 2016. The Netherlands was second with 18.9% market export share, followed by Spain (12.6%) and Morocco (6.1%) (Directorate Marketing, 2017). The Dutch tomato exports are reported to have higher economic value than those Mexico (Heuvelink, 2018). Globally, USA, Germany, France, Russia and the UK recorded the highest tomato import volumes in 2016 (FAOSTAT, 2019). SA recorded low import volumes in 2016 (FAOSTAT, 2019), showing that it is a self-sufficient country in tomato production.

2.4. Economic and Nutritional importance of tomato

Tomato is grown in all parts of the world, for domestic use or export (OECD, 2017). With the advances in the modern technology, tomato is now grown in all seasons and geographical zones, in the outdoor fields, home gardens or greenhouses, small-scale agricultural patches or as large-scale urban market productions (OECD, 2017). Better controlled environment conditions have been the main factor responsible for the increase in tomato production (OECD, 2017). As a result, the economic importance of the fruit has increased and it has become a source of income in developing countries (OECD, 2017). In SA, producers sell high-quality tomatoes as fresh produce while low-quality tomatoes are used for processing, thus, the best income is generating from the fresh produce consumer market (Malherbe and Marais, 2015). Tomato production has created job opportunities to more than 25 000 people in SA, with a larger number of employees in summer months where production volume reaches a peak (PHI, 2017).

Tomato is consumed in many different ways, either raw or processed into sauces, canned tomatoes, pastes, juices and ketchup (Mujtaba and Masud, 2014; Pinheiro *et al.*, 2014). Tomato is incorporated in many dishes and its consumption is interwoven into different cultures from different communities, which explains its global appeal (Beckles, 2012). Like any other fruits and vegetables, tomato is a good source of Vitamins A, C and E, carbohydrates, minerals, β-carotene, lycopene, fibres and phenolic compounds. Lycopene, a carotenoid, is an effective antioxidant and may provide protection from many kinds of cancer, and cardiovascular, hepatic and renal diseases (Toor and Savage, 2006; Pinheiro *et al*., 2013; Arah *et al*., 2015; Lydia, 2015).

2.5 Postharvest losses of tomato

The increase in population size, consumption per capita, urbanization per capita income and the income elasticity have caused a continued increase in the demand for tomatoes worldwide (Directorate Marketing, 2017). The campaign for a healthy diet and lifestyle has a positive impact on the tomato industry (Heuvelink, 2018). However, regardless of the consumer preferences for high-quality tomatoes, producers have been more focused on large volume production at low cost (Heuvelink, 2018). Although the emphasis in the tomato research has shifted from quantity to quality, there have been hardly any improvements in the quality of commercially produced tomato

varieties (Arah *et al*, 2015). Tomato quality is an important factor that ensures consistent marketability of the fruit. Despite the numerous benefits that can be derived from the production of tomatoes, postharvest losses can make its production unprofitable (Arah *et al*, 2015). Postharvest losses are both quantitative and qualitative. The qualitative losses, which impact the nutrient quality, consumer acceptability and the financial income of producers, are more difficult to assess than the quantitative losses (Kader and Rolle, 2004; Arah *et al*, 2015).

Worldwide, postharvest tomato losses have been estimated to reach 40% of the total yield (Figure 1) (Ukeh and Chiejina, 2012; Etebu et al., 2013; Pinheiro *et al.*, 2013; Sibomana *et al*., 2016). Losses are more substantial in developing countries due to the lack of knowledge and sophisticated storage facilities (Ukeh and Chiejina, 2012; Sibomana *et al*., 2016). Although there is little information on postharvest losses of tomato in South Africa, postharvest losses have been estimated to exceed 9% (Figure 1) (FAOSTAT, 2019). Losses of up to 50% of the harvested tomato crop have been reported in developing countries as the results of a high rate of bruises, water loss and subsequent decay by disease-causing postharvest pathogens (Lydia, 2015). Postharvest pathogens passively infect fruit via wounds or natural openings. Some, however, can actively penetrate the outer layer of tomatoes. Postharvest losses mainly occur during the ripening stage of the fruit after harvest, and are affected by the postharvest handling methods, sanitation, packaging, transportation facilities and storage conditions. However, various pre-harvest practices can aggravate postharvest losses (Sibomana *et al*., 2016). According to Arah *et al*. (2015), tomato quality can never be improved after harvest but can be maintained. Therefore, various pre-harvest practices, and harvest and postharvest handling techniques play important roles in the postharvest qualities and shelf-life of tomato fruits (Kader, 1984; Kader, 2000; Etebu et al., 2013; Arah *et al*., 2015).

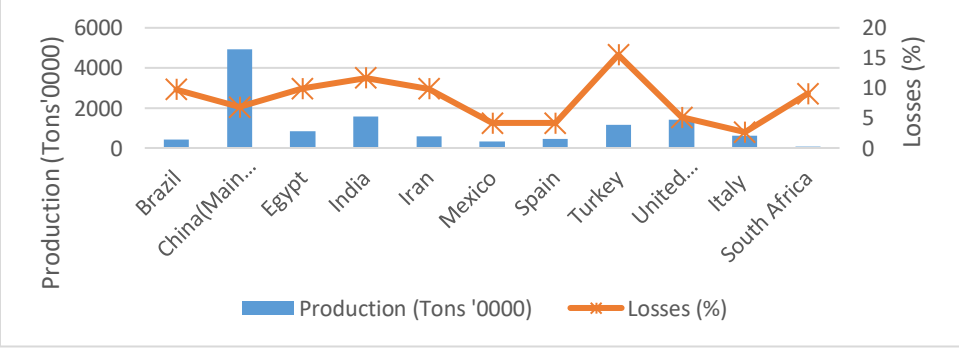

Figure 1: The percentage of tomato losses of the total production recorded in 2017 for the respective countries (FAOSTAT, 2019).

2.5.1 Effect of pre-harvest practices on the postharvest quality and shelf-life of tomato

Quality, defined as the degree of excellence or superiority, is a complex of external and internal traits with multiple attributes. External traits include the general attractiveness of the fruit (colour, size, shape) and the firmness, with no signs of bruises, shrivelling or physical and/or mechanical damages. The internal traits comprise the biochemical traits such as the taste (sweetness, acidity, aroma and flavour), the texture, the shelf-life and the nutritional value (Kader and Rolle, 2004; Heuvelink, 2018). The external traits are more likely to influence the consumer's decision to purchase rather than to reflect on the actual quality of the produce (Kader and Rolle, 2004; Hewett, 2006). All these attributes are developed before harvest (Hewett, 2006). Any defects occurring during the growth of the plant may influence the overall quality of the fruit, which in turn compromises the harvest and the postharvest handling conditions and quality of the fruit (Thompson, 2008; Kader, 1984; Sibomana et al., 2016). Climatic conditions and other cultural practices such as the application of fertilizers, irrigation and choice of cultivars can be the major pre-harvest factors that affect the quality and shelf-life of fresh tomato products (Pinheiro *et al*., 2013; Arah *et al*., 2015; Lydia, 2015; Sibomana et al., 2016).

Climatic conditions

Tomato is a diploid, self-pollinating, tender, herbaceous, perennial plant with an optimum growth temperature of 21–23°C. It has a perfect flower with both male and female functional parts. The fruit maturation (from pollination to ripening) varies from 6–10 weeks, depending on the environmental conditions and the variety. The environmental conditions can significantly influence the growth rate, fruit set, yield, and quality of fruit (Gnanamanickam, 2002).

Temperature, light intensity, carbon dioxide (CO_2) levels, relative humidity and water availability are the main climatic and environmental factors responsible for the quality and nutritional contents of fresh produce after harvest. Environmental factors may have a direct or indirect effect on the general bioactive compounds; indirectly by providing the prerequisites for photosynthesis thereby, providing the energy or precursors for the synthesis of the bioactive compounds. Environmental factors such as water availability and soil fertility, which vary in time and places, affect the final

quality of the fruit (Hewett, 2006). The atmospheric conditions such as the relative humidity, the CO_2 concentration and temperatures are hard to control or manage under field conditions and are reported to have a durable impact on the quality of the fruit (Weston and Barth, 1997). Temperatures influence the uptake and metabolism of minerals by plants. It is an important factor, not only during the juvenile stages of plant growth but also for sexual development as it is a flowering stimulus (Kader, 2000).

Light intensity also has a major effect on tomato quality (Weston and Barth, 1997; Kader, 2000). Adequate exposure to the sun provides plants with photosynthates, which are needed for plant growth (Hewett, 2006). Furthermore, light is required for the formation of carotene and increase the ascorbic acid concentration in tomato fruits. Tomato fruit grown in full light have more sugar and dry matter content than those grown in shaded areas or fields (Weston and Barth, 1997; Kader, 2000).

Cultural practices

- **Application of fertilizers**

Inorganic mineral nutrients are important for the growth and development of the tomato plant, as well as for disease resistance or control. The selection of adequate fertilizers and their timely application, at the appropriate maturity stage, are crucial for increased yield, nutritional content, quality, storage and shelf-life of fresh produce postharvest (Arah *et al*., 2015). Improper nutritional balance (excess/ deficiency) will affect the fruit quality in many ways and may result in fruits with physiological disorders (Hewett, 2006). The application of adequate potassium fertilizer has been reported to improve the colour and reduce the incidence of yellow shoulder of tomatoes in the stem scar. It has also been reported to increase the total titratable acidity of the fruits, which favour Vitamin C. An insufficient supply of potassium may result in a ripening disorder in tomato fruits (Arah *et al*., 2015). Furthermore, the application of nitrogen fertilizer above certain threshold levels will reduce fruit quality and other traits such as the glucose and fructose concentrations and the pH (Arah *et al*., 2015). Unlike nitrogen, a high calcium content in tomato fruit is related to a long shelf-life after harvest, due to a decreased rate of transpiration and ethylene production, which lead to a firm fruit with delayed ripening, and decreased disease and disorder incidence on the fruits (Arah *et al*., 2015). A calcium deficiency has been reported to cause blossom end rot in tomato fruits (Hewett, 2006). Inadequate application of other micronutrient fertilizers such as

boron, copper and molybdenum affect the fruit firmness, making it susceptible to rapid physical and/or microbiological damage (Kader, 1984; Weston and Barth, 1997; Hewett, 2006).

- **Irrigation**

Tomato is a perishable fruit that is not resistant to drought and high temperatures. Therefore, proper water irrigation schedules should be implemented for efficient water management and to maintain the quality and yield of the crop (Arah*et al*., 2015). Insufficient water during the growing season has been reported to cause fruit softening or fruit dehydration, making it prone to physical damage and decay during storage (Weston and Barth, 1997). Excessive water, on the other hand, has also been reported to increase turgidity and cracking of fruits, resulting in reduced firmness, delayed maturity and increased susceptibility to physical damage and decay (Kader, 2000).

The use of untreated water for irrigation causes significant tomato loss due to contamination from faeces. Such incidences have been reported in Sub-Saharan Africa due to the resource challenges for small-scale growers. Use of unclean irrigation water is one of the main sources of enteric human pathogens deposited on tomato fruit. However, this challenge can be resolved by chlorine pre-treatment of the water during the growing seasons and/or decontamination of fruits after harvest (Sibomana *et al*., 2016).

- **Choice of cultivars**

The quality and shelf-life of tomato after harvest depend also on the cultivar type. New cultivars are normally developed to improve adaptability to the different environmental conditions, disease resistance and quality of produce (Weston and Barth, 1997). Desirable cultivars with potential qualities should be selected in order to reduce the susceptibility of fruits to environmentally or microbial induced decay. The choice of cultivars has further been reported to increase the number of high-quality fruits after harvest with prolonged shelf-life (Arah *et al*., 2015). Therefore, the selection is critical to the postharvest storage conditions of the tomato fruit.

2.5.2 Effects of harvest and postharvest handling on the loss of tomato

Maturity stage at harvest

Tomatoes can be harvested at different stages from green mature, half-ripe to red-ripe, depending on the production and market demand. Fruits harvested at the mature green stage have been reported to have a longer shelf-life than those harvested at the half-ripen or the red-ripen maturity stages. Even though the shelf-life is the most important factor in postharvest technology, other aspects of the fruit can also be affected if tomatoes are harvested at the mature green stage. When harvested at the green stage, the sugar transport into the fruit will cease, facilitating the degradation of starch, which is undesirable. At the same time, fruits harvested at a later maturity stage have been also reported to have accumulated a high sugar content, which makes them susceptible to physical damage, with a short shelf-life (Arah *et al*., 2015). According to Kader (2000) tomato fruits should be harvested at a half-ripe stage to provide consumer flavour and quality satisfaction.

Harvesting and postharvest handling techniques

Typical industrial techniques associated with tomato production include mechanical harvesting, packing, sorting, grading, washing, and long distances transportation. Mechanical injury due to bruising, scarring, scuffing, cutting, or puncturing the fruits may occur at any stages (Arah *et al*, 2015). Injuries that are equivalent to or greater than the yield point lead to a total breakdown of fruit cells and are accompanied by unwanted metabolic activities such as an increased ethylene production, accelerated respiration rates and ripening, which in turn results in either reduced shelf-life or poor quality. Therefore, it is important to handle tomato fruit with care during the harvest and postharvest in order to minimise postharvest losses (Arah *et al*, 2015).

Tomato fruit may experience strong compressive and puncture forces during harvest. Excessive exertion of these forces results in the fruit physical/mechanical damages, which include bruising, breakage and cuts (Pinheiro *et al*., 2013; Sibomana *et al*., 2016). The use of inappropriate harvesting containers by small scale tomato growers is also reported to result in injuries during harvest (Arah *et al*, 2015). According to Kader (2000), physical injuries accelerate both water and Vitamin C losses from the fruit and increase the fruit susceptibility to various postharvest disease-causing pathogens. The handling and management of harvesting techniques influence the severity and incidence of injuries.

Tomatoes should be harvested at a mature green stage to be ready for the fresh market. However, workers should be experienced enough to recognize the maturity of tomato fruit from their skin

colour. Tomatoes harvested in an immature stage ripen poorly and are unable to withstand compressive forces (Kader, 2000; Sibomana *et al*., 2016).

Jung *et al*., (2014) and Sibomana *et al*., (2016) have reported that improper stalking and rough handling of fruits during transportation also accelerate physiological and mechanical damage. The containers used during packaging and transportation may also be sources of physical damage and microbial contamination. Overloading, poor transportation facilities and poor road conditions during shipment are also other causes of postharvest losses. Advanced packaging materials can be used to provide modified atmospheres and protection to fruits. Packaged fruits in a controlled atmosphere (CA) experience levels of 20.30 to 20.40% oxygen, and 1.13 to 2.20% CO_2. As a result, CA packaged fruits have fewer firmness losses than unpackaged ones, and the high relative humidity also results in a lower weight loss of packaged fruits (Jung *et al*., 2014; Sibomana *et al*., 2016).

The role of transportation is significant in relation to the microbial infection of freshly harvested fruits (Shewfelt, 1992). Fresh produce is transported from the point of harvest to the market. The agitation and bouncing of the fruits during transportation may cause the fruits to be bruised, crushed and abraded. All these injuries increase the chances for microorganisms to penetrate the fruit, and to cause spoilage and postharvest losses (Shewfelt, 1992). The lack of proper transportation infrastructure and refrigerated trucks are a major challenge for both tomato producers and distributors in most developing countries (Arah *et al*, 2016).

Sanitation of workers should also be taken into consideration as cross-contamination may occur through contact. Clean harvesting equipment and containers should be used and stacking of containers on top of the soil and also on the top of each other should be avoided in order to control contamination as well as excessive pressure on tomato fruits (Sibomana *et al*., 2016).

Postharvest Storage conditions

In order to maintain postharvest tomato quality and minimize deterioration and decay after harvest, tomato fruits should be stored at optimum temperatures. Right after harvest, the fruit have high temperatures due to the field heat and need to be stored in a cold room immediately after harvest (Arah *et al*., 2015). High temperatures are reported to accelerate the rate of respiration (CO_2 production). This results in an increased production of ethylene, followed by premature fruit ripening and senescence (Arah *et al*., 2015). Rapid cooling of tomato fruits to about 12.5℃ immediately after harvest removes heat and retards ripening, resulting in prolonged storage and shelf-life, with reduced water loss and disease incidence (Rees and Orchard, 2012). Recommended postharvest temperatures and relative humidities of tomato are 10.0-12.5℃, 90-95%, respectively, with ripening expected at temperatures above 14.0℃ (Sibomana *et al*., 2017). A delay in cooling by one hour has been reported to cause a one-day loss of shelf-life of the fruit (Jung *et al*., 2014; Arah *et al*., 2015). However, fruits exposed to extremely cold temperatures are also reported to suffer from chilling injuries (CI). The optimal cooling storage temperature of 10℃ maintain quality without any significant damage to tomato fruit (Arah *et al*., 2015). According to Masarirambi *et al*. (2009), CI affected fruits fail to ripen and develop full colour and flavour. Other consequences such as irregular colour development, shrivelling, softening, surface pitting and increased susceptibility to diseases have also been reported (Masarirambi *et al*., 2009). CI is even worst on fruits harvested before physiological maturity (Rees and Orchard, 2012). According to Rees and Orchard (2012), the sensitivity of fruits to CI varies depending on the temperature, length of the exposure period, maturity of fruit and variety (Table 1). Therefore, ideal cooling temperatures should be used before shipment since the choice of the temperature may lead to the physiological stress of the fruits and loss of quality and shelf-life. All members in the harvesting and distribution chain should know about the optimum storage conditions for tomato fruit, in order to make right decisions as to the choice of temperature (Jung *et al*., 2014; Arah *et al*., 2015; Sibomana *et al*., 2016).

Table 1: Temperature and storage conditions for different maturity and ripeness classes of tomato based on their susceptibility to chilling injury

Class	Temperature ( )	Storage duration (days)
Mature-green	12.5-15	Up to 28
Pink	10-12.5	7-14
Light-red	9-10	4-7
Firm-ripe	7-10	3-5
Pink-red, Firm-red or vine-ripe	7	2-4

Source: (Rees and Orchard, 2012)

2.5.3 Postharvest physiology of tomato affecting its postharvest life

After germination, the life of fruits and vegetable plants is divided into three major physiological stages, namely growth, maturation and senescence. Growth and maturation are collectively referred as the developmental phase of the fruit while senescence is a phase where the anabolic (synthetic) biochemical processes give way to catabolic (degradative) processes, leading to ageing and finally death of the fruit (Wills *et al.*, 2007). Ripening is an irreversible event, which marks the completion of the developmental phase of the fruit and the commencement of senescence (Wills *et al.*, 2007). Ripening and senescence of climacteric fruit continue after the fruits are detached from the plant and they undergo postharvest physiochemical changes (Table 1), which will determine their marketable quality (Toor and Savage, 2006; Wills *et al.*, 2007; Pinheiro *et al.*, 2013). Once harvested, the life of the fruit depends on the reserves, which once exhausted, cause the fruit to undergo accelerated ripening and ageing, with a subsequent deterioration. Ripening begins during the later stages of the fruit maturation and the first stage of senescence (Wills *et al.*, 2007). During the onset of ripening, the fruit's respiration rate and ethylene production, a regulatory hormone responsible for the major physiological stages, has been reported to increase while the transpiration decreases. The accelerated ripening of the fruit after harvest results in increased fruit susceptibility to pathogenic attack, and a reduced shelf-life, which increases consumer rejection and postharvest losses (Toor and Savage, 2006; Pinheiro *et al.*, 2013). However, the origin of pathogens in fruit is mainly due to the harvesting techniques and subsequent handling and storage conditions (Jung *et al.*, 2014; Sibomana *et al.*, 2016).

Physiochemical changes that may occur during the ripening of fleshy fruit
Seed maturation
Colour change
Abscission (Detachment from parent plant)
Change in respiration rate
Change in ethylene production
Change in tissue permeability and cellular compartmentation
Softening: change in the composition of pectic substances
Changes in carbohydrate composition
Organic acid changes
Production of flavour volatiles
Development of wax on skin

Source: (Wills *et al*., 2007)

2.6 Postharvest fungal diseases of tomato

Postharvest losses have been reported to occur at all stages from the growth and development to harvesting and postharvest stages of the fruit (Coates and Johnson, 1997). Up to 50% of harvested tomato is lost as a direct consequence of disease-causing postharvest pathogens (Lydia, 2015). Postharvest pathogens passively infect fruit via wounds or natural openings. Some, however, are able to actively penetrate the outer layer of tomatoes (Cooper *et. al.*, 1978). Some infections are latent (quiescent) at harvest and enter a dormant stage until the physiological status of the fruit starts changing (Etebu *et al*., 2013).

Postharvest diseases, especially those caused by fungal pathogens, cause significant economic losses (Klein and Kupper, 2018) because of their abundance, spore formation and resistance to several drying and environmental stress factors (Etebu *et al*., 2013). Approximately 94% of a ripe tomato fruit is water, and this large amount of water, coupled with the perishable nature of the fruit, makes tomato fruit prone to both pre-harvest and postharvest damage, and susceptibility to spoilage by fungi (Auret, 2007). Fungi generally invade damaged or senescent tissue; therefore, they are referred to as opportunistic pathogens (Cooper *et al.*, 1978). Fungal pathogens, in general, execute a serious of sequential steps in order to infect and cause disease. Those steps include host

recognition and attachment; germination, colonization and nutrient derivation from the host; disruption of host defence responses; reproduction, exit and dispersal; and finding another host (Sexton and Howlett, 2006). Anthracnose and sour rot caused by *Colletotrichum* and *Galactomyces* species, respectively, are major fungal pathogens associated with tomato crop losses after harvest (Wolf-Hall, 2010).

2.6.1 Sour rot

Sour rot is caused by *Galactomyces* species and is one of the most unpleasant economically important postharvest disease of fruits and vegetables worldwide. It is an ubiquitous organism found in the air, water, silage and the soil (Agrios, 2005, Thornton, *et al.,* 2010). It causes significant losses in tomatoes, carrots, citrus fruits and other fruit and vegetables pre- and postharvest (Agrios, 2005). *Galactomyces* sp. are also found in foodstuffs such as milk, cheese and fermented milk products where it causes food spoilage and off-flavours (Botha, 1999, Thornton, *et al.*, 2010). In addition, it is a health hazard to immunocompromised individuals (Botha, 1999). Some strains of *Galactomyces* have been associated with infections of blood, cornea, ileum, tongue, skin and nails (Botha, 1999). This pathogen is considered to be a wound pathogen and cannot penetrate the fruit epidermis directly. It infects fruits and vegetables during harvest and postharvest handling procedures (McKay *et al*., 2012). Tomato fruits infection by *Galactomyces* may occur at the mature-green stage, but ripe and overripe fruits, as well as fruits which are stored in plastic bags or packages, are more susceptible to infection by *Galactomyces* (Agrios, 2005). Although there are many species of this organism, *G. candidum* is the only species which causes a significant loss in foods and postharvest fruits (Bullerman, 2003).

Symptoms

Sour rot is characterized by thick lesions with white, soft, creamy yeast-like colonies (Bullerman, 2003; Etebu et al., 2013). It has a septate mycelium that readily fragments into arthrospores, which are the organism's primary means of reproduction, and can be observed under the microscope (Figure 2b) (Bullerman, 2003; Blancard, 2012). If the arthrospores penetrate a lesion in tomato fruit, they cause rotting inside the fruit and eventually to the whole fruit. Over time, the skin cracks open, releasing a white, cheesy water-soaked juice with a sour, yeasty odour (Figure 2a) (Bullerman, 2003; Agrios, 2005). Green fruits affected by *Galactomyces* may remain firm for

longer, but they eventually develop similar symptoms to ripe fruits when fully colonized (Blancard, 2012).

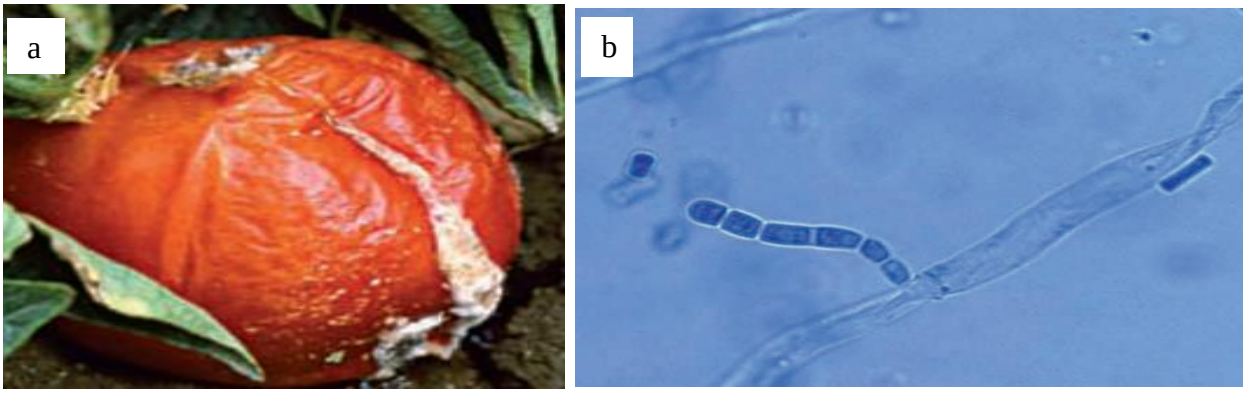

Figure 2: Sour rot lesions with a soft, creamy juice; (b) cylindrical *Galactomyces* conidial column with a septate mycelium, under a microscope. Source: (Blancard, 2012).

Disease cycle

Galactomyces is a ubiquitous soil- inhabitant and can be dispersed by wind, water or insects (Botha, 1999; Blancard, 2012). It is strictly a wound based pathogen that penetrates fruits via the stem scars, skin cracks or injuries from insects causing mechanical damage. Conidia are either splashed onto fruit by rain or irrigation water, or they are carried by flies or other insects. Farm workers may also disseminate conidia during cultural operations onto freshly harvested fruits from infected plant material (Botha, 1999; Blancard, 2012). *Galactomyces* infection occurs both in the field and postharvest settings. In the fields, it infects fruits with tissue damage, or spreads on overly vine-ripe fruits as a saprophyte. Once on the fruit, *Galactomyces* grows rapidly and starts to multiply in large quantities and spreads in the internal tissue (Blancard, 2012), causing rotting inside the fruit and a foul odour. Infected fruit acts as a source of inoculum and attracts house flies, which further spread the pathogen within the crop (Bullerman, 2003; Agrios, 2005). In the postharvest setting, infected fruit may spread the disease to other fruits in storage through contact and are usually is accompanied by bacterial soft rot. Infection of tomato fruit by *Galactomyces* was first reported in 1923 by Prichard and Porte (Fiedler, 2014).

Epidemiology

Galactomyces infects tomatoes in all growing seasons, particularly after periods of heavy rainfall during the autumn harvest, and is favoured by high relative humidity and temperatures (>10°C) (Barkai-Golan, 2001) but may also be active at temperatures as low as 2°C (Thornton et al., 2010). Disease progression is rapid at temperatures of 25-30°C (Baudoin and Eckert, 1982). Rapid epidemics are associated with fruits injured by insects or mechanical means during harvest and postharvest. Physiological change in fruits, duration of storage and packaging may all increase the susceptibility of fruits to sour rot (Baudoin and Eckert 1982). In storage, *Galactomyces* may cause complete spoilage and liquefaction of infected fruit. Juices dripping from the infected fruit may result in the spread of the pathogen to healthy fruits. Unclean equipment also provides a favourable environment for its rapid growth (McKay et al., 2012).

2.6.2 Anthracnose

Anthracnose is caused by *Colletotrichum* species, which belongs to the Kingdom Fungi; Phylum *Ascomycota*, Class *Sordariomycetes*; Order *Phyllachorales*; and Family *Phyllachoraceae* (Than *et al.*, 2008). Anthracnose is globally distributed (Gnanamanickam, 2002). It causes significant losses in tomatoes, strawberry, mango, citrus, avocado, banana and other crops (Blancard, 2012; Cannon et al., 2012). It is one of the most successful postharvest pathogens because it has an efficient stage of latent infection. Typically, the pathogen infects immature fruit before harvest but becomes active during storage as the fruit ripens, and appearing as visible lesions on ripe fruit on the market shelves. Anthracnose is reported to cause up to a 100% loss in stored fruits (Dean et al, 2012).

Symptoms

Anthracnose symptoms first become visible on ripe or ripening tomato fruits as small circular, slightly sunken lesions on the skin, which later expand to 2-3 cm and develop dark concentric rings and develop a water-soaked appearance directly beneath the skin (Figure 3a). In moist weather, the acervuli produce conidial masses, which can be seen as distinctive black specks and unicellular hyaline conidia under a microscope (Figure 3b). Several lesions may coalesce and cause an extensive decay on the fruit. At this stage, the fungus has penetrated into the tomato flesh and the fruit may rot completely due to attack by secondary microorganisms through anthracnose spots

(Agrios, 2005). Decay may be dry and firm at the beginning, but as the disease progresses, the skin becomes greyish and eventually, a soft rot appears (Rees and Orchard, 2012).

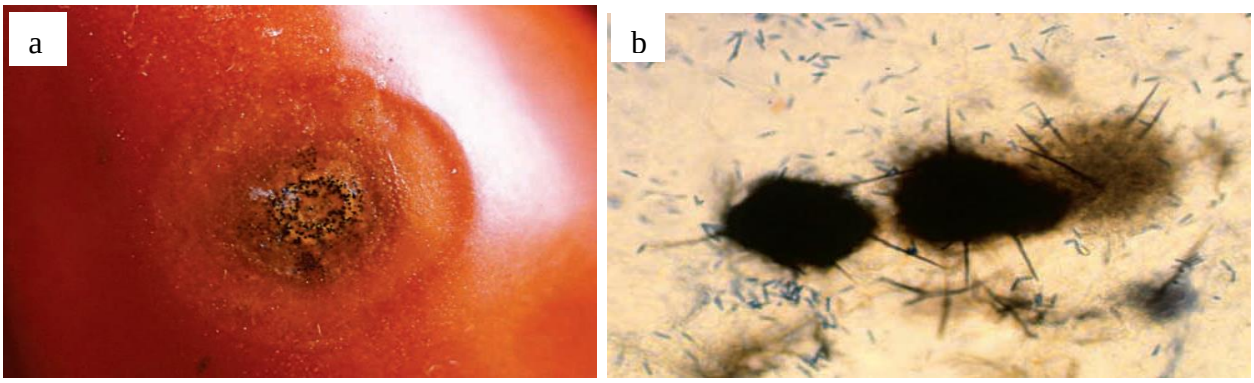

Figure 3: (a) Anthracnose lesions with dark concentric rings developed on the surface of tomato fruit. (b) Acervuli with distinctive black specks and unicellular hyaline conidia. Source: (Blancard, 2012)

Disease cycle

Colletotrichum survives in infected plant debris and in the soil as micro-sclerotia. It is known to infect plant at any growth stage and may infect the leaves, stem and roots, but the most visible symptoms occur on fruits (Delahaut and Newenhouse, 1997; Agrios, 2005). According to Delahaut and Newenhouse (1997) and Tsitsigiannis *et* al., (2008), lower leaves and fruits that come in contact with soil may act as the initial point of infection. Fungal conidial masses are splashed by rain, overhead irrigation or carried by insects from infected plant debris or the soil onto healthy fruit and foliage (Sherf and MacNab, 1986; Agrios, 2005; Than *et al.*, 2008). The conidia germinate on the healthy fruit and foliage and produce appressoria, which enable them to adhere to the plant surfaces. Once the appressoria are pigmented, the fungus penetrates the fruit skin directly or through wounds from insects or natural openings. After germination, fruits become infected within 24 hours at 100% RH and 38 to 42°C and symptoms develop within 6 to 10 days of inoculation. New conidia develop on fresh acervuli and serve as inoculum sources for secondary infections, continuing the disease cycle (Sherf and MacNab, 1986). Appressoria that are formed on immature fruits may remain quiescent until ontogenic changes such as ripening and senescence occur in the physiological state of the maturing fruits. Under severe conditions, lesions may coalesce, causing extensive decay on the fruit. Eventually, the lesions get invaded by secondary

microorganisms, causing a complete rot of the fruit (Figure 4). Many studies have concluded that this disease is extremely difficult to manage under conducive environmental conditions (Than *et al.*, 2008).

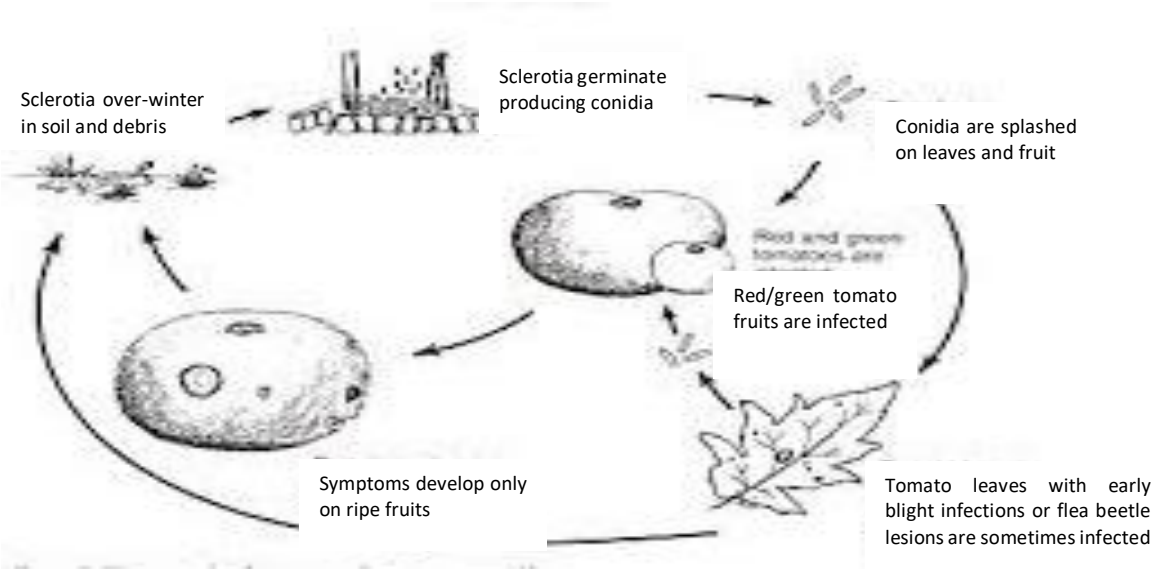

Figure 4: Tomato anthracnose disease cycle. Source: (Dillard, 1987).

Epidemiology

Many postharvest diseases of fruit exhibit the phenomenon of quiescence whereby the symptoms develop after the fruit ripens. *Colletotrichum* species are the most important pathogens that cause latent or quiescent infections (Than *et al.*, 2008). Anthracnose can occur on leaves, stems, and on both pre- and postharvest fruits. It is favoured by high temperatures (>27°C), high relative humidity (80%) and frequent rainfall (Than *et al.*, 2008; Agrios, 2005).

2.7 Cultural practices to control postharvest diseases of tomato

Staking tomatoes in the field improves air movement and may reduce the occurrence of favourable environmental conditions for infection (Kennelly, 2009). Pre-harvest mulching of tomato plants prevents splashing of spores from the soil onto the fruits. Avoiding overhead irrigation and removal of infected or rotting fruits from the plant also decrease fungal infection (Kennelly, 2009). Cultural practices to control postharvest pathogens involve careful harvest and postharvest

handling techniques, manipulation of postharvest environment and sanitation practices (Coates and Johnson, 1997; Barkai-Golan, 2001). Minimizing fruit injuries or wounds by careful harvesting, sorting, packaging and transportation, including preventing the fruit from falling at all stages, may reduce the numbers of wound pathogens and suppress disease development (Barkai-Golan, 2001).

Once harvested, proper sanitation practices should also be implemented within the packaging house (Barkai-Golan, 2001). Fresh fruits arriving the packaging house may encounter pathogenic spores carried on fruit containers, the equipment in the packinghouse, as well as on the workers' hands and tools. The air in the packinghouse may also carry an abundance of pathogenic spores originated from infected fruit and plant debris in the packinghouse or its surroundings, which serve as substrates for many pathogenic fungi (Barkai-Golan, 2001). Removing rejected fruits from the packhouse or storage environment, as well as filtering or frequently changing the water used to wash fruits or for cooling purposes will reduce the inoculum of postharvest pathogens in the packhouse (Coates and Johnson, 1997; Barkai-Golan, 2001).

Temperature influences the rate of pathogen growth and fruit ripeness. Postharvest disease development is associated with the ripeness of fruit, therefore, the temperature used in the storage should be manipulated in a way that delays both the ripeness and disease development. High humidity is often used in a storage environment in order to prevent water loss from produce, but this may increase the level of disease, and therefore it should be manipulated to be unfavourable for pathogens (Coates and Johnson, 1997).

Disinfestation of fungal conidia on working surfaces is possible by using appropriate disinfectants. Disinfecting fruits, packaging house atmosphere and boxes are frequently treated with an active solution of hypochlorous acid or steam to remove inoculum of pathogens (Eckert, 1990).

2.8 Chemical control of postharvest diseases

Postharvest losses are traditionally controlled by the application of synthetic fungicides pre-harvest and the rinsing of fresh tomato fruits with chlorinated water, postharvest. Synthetic chemicals are relatively inexpensive and easy to apply (Palou, 2013). They have curative action against pre-existing or established infections and persistent preventive action against potential new infections, and many also inhibit the sporulation from lesions on decaying fruit (Palou, 2013). Consistent

level of control can be achieved with some synthetic fungicides especially with those which have systematic activity (Auret, 2007). These chemicals include systemic and protectant fungicides, fumigants and sanitation chemicals. Unlike protectant fungicides, systemic fungicides are used to control infection that already been established (Chavan and Pawar, 2012). Fumigants can be applied after harvest to prevent or reduce insect infestation and decrease disease incidence. This approach is reported to be effective because insects not only transmit fungal spores but they also create wounds on fruit which serve as infections site (Coates and Johnson, 1997). These chemicals are generally applied as dips, sprays, fumigants, treated wraps and box liners or in waxes and coatings (Narayanasamy, 2006). Table 2 summarises some of the fungicides used for postharvest decay control, their mode of action with their limitations.

Synthetic fungicides are primarily used to control postharvest losses. However, these applications are associated with the accumulation of chemical residues as well as the production of secondary effects on fruit qualities (Weston and Barth, 1997; Sibomana *et al*., 2016). In addition, the loss of effectiveness of conventional fungicides due to the appearance of resistant strains has increased the search for low cost, non-chemical approaches for the control of postharvest diseases (Weston and Barth, 1997; Liu *et al*., 2013; Bhattacharjee and Dey, 2014; Sibomana *et al*., 2016). The costs of registration of new products is another problem affecting the development of new fungicides due to the increasing requirements for tests for their toxicity and environmental impact (Auret, 2007).

Table 2: Registered fungicides currently available in SA to control postharvest diseases of fruit crops

Active ingredient, trade name	**Fungicide class**	**Mode of action**	**Main crops**	**Target pathogens**	**First reported resistance**	**Other Problems**	**Reference**
azoxystrobin Amistar®	Strobilurin (QoI)	Systematic, single-site, preventative, early curative, anti-sporulant	Mango, citrus, avocado, tomato	Sour rot, anthracnose, green and blue mold	1998	Phytotoxicity	Erasmus, 2014
imazalil Magnate® Fungaflor®	Imidazole (DMI)	Systematic, single-site, curative and protective	Citrus	Green and blue mold	1986	Nephrotoxicity, teratogenicity, carcinogenicity suboptimal residue level	Eckert, 1990; Holmes and Eckert, 1995; Gupta, 2018
fludioxonil Scholar®	Phenylpyrrole	Non-systematic, contact, multi-site, protective, anti-sporulant	Citrus, pome fruit	Green and blue mold, stem end rot	2013	Eye irritation, long term exposure may cause liver necrosis, kidney nephropathy and mild anaemia	Cosseboom, 2018 Gupta, 2018
guazatine Zanoctine® Panoctine®	Guanidine	Non-systematic, contact, multi-site, curative and preventative		Sour rot, blue and green mold	1983	Eye and skin irritation, residue level	Wild, 1983; Scordino *et al*., 2008
prochloraz Sportak® Omega®	Imidazole	Non-systematic, contact, multi-site, translaminar, protective and curative	Avocado, citrus, mango	Sour rot, anthracnose, *Alternaria*, *Botrytis*, *Fusarium* spp	1990	Impurity hazard; toxic to aquatic species	Danderson, 1986; Stafford, 1996; FAO, 2009
pyrimethanil Philabuster®	Anilinopyrimidine	Systematic, Contact, single-site, curative	Apple, pome fruit	Green mold	2009	Toxicity to aquatic species	Kinay et al., 2007; Caiazzo *et al*., 2014; Araújo *et al*., 2015
SOPP Preventol®	Substituted phenols and salts	Non-systematic, multi-site, curative	Pears, citrus	Blue mold only	1962	Phytotoxicity, carcinogenic	Harding 1962; Kinay *et al*., 2007; Erasmus, 2014; Xue *et al*., 2016; Palou and Smilanick, 2019
Thiabendazole Vorlon® Tecto®	Benzimidazole (MBC)	Systematic, single-site, curative	Stone and Pome fruit, citrus	Green mold, stem end rot, anthracnose, brown rot	1970	Nephrotoxicity, teratogenicity, carcinogenicity	Harding,1962; Kinay *et al*., 2007; Palou and Smilanick, 2019

DIM: Demethylation inhibitor; QoI: Quinone outside inhibitors; MBC: Methyl benzimidazole carbamates; SOPP: Sodium ortho-phenylphenate

2.9 Physical control of postharvest diseases

In recent years, finding alternative methods that are safe and effective in reducing postharvest losses of harvested commodities has been the focus of much research because of the strict regulations of the use of new and existing fungicides, combined with pathogen resistance development (Wisniewski *et al.*, 2016). Physical methods have been used as an alternative method to synthetic fungicides due to the absence of residues on treated fruits, with minimal health and environmental impact (Palou, 2013; Zhang *et al*., 2017). In general, physical applications can be grouped into non-thermal or thermal treatments.

2.9.1 Non-thermal physical control

Non-thermal treatments involve the application of edible coatings, UV-C irradiation, ozone treatment, modified atmosphere, controlled atmosphere and plant extracts (Zhang *et al*., 2017). Table 3 summarises some examples of successful non-thermal physical treatments available to control postharvest losses, with their possible limitations.

2.9.2 Thermal (heat-based) physical control

Thermal (heat) treatments have been used to control postharvest decay and pests in fruits and vegetables since the 1920s. However, they became economically unattractive with the discovery of new fungicides and pesticides. Heat treatments are the most important and popular alternative postharvest disease control measures due to their complete safety with no concern during application and zero residue on fruit, and ready implementation without the requirement of registration (Lurie and Pedreschi, 2014; Spadoni *et al*.; 2015). Postharvest heat treatments may be used to eradicate pathogens or pests that are present on the fruit surface, to modify the fruit response to other stresses and to maintain the overall fruit quality during storage and the supply chain (Lurie and Pedreschi, 2014; Spadoni *et al*.; 2015). On the other hand, heat treatments can cause physical damage and physiological disorder to fruits and vegetables. Furthermore, damage incidence can increase with increasing treatment temperatures and duration, as well as prolonged cold storage. The use of heat treatments depends on two parameters: the temperature used, normally between 37 and 65°C, and the exposure time, which varies from few seconds to several days (Rodoni *et al*., 2016).

Table 3: Examples of successful non-thermal physical treatment methods used to control postharvest loss

Treatment	Fruit	Pathogen	Limitation	Reference
Ultraviolet-C (UV-C)	Tomato Papaya	*Rhizopus stolonifer, Botrytis cinerea, Colletotrichum gloeosporioides*	Lack of penetration	Stevens *et al*., 2004; Cia *et al*., 2007; Charles *et al*., 2009
Modified Atmosphere	Peach Apple	*Penicillium expansum B. cinerea*	Condensation inside packaging, microbial growth and decay	Karabulut and Baykal, 2004
Ozone	Tangerine Longan	Naturally occurring decay *Penicillium digitatum*	No penetration of natural openings	Whangchai *et al*., 2010; Boonkorn *et al*., 2012
Edible coatings	Tomato Strawberry	*P. expansum, B. cinerea*	Lack of edible materials with desired protein, regulatory challenges	Liu *et al*., 2007; Feliziani *et al*., 2015
Thyme oil, Lemongrass oil	Avocado Peach	*C. gloeosporioides, R. stolonifer, B. cinerea*	Possible irritation and toxicity	Arrebola *et al.* 2010; Sellamuthu *et al*., 2013; Mbili, 2015
Gamma radiation	Grapes	*C. gloeosporioides*	Capital intensive, low consumer acceptance due to perceived association with radioactivity	Cia *et al*., 2007
Controlled atmosphere (CA)	Apple	*Colletotrichum acutatum*	Capital intensive; needs a high volume of fruits	Janisiewicz *et al*., 2003

Heat treatments can be applied as hot water treatment (HWT), vapour (moist) heat treatment (VHT) and hot (dry) air treatment (HAT), far-infrared radiation, or electromagnetic energy (Geysen *et al*., 2005). HWT, VHT and HAT are the conventional heating methods used as postharvest treatments of fruit (Geysen *et al*., 2005). Applications are by means of a batch, continuous or drainage systems (Sivakumar and Fallik, 2013).

VHT has gained commercial acceptance in many countries as quarantine purpose for many tropical and subtropical fruits such as mango and papaya (Siddiqui, 2018). Heat transfer is accomplished by the condensation of water vapour on the relatively cool fruit surfaces (Geysen *et al*., 2005). VHT can be long or short, depending on the sensitivity of the fruit to temperature (Siddiqui, 2018). During VHT, the interior region of the fruit is heated to the desired temperature for long enough to kill the insect. Fruits then are cooled immediately after the holding period in order to prevent fruit heat injury due to high humidity.

HAT applications are mainly used for quarantine purposes of subtropical fruits against insects. HAT times are considerably longer (12-96 hours) compared to HWT or VHT, at temperatures ranging from 38°C to 46°C (Geysen *et al*., 2005; Siddiqui, 2018). Treatments are accomplished by placing the fresh produce in a heating chamber and passing hot air (without steam) over the commodity (Lurie, 1998; Siddiqui, 2018). The heat transfer can be improved by controlling the air circulation using ventilation fans in the heating chamber or by applying forced hot air where the speed of air circulation is precisely controlled (Geysen *et al*., 2005; Siddiqui, 2018). Owing to its slow heat transfer and lower humidity than VHT and HWT, HAT has been proposed as a safer treatment with a reduced risk of damage to the fresh produce (Siddiqui, 2018). HAT prevents condensation in the treatment areas and fruit surfaces, preventing fruit desiccation and scald (Collin *et al*., 2007).

HWT is the most important and popular postharvest disease control measures because it is relatively effective, simple, cheap, easy to apply and can be combined with other disease control methods (Geysen *et al*., 2005; Palou, 2013). Water is the most efficient medium for delivering thermal energy to the fruit surface (Geysen *et al*., 2005; Pareek, 2017). This will be discussed in detail in the next section, Section 2.8.3. Table 4 summarises successful heat treatments on fresh produce, with their intended purpose.

Table 4: Successful heat treatment methods on selected fresh harvested produce and their aim

Crop	Treatment	Optimal temperature x time	Aim	Reference
Date fruits	HAT	55°C, 30 min 60°C, 15/20 min	Quarantine	Ben-Amor *et al*., 2016
Peach	HAT	38°C, 3 h	Maintain quality	Huan et al., 2017
Basil	VHT	38°C, 8 h	Control decay and chilling injury	Aharoni *et al*, 2010
Mango	VHT	48°C, 20 min		Gan-Mor *et al*., 2011
Tomato	HWD	39 or 45°C, 60 min	Chilling resistance, decay control	McDonald *et al*., 1999
Broccoli	HWD	50°C, 3 min	Maintain and enhance quality	Perini *et* al, 2017
Peach	HWD	60°C, 1 min		Spadoni *et al*., 2014
Papaya	HWD	48/50°C, 20 min	Anthracnose and stem end rot control	Martins *et al*., 2010
Strawberry	HWRB	60°C, 20 s	Decay control, quality maintenance	Jing et al., 2010
Apple	HWRB	55°C, 20/25 s	Control storage rots	Maxin et al., 2012
Tomato	HWRB	52°C, 15 s	Decay control, ripening inhibition, chilling and decay resistance	Ilic *et al*., 2001; Fallik et al., 2002
Tomato	HWRB		Cleaning	Fallik *et al*., 1996
Banana	HWT	45°C, 5 min	Quality maintenance and shelf-life extension	Siddiqua *et al*., 2018

2.9.3 Rapid hot water treatment (rHWT) for the control of postharvest pathogens

HWT was originally used to control fungal pathogens, which are typically found on the surface or in the first few cell layers under the peel of the fruits, but its use was later extended for insect disinfestation (Geysen *et al*., 2005). It is accomplished either through spraying, dipping, or rinsing and brushing (Pareek, 2017).

Hot water dips (HWD)

HWD for postharvest decay control is done at a comparatively low temperature (50–60°C) and for shorter period (up to 10 min), while HWD for insect disinfestations can last up to 1h or more at temperatures below 50°C. The difference in the temperature and exposure time is due to the fact that hot water treatments for decay control need only surface heating in order to trigger a cascade of host resistance responses, which then produce antifungal compounds and pathogenesis-related proteins, which may reduce the pathogen propagules while treatments targeted for pest control are required to bring the total commodity to a desired proper temperature (Pareek, 2017). Typically,

the treatment tank has a heat exchanger unit with a water circulation system, and a temperature controller unit to ensure that there is a uniform and consistent temperature profile throughout the treatment tank, at or slightly above the set point temperature (Tsang *et al*., 1995).

Hot water rinsing and brushing (HWRB)

HWRB for cleaning and postharvest decay control of freshly harvested produce is done at relatively high temperatures of 45-62°C for a very short time, lasting 15-25s, whereby the temperature, intensity and duration of the treatment are controlled by varying the speed of the brushes and the number of spouts (Fallik, 2011). It is an improved technique over the HWD in which the machine is incorporated into a sorting line with produce first being rinsed from above with non-heated tap water, before passing over revolving brushes while being sprayed with hot water at the optimized temperature with a short exposure time (Pareek, 2017). HWRB was commercially introduced in 1996 (Fallik, 2004) and has been applied to several fruits and vegetables such as apple, grapefruit, litchi, kumquat, sweet pepper, tomato and citrus fruits to control decay and to maintain fruit quality after prolonged storage and shelf-life (Fallik, 2004, 2011).

Besides its effectiveness against fungi and insects, and its impact on fruit quality, the economic feasibility of HWRB in terms of cost, time and equipment complexity is the major factor limiting the commercial applicability (Pareek, 2017). Operating HWRB requires additional equipment, energy costs and packhouse space. HWT treatments are considerably cheaper than HWRB and other heat treatment methods (Fallik, 2004). According to Fallik (2004), the cost of commercial HWD technology is about 10% of commercial VHT technology. The occurrence of superficial brush injuries is the main disadvantages of HWRB over HWD treatment methods (Smilanick *et al*., 2003). HWD is easy to assemble, simple to operate and affordable (Tsang et al., 1995).

2.9.4 Mode of action of heat treatments

The effect of heat treatments on the reduction of postharvest diseases is a combination of a direct effect on the pathogen and indirect effect on the fruit host. The heat directly affects the conidia or the pathogen hyphae which are present on the rind wound depending on the temperature and treatment duration (Palou, 2013). The variation in heat sensitivity is significant among fungal species and is also dependent on their life stages such as mycelium, dormant conidia or germinating

conidia (Geysen *et al.*, 2005). According to Geysen *et al.* (2005), non-germinated conidia are more heat tolerant than germinated conidia or mycelium. Other factors such as the moisture content of the conidia, age of the inoculum and the inoculum concentration can also affect the response of fungi to heat (Geysen *et al.*, 2005). The general direct effects of heat on the pathogenic structures include: changes in the nuclei and cell wall structures, protein denaturation, destruction of mitochondria or outer membranes, disruption of vacuolar membranes, formation of gaps in the cytoplasm, lipid liberation, destruction of hormones, asphyxiation of tissue, depletion of food reserves, or metabolic injury with or without accumulation of toxic intermediates which lead to reduced inoculum levels and decay control (Palou, 2013). It has been reported that more than one of these mechanisms can be triggered at the same time, to a different extent (Palou, 2013).

The indirect effect of heat treatment is based on constitutive and induced defence mechanisms against pathogens and pests in fruit. The effect of HWT treatment on the rearrangement of the outer epicuticular wax layer has been reported as part of the constitutive defence mechanism of heat-treated fruit which reduce cuticular cracks and acts as a barrier for pathogen penetration (Lu et al., 2007). Induced defence mechanism involves complex interactions, which trigger physiological and pathological responses such as the production of antimicrobial chemical compounds and pathogenesis-related proteins (Pareek, 2017). Apart from the nature and characteristics of heat treatment, this induction, however, depends greatly on the genotype and physiological condition of the fruit during the application of the treatment (Palou, 2013).

Fruits are exposed to low temperature in order to reduce the normal respiration and delay ripening and senescence, and consequently, extend the shelf-life of produce. However, many commodities including tomatoes are reported to develop chilling injury (CI) if the temperatures are too low and below critical temperatures (Lurie and Pedreschi, 2014). The effect of pre-heat treatments on the induction of chilling tolerance and inhibition/delay of ripening has been reported in many horticultural crops. Induction of resistance to CI on heat-treated commodities is associated with the presence of heat shock proteins (HSPs) present in their tissues and the protective effect they exert. This is an irreversible action in which HSPs increase as a result of heat stress and generally disappears rapidly when the plant is returned to ambient temperature (Lurie and Pedreschi, 2014). Metabolic profiling of tomato studies by Luengwilai *et al.*, (2012) has shown that when comparing control fruit to heat-treated fruit at 40°C HWT for 7 min 'Heat-Shock' treatment before cold

storage at 2.5℃ for 14 days showed that the heat treatment provided protection from chilling by altering the levels of fruit metabolites. Treated fruits had low levels of arabinose, fructose-6-phosphate, valine and shikimic acid. They had higher levels of four sugars, three organic acids, one fatty acid, one amino acid, as well as allantoin and putrescine, relative to the control, even after cold storage. They concluded that these changes were associated with heat-shock proteins (HSPs) that induced chilling tolerance (Luengwilai *et al*., 2012). Zhang *et al*., (2013) also investigated the role of the products of the arginine pathway in contributing to resistance to chilling injury. HAT treated tomato fruits at 38℃ for 12 h and stored at 2℃ for 28 days showed higher levels of arginine, proline and putrescine, as well as increased activities of the antioxidative enzymes SOD, CAT and APX. Arginase induction was indicated to be partly involved in HA-induced chilling tolerance in tomato fruit, possibly by a mechanism involving activation of antioxidant enzymes and an increase in proline levels (Zhang *et al*., 2013). Heat treatments applied prior to low-temperature storage has also been reported to activate the antioxidant properties of tomato fruit, thereby protecting the fruit from the damaging effects of reactive oxygen species that are associated with chilling injury (Rees and Orchard, 2012). The inhibition of ripening by heat treatment is mediated by its effect on the ripening hormone, ethylene, and cell wall degrading enzymes (Lurie and Pedreschi, 2014). During heat stress, polyribosomes disassociate rapidly, protein synthesis stops briefly and then resume with a new set of proteins, including HSPs (Lurie and Pedreschi, 2014). As a result, the normal ripening processes of the fruit are inhibited. The inhibition of ripening will persist for some time if the treated commodity is kept at a low temperature, whereas ripening occurs when the commodities are rewarmed after storage (Lurie and Pedreschi, 2014). Table 5 summarises successful heat treatments and possible mode of action to control postharvest diseases of fruits and vegetables.

Table 5: Examples of successful heat treatments and their mode of action to control postharvest diseases of selected fruits

Treatment	**T X t**	**Crop**	**Disease or pathogen**	**Mode of action**	**Reference**
Slow HWTs (>10 minutes to hours)					
HAT	38°C, 36 h	Tomato cherry	*Pichia guilliermondii*	Plant defence response due to increased levels defence-related genes (PAL and GNS)	Zhao *et al*., 2009
HAT	44°C; 1 h 54 min	Sweet cherry	*Penicillium expansum*	Induction of host resistance	Wang *et al*, 2015
HWD	43°C, 30 min	Pear	*B. cinerea*	Direct inhibition by ROS generating NoxA gene expression causing oxidative damage to spores and germ tubes	Zhao *et al*., 2014
HWD	45°C, 25 min	Lemon	Fusarium rot	Direct fungal inhibition with the elicitation of defence response	Sui *et al*., 2014
VHT	52.5°C;20/24 min or 55°C; 18/21 min	Table grape	*B. cinerea*	Inhibition of fungal growth	Lydakis and Aked, 2003
HWD	48°C, 12 min	Peach	*Monilina laxa*	Inhibition of spore germination and fungal growth	Jemric *et al*., 2011
Quick HWTs (>5 minutes to 10 minutes					
HWD	48°C, 10 min	Peach	Brown rot	Stress-related protein synthesis, ROS activation, sHSPs gene expression leading chilling tolerance and extended shelf-life	Huan *et al*., 2017
HWD	40°C, 10 min	Tomato	Reduced decay	Activation of antioxidant enzymes (increased levels of POD and CAT, decreased level of PPO)	Boonkorn, 2016
HWD	40°C, 10 min	Peach	*Monilinia fructicola*	Inhibition of spore germination and germ tube elongation; induction of defence-related genes such as CHI, GNS and PAL	Liu *et al*., 2012
HWD	48°C, 6 min	Nectarine	*M. laxa*	Inhibition of conidial germination and fungal growth	Jemric *et al*., 2011

Very quick HWTs (1 to 5 min)					
HWD	55°C; 5 min	Mango	*Colletotrichum gloeosporioides*	Host resistance induction (PAL, GNS)	Benitez *et al*, 2006
HWD	54°C, 4 min	Papaya	*C. gloeosporioides*	Enhanced host resistance	Li et al., 2013
HWD	55°C, 3 min	Mango	*C. gloeosporioides*	Unclear	Chiangsin *et al*., 2016
HWD	56°C, 2 min	Orange	*Guignardia citricarpa*	Directly killing the pathogen or indirectly inducing fruit disease-resistance mechanisms	Yan et al., 2016
HWD	60°C, 1 min	Peach	Brown rot	Inhibition of conidial germination	Spadoni *et al*., 2013
Rapid HWTs (<1 min)					
HWRB	55°C, 15/20 s	Mango	*Alternaria alternata*	Induced host resistance	Lurie *et al*., 2014
HWD	56°C, 20 s	Orange	*Penicillium digitatum*	Inhibition of conidial germination due to increased levels of oxygenated monoterpenes, esters and aldehydes	Strano et al., 2014
HWD	60°C, 20 s	Peach	*M. laxa*	Enhanced the expression levels of PAL, HSP70, APX, MNSOD, CAT and GR led to reduced expression of cell wall genes mainly involved in ripening	Spadoni *et al*., 2014
HWB	62°C, 20 s	Grapefruit	*P. digitatum*	Accumulation of CHI and GNS proteins, induction of host resistance	Pavoncello *et al*., 2001

The overall quality of fresh produce treated with optimized HWTs is significantly better than untreated produce as has been found by a significant reduction in decay incidence and maintenance of the quality of several fruits. However, in spite of all the achievements of heat treatments, most previous studies have been performed at relatively low temperatures with long exposure times. Temperature values and treatment time are the most critical factors for an effective and successful outcome (Fallik, 2004). Fruits and vegetables can tolerate high temperatures up to 75℃, however, there could be heat damage to the sensitive tissues of the commodities if they are treated with high temperature combined with long exposure times (Tuan *et al*., 2004; Palou *et al*., 2001). In recent studies, higher temperatures with shorter exposure time combinations have been found to be more effective (Tuan et al., 2004; Strano et al., 2014; Wang *et al*., 2017). Strano *et al*., (2014) studied the effectiveness of two hot water treatments to control *P. digitatum* in citrus fruits. The first treatment was at 52℃ for a long exposure time of 180s, while the second treatment was at a higher temperature of 56℃ for a shorter time of 20s. The results were compared with fruits treated with an effective standard fungicide, imazalil, and a non-treated control. The results showed effective inhibition of *P. digitatum* at treatments which had lower temperatures with longer exposure time (52℃, 180s). However, better disease management was recorded with the treatments of high temperature and short exposure time (56℃, 20s). There was no surface damage and colour change on the fruits, and the treatment had no effect on the internal quality parameters of the fruit (Strano et al., 2014). Similar results have also been recorded for mangos (Wang *et al*., 2017). The effects of three hot water treatments (50℃, 10 min; 60℃, 1 min and 70℃, 5s) on the physical, physiological and biochemical quality of ivory mangoes were studied. The results showed that all hot water treatments applied to ivory mangos improved the quality during storage. However, the 60℃ x 1 min treatment was the most effective method (Wang *et al*., 2017). In another study on cherry tomato fruits, fruits treated with low temperature and long exposure time treatments developed heat damage, leading to ripening abnormalities. The low temperature/long exposure time treatment did not affect lycopene synthesis because there was no colour delay recorded on fruits. The fruits also developed low acidity levels after the low temperature/long exposure treatments due to an increase in the respiration rate, which had a negative impact on the quality of cherry tomato fruits (Tuan *et al*., 2004).

Although high temperatures may cause heat damage, when combined with short exposure times, they may provide an advantage in the postharvest processing technology in terms of energy cost and efficiency. The period or duration for fruit immersion is an obstacle in

packhouses where large volume of products needs to be processed quickly (Strano *et al*., 2014). Therefore, high temperature/short exposure treatments may eliminate the delay in processing time, while controlling postharvest decay and maintaining the overall fruit quality. A rapid hot water treatment (rHWT), equivalent to pasteurization, represents a promising alternative to synthetic fungicides for the management of postharvest diseases.

2.10 Biological control of postharvest diseases

Since 2000, the use of microbial antagonists has emerged as an important component of postharvest disease control, as an alternative to synthetic fungicides for reducing decay losses in harvested commodities. Biological control means the use of beneficial microorganisms or biocontrol agents (BCAs), and products to suppress organisms causing disease, through direct or indirect manipulation of the organisms and their host environment (Sharma *et al*., 2009). Many microbial antagonists, including yeasts, fungi and bacteria, have been developed as successful BCAs. Their effectiveness as BCAs results from their ability to compete for nutrients and space that allows for the rapid establishment within the stable microflora in the host plant, and to improve plant health and stimulate root growth (Sharma *et al*., 2009). Naturally occurring yeast antagonists isolated from fruit surfaces or artificially introduced antagonists have been reported to be effective for postharvest disease management (Sharma *et al*., 2009). Yeasts have been of a particular interest among these antagonists because they have simple nutritional requirement; they can adapt to the fruit microenvironment; they colonize wound sites after extended periods under dry conditions; they can survive a wide range of environmental stresses; they grow rapidly on an inexpensive media; and they are easy to produce in large quantities without producing toxic metabolites (Sharma *et al*., 2009; Stocco *et al*., 2019).

2.10.1 Mode of action of biocontrol agents

Several yeast biocontrol agents have been reported to effectively control postharvest diseases for fruits and vegetables (Pal and Gardener, 2006; Sharma *et al*., 2009; Spadaro and Droby, 2016). The mode of action of yeast biocontrol agents is categorized into direct and indirect microbial effect against target pathogens (Table 6). Microorganisms achieve antagonistic interaction by occupying the same ecological niche as the pathogen and directly suppress it. The indirect effect of microbial antagonists on the target pathogen is achieved by interacting

with the host tissue, inducing host resistance which provides protection against the pathogen (Köhl *et al*., 2019).

Table 6: Mode of action of biocontrol agents

Direct antagonism	**Indirect antagonism**
Antibiotic production (Ren *et al*., 2012) Lytic enzyme production (Ferraz *et al*., 2016) Parasitism (Aguirre-Güitrón *et al*., 2019) Attachment and colonization of fungal hyphae and inhibition of conidial germination (Nantawanit *et al*., 2010; Xu et al., 2013; Chen *et al*., 2018)	Competition for space and nutrient and space (Aguirre-Güitrón *et al*., 2019) Induction of host resistance (Droby *et al*., 2002)

Understanding the host-pathogen-microenvironment-antagonist interaction system is crucial for successful biological control strategies to be developed (Spadaro and Droby, 2016). A schematic representing this interaction is presented in Figure 5. The wound site, which is the court for pathogenic infection, is of particular interest in studying the mechanism of action of the microbial antagonists. During infection, the pathogen is reported to release pathogen-associated molecular patterns molecules (PAMPs) that can be recognized by specific plant recognition receptors, which trigger the initial immune response by the fruit that is associated with a small oxidative burst. The response varies depending on the fruit species, cultivar and also the physiological stage. However, pathogen may overcome the fruit's first line of defence by releasing effectors. These suppress further defence mechanisms of the fruit, making the fruit surface or tissue susceptible to infection. In the cases where the pathogen is unable to manipulate the fruit defence mechanisms, the fruit will typically respond by triggering a strong oxidative burst followed by the synthesis of phytoalexins and pathogenesis-related proteins. Some pathogens are reported to actively stimulate an oxidative burst, which may lead to cell death, necrosis, and colonization of the dead tissue. Pathogens then start to release cell wall degrading enzymes and/or phytotoxins, which are regulated by host pH modification (Spadaro and Droby, 2016).

Injured fruit surfaces and wound sites are rich in nutrients, such as glucose, and are readily available for pathogens. Damaged fruits are reported to release damage-associated molecular pattern molecules (DAMPS) in order to trigger secondary defence mechanisms that are regulated by the jasmonate signalling pathway. This activates wound healing processes. Further

release of a strong oxidative burst, synthesis of phenolics, and the formation of corky cells then aid in protection against pathogen invasion (Spadaro and Droby, 2016). The growth of pathogens that gain access to the wounded fruit surface can be inhibited by plant substances that are either present or induced in response to the injury or infection. This may lead to oxygen depletion in the wound microenvironment due to plant cell respiration and rapid colonization by microorganisms that are able to tolerate an oxygen deficiency in the environment (Spadaro and Droby, 2016).

Wounded fruit also responds to various yeast elicitors by regulating the yeast population density through changes in pH, the production of oxidative compounds, and inducing a change in yeast morphology. In turn, yeasts adhere to host tissues or pathogen cell walls and exert their antagonistic effect through a direct effect on the pathogen or indirectly by interacting with the host tissue, inducing host resistance, which provides protection against the pathogen (Liu et al., 2013).

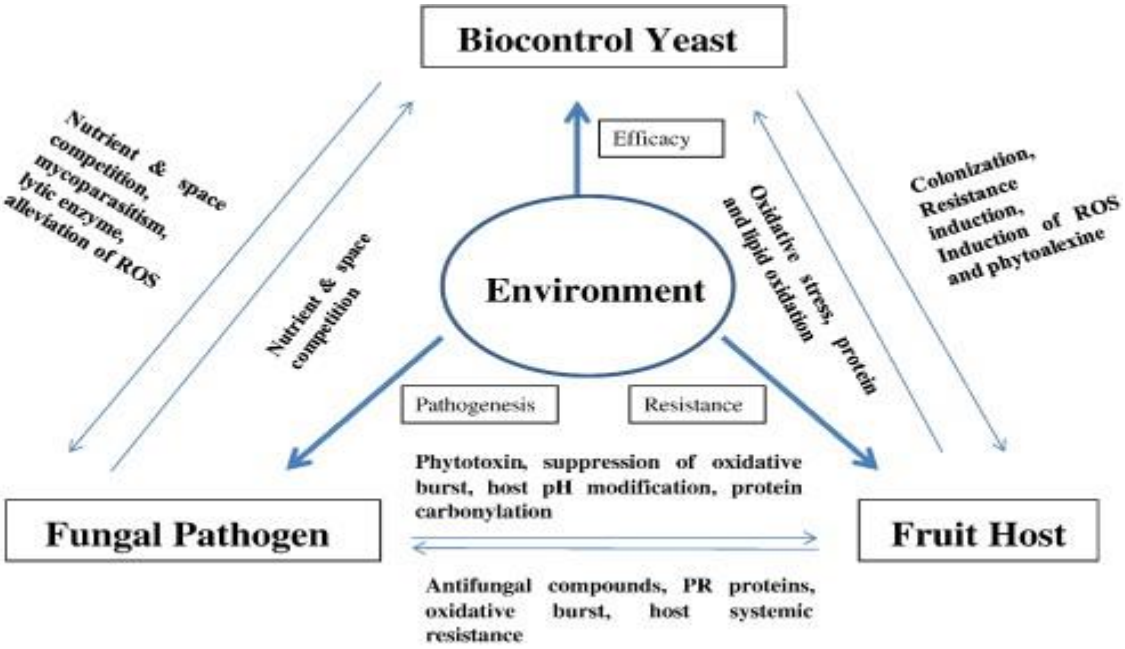

Figure 5: Possible interactions between host, pathogen, antagonist and the environment (Liu *et al*., 2013)

2.10.2 Yeasts: potential microbial antagonists

Several yeast antagonists have been described as successful biocontrol agents (BCAs) in pre- and postharvest treatments. However, they work best in postharvest applications. In postharvest applications, yeast antagonists have a positive effect that lasts from the time of treatments to consumption. However, when applied as a pre-harvest, they can protect the fruit for long

periods but the unpredictable and highly variable conditions in the field makes pre-harvest applications of yeasts less reliable (Wisniewski and Wilson, 1992; Sharma *et* al., 2009). According to Wisniewski and Wilson (1992), postharvest facilities have controlled environments, which favours the reliable performance of BCAs. BCAs are generally applied on wound sites, and are active for short periods, which allows postharvest yeast BCA applications to be effective, practical and useful for decay control (Wisniewski and Wilson, 1992; Sharma *et* al., 2009). They are applied as postharvest sprays or dips (Sharma *et al*., 2009). Table 7 summarizes some of the successful biocontrol agents that have been developed for biological control of postharvest diseases of various crops.

Table 7: Examples of microbial antagonists used for successful control of postharvest diseases of fruits and vegetables and possible mode of action

BCA	**Disease**	**Fruit**	**Mode of action**	**Reference**
Debaryomyces hansenii	*Galactomyces candidum*	Citrus	Competition and Induction of host resistance	Wilson and Wisniewski, 1989
D. hansenii	*G. candidum*	citrus	ND	Chalutz and Wilson, 1990
Candida oleophila (I-182)	*Penicillium digitatum*	Grape	Induction of host defence	Droby *et al*., 2002
C. oleophila	*Colletotrichum gloeosporioides*	Papaya	ND	Gamagae et al., 2003
Pichia membranaefaciens	*Rhizopus stolonifer*	Apple	Attachment and lytic enzyme secretion	Chan and Tian, 2005
Metschnikowia pulcherrima	*Botrytis cinerea*	Apple	Iron depletion	Saravanakumar *et al*., 2008
Candida guilliermondii, Cryptococcus laurentii	*Monilinia fructicola*	Peach	Alleviation of oxidative damage of fruit host	Xu *et al*., 2008
Pichia pastoris	*Galactomyces citri-aurantii*	Citrus	Antibiosis	Ren *et al*., 2012
Cystofilobasidium infirmominiatum	*Penicillium expansum B. cinerea*	Apple	ROS tolerance	Liu et al., 2012
Candida azyma	*G. citri-aurantii*	Citrus	killer activity and hydrolytic enzyme production	Ferraz *et al*., 2016
Meyerozyma caribbica (fresh or dry formulation)	*C.gloeosporioides*	Mango	Competition, hydrolytic enzyme production, biofilm formation, parasitism	Aguirre-Güitrón *et al*., 2019

ND: Not determined

Although many different yeasts, isolated from a variety of sources, have been reported as good postharvest biocontrol agents, only a few yeast-based biological control products have been

developed and commercialized (Table 8). These products are registered for use on several different commodities to control several different pathogens. The ability of the antagonists to control different pathogens on different commodities is essential for the economic viability of a postharvest biocontrol product (Liu *et al*., 2013; Sharma *et al*., 2009).

Table 8: Examples of biological products developed to control postharvest diseases of produce

Product name and BCA	**Country**	**Target pathogen**	**Reference**
Aspire *Candida oleophila*	Ecogen, Inc., USA	blue, grey, and green moulds	Mercier and Wilson, 1994; Wisniewski *et al*., 1995
Avogreen *Bacillus subtilis*	South Africa	anthracnose	Korsten *et al*., 1997; Janisiewicz and Korsten, 2002
Biosave *Pseudomonas syringae*	EcoScience Corporation, USA	blue and grey mould, *Mucor*, and sour rot	Janisiewicz and Korsten, 2002; Droby, 2006
Serenade *Bacillus subtilis*	Agro Quess Inc., USA	powdery mildew, late blight, brown rot	Pusey and Wilson, 1984
Shemer *Metschnikowia fructicola*	Bayer/Koppert The Netherlands	*Penicillium, Botrytis, Rhizopus, Aspergillus*	Ferraz and Lucas, 2019
Yieldplus *Cryptococcus albidus*	Anchor Yeast, South Africa	*Botrytis, Penicillium, Mucor*	Droby, 2006

2.11 Integration of rapid hot water treatments and biocontrol agents for the control of postharvest pathogens of tomato

The absence of chemical residues in/on fruits is a major advantage of heat treatments for the control of postharvest diseases. Their minimal impact on the environment is important due to the growing need to implement non-polluting antifungal treatments as an answer to regulatory and consumer demands (Palou, 2013). However, the potential acquisition of thermotolerance and the development of heat resistance by pathogens; lack of preventive activity; the low persistence and the inconsistency associated to the nature and the mode of action of these treatments have been the main limiting factors in postharvest heat treatments (Palou, 2013). Long heat exposure treatments cause internal damage such as poor colour development, flesh softening, and the development of internal cavities, as well as external damage: scalding, shrivelling and failure to softening, which may result in increased susceptibility to decay (Sivakumar and Fallik, 2013; Pareek, 2017). The use of heat treatments is effective against prior infections, but it does not provide a residual action, and is therefore inadequate to protect fruits from future decay (Pareek, 2017; Schirra *et al*., 2000). Biocontrol agents are also

important alternatives to chemicals, however, currently available microbial antagonists do not control previously established infections and they often fail to consistently control postharvest infections or to provide acceptable levels of control of fruit diseases (Karabulut *et al*., 2002; Abd-Alla *et al*., 2007).

Due to all these limitations, the use of hot water treatments and biocontrol agents as standalone treatments have failed to effectively control postharvest pathogens (Abraham, 2010; Palou, 2013). Therefore, considerable attention has been given to their integration to control postharvest diseases. In general, integration of treatments can pursue three different objectives:

- Additive and/or synergistic effects to increase the effectiveness and/or the persistence of individual treatments;
- To provide complementary effects, combining their curative and preventive activities, respectively;
- Potential commercial implementation of effective treatments that are too impractical, costly, or risky to apply as single treatments (Palou, 2008).

Preliminary studies have shown that the two treatment regimes complement each other (Palou, 2009; Sharma *et al*., 2009; Palou, 2013). HWT typically offers curative activity against existing or incipient pathogenic infections but does not effectively protect the fruit from future infections. Biocontrol agents can colonize rind infection sites and offer effective preventative activity against pathogens that may reach the treated fruit during storage or marketing. Both curative and preventative activity against postharvest pathogens can then be achieved at the same time if they are integrated, which may provide successful postharvest disease management without the application of synthetic chemical fungicides (Palou, 2009).

Despite all the modern storage facilities and technologies available in the developed countries, postharvest losses of tomato are estimated up to 30 to 40% of the total yield. Losses are more severe in undeveloped countries. Disease-causing fungal pathogens are the main cause for the postharvest decay of tomato. Considering the perishability, microbial susceptibility and short shelf-life of tomato fruits, the application of rHWT combined with BCAs may provide an effective and safe disease management strategy for tomato fruits, postharvest.

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Chapter 3: Isolation, and morphological and molecular characterization of pathogens causing tomato anthracnose and sour rot diseases

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Abstract

Tomato (*Solanum lycopersicum* L.) is one of the most widely grown and extensively consumed vegetable worldwide. Anthracnose and sour rot caused by *Colletotrichum* and *Galactomyces* species, respectively, are major fungal pathogens associated with tomato crop losses after harvest. The objectives of the presented study were to isolate and identify two primary pathogens of tomato in South Africa causing anthracnose and sour rot after harvest. A total of 55 isolates were recovered from symptomatic tomato fruits with typical symptoms of anthracnose and sour rot. The cultural and morphological characteristics of all isolates were observed and compared with standard scripts to establish their identity. Pathogenicity tests were performed, and the effect of wound and non-wound inoculation methods were studied for each isolate by scanning electron microscopy. The most pathogenic *Colletotrichum* and *Galactomyces* isolates were sent to Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa) for DNA extraction, PCR and sequencing in order to confirm their identities. Out of the 55 isolates, 33 were *Colletotrichum* spp., accounting for 60% of the isolates. The other 22 isolates were *Galactomyces* species. *Colletotrichum* isolates were further classified into *Colletotrichum gloeosporioides* and *Colletotrichum acutatum*, based on cultural and morphological analyses. All the *Galactomyces* isolates were morphologically identical and were identified as *Galactomyces candidum*. Among the isolated strains, C24 and C37A from the *Colletotrichum* isolates, and G18, G23 and G29 from the *Galactomyces* isolates, were extremely pathogenic. All wound and non-wound *Colletotrichum* inoculated fruits developed anthracnose, while non-wound *Galactomyces* inoculated fruits failed to develop sour rot, indicating that *Galactomyces* is strictly a wound pathogen. Molecular analyses confirmed the identities of these pathogens as *Colletotrichum gloeosporioides* (Penz.) and *Galactomyces candidum* (Link.). These results will contribute towards the development of effective control strategies.

***Keywords*:** *Colletotrichum gloeosporioides; Galactomyces candidum; Identification; Postharvest; Tomato.*

3.1 Introduction

Tomato (*Solanum lycopersicum* Mill.) is one of the most widely grown and extensively consumed vegetable worldwide (Sandoval *et al*., 2015). However, the climacteric ripening and high perishability of the fruit is affected by various pre-harvest practices. In particular, the treatment of the fruit during harvest and postharvest processes expose tomato fruit to various pathogenic microorganisms, leading to the fruit's rapid deterioration and loss after harvest (Arah *et al*., 2015). An estimated 50% of the loss of harvested tomato fruit is believed to be caused by microbial pathogens (Abd-Alla *et al*., 2009; Pinheiro *et al*., 2013; Sibomana *et al*., 2016). Postharvest diseases, especially those caused by fungal pathogens, cause significant economic losses (Klein and Kupper, 2018) because of their abundance, spore formation and resistance to several drying and environmental stress factors (Etebu *et al*., 2013). Fungi generally invade damaged or senescent tissues; therefore, they are referred to as opportunistic pathogens (Cooper *et al.*, 1978). Fungal pathogens, in general, execute a series of sequential steps in order to infect and cause disease. Those steps include fungal spread by wind, water or insects and come into contact with an appropriate host plant; spore germination, fungal attachment to host structures and recognition triggered by signals from the host and environmental factors; pathogen entry through wounds, natural openings or by direct penetration; pathogen infection and invasion of the host, pathogen reproduction and spore production in infected host tissue; and spore dissemination of these pathogens to other susceptible host or new plant (Sexton and Howlett, 2006). Anthracnose and sour rot caused by *Colletotrichum* and *Galactomyces* species, respectively, are major fungal pathogens associated with tomato crop losses after harvest (Wolf-Hall, 2010).

Anthracnose is one of the main economic constraints in tomato production and is responsible for significant losses worldwide (Gnanamanickam, 2002; Živković *et al*., 2010). *Colletotrichum* spp are among the most successful postharvest pathogens because they have an efficient stage of latent infection (Mello *et al*., 2004). Among the various species of *Colletotrichum*, *C. gloeosporioides* is one of the most important and devastating pathogens, causing tomato anthracnose (Živković *et* al., 2010). Typically, the pathogen infects fruit before harvest but becomes active during storage as the fruit ripens, causing visible lesions on fruit on market shelves. Symptoms appear as small circular, slightly sunken lesions on the fruit, which later develop concentric rings, with a water-soaked appearance beneath the skin (Agrios, 2005).

Anthracnose may cause up to a 100% loss in storage (Dean *et al*., 2012). Disease progression is rapid at temperatures of 25-28°C and a pH 5.8-6.5 (Mello *et al*., 2004).

Sour rot is caused by *Galactomyces* species, resulting in a rot with a foul smell, and it causes significant losses in tomato, carrot, citrus and other fruit and vegetable crops, pre- and postharvest, worldwide (Agrios, 2005; Bourret *et al*., 2013). Although there are many species in the genus, *G. candidum* is the only species that causes significant postharvest losses in fruit and vegetable crops (Bullerman, 2003). *Galactomyces* is strictly a wound-based pathogen, which penetrates fruits via the stem scars, skin cracks or injuries from insects causing mechanical damage. It infects fruits and vegetables during harvest and postharvest handling procedures (McKay *et al*., 2012). Infection of tomato fruits by *Galactomyces* may occur at the mature-green, ripe and overripe stages. Fruit that is stored in plastic bags or airtight packages are more susceptible to infection by *Galactomyces* (Agrios, 2005). Symptoms on tomato fruits appear as thick lesions with white, soft, creamy yeast-like colonies (Bullerman, 2003; Etebu *et al*., 2013). Disease progression is rapid at temperatures of 25-30°C and pH 2.0-8.5, and it can be spread by contact (Baudoin and Eckert, 1982; Talibi *et al*., 2014; Featherstone, 2015). However, it may also be active at temperatures as low as 2.0°C (Thornton *et al*., 2010).

Many species of *Colletotrichum* and *G. candidum* causes significant losses on a wide range of host crops. Identification among species were done by classical techniques based on morphological and cultural characteristics such as the colony colour, size and shape of conidia, growth rate, presence or absence of setae, etc. However, it is difficult to distinguish among species of a genus or between strains due to cultural similarities, minimal morphological variability among isolates and the broad host range of the pathogens. Therefore, these techniques should be complemented with molecular techniques to overcome the limitations of morphological identification (Alsohaili and Bani-Hasan, 2018).

The objectives of the present study were:

1. To isolate and identify primary pathogens of tomato causing anthracnose and sour rot using classical and molecular techniques.
2. To test their pathogenicity on healthy tomato fruits.
3. To study the effect of wound and non-wound inoculation methods on infection process and disease development.

3.2 Materials and Methods

3.2.1 Colletotrichum and Galactomyces isolation and maintenance

Tomato fruits with typical symptoms of the target pathogens were collected from different shops in Pietermaritzburg and brought to the Plant Pathology Laboratory for fungal isolation. Typical symptomatic fruits selected for anthracnose tests developed small circular, slightly sunken lesions on the skin with dark concentric rings and a water-soaked appearance directly beneath the skin (Agrios, 2005). For sour rot, fruits displaying thick lesions with white, soft, creamy yeast-like colonies were selected (Etebu *et al*., 2013).

The direct single spore isolation method, as described by Goh (1999), was used with slight modifications to isolate the target pathogens from the infected tissues of tomato fruits. Individual spores were removed from the surface of the infected tomato fruits using a sterile needle and were transferred directly onto potato dextrose agar (PDA) plates. The plates were incubated at 25°C for 7 days. Pure cultures were prepared by sub-culturing cuttings of 3 mm diameter agar plugs onto fresh PDA plates incubated at 28°C for 7 days. Agar plugs of 2 mm x 2 mm were transferred into double sterilized distilled water in McCartney bottles and stored at 4°C for medium-term storage and current use. For long term storages, the cultures of each isolate were maintained in glycerol stock (30% glycerol into double sterilized distilled water) in microfuge tubes and stored at -80°C. To re-activate the conidia and verify their virulence, the pathogens were wound inoculated into tomato fruit. After symptom development and sporulation, conidia from diseased fruit were transferred onto PDA plates to create fresh colonies.

3.2.2 Preparation of a spore suspension

All the isolates were cultured on freshly prepared PDA plates and were incubated at 25°C for 10 days. To prepare a fungal spore suspension, culture plates were washed off with distilled water and the suspension was filtered through 4 layers of cheesecloth to remove mycelia. Then the conidial concentration was determined using a haemocytometer and adjusted to the desired concentration with distilled water.

3.2.3 Cultural and morphological tests

All the isolates were cultured on freshly prepared PDA plates and they were incubated at 25ºC for 10 days. The conidial suspensions of isolates were prepared by the procedure mentioned in

Section 3.2.2. A drop of the suspension was placed on a glass slide and covered with the covering slip and was viewed under a light microscope for morphological analysis. All the plates were first observed for their cultural characteristics before the conidial suspension was prepared. The culture colour, conidial shape and size were used to differentiate each isolate morphology. The results were compared with standard scripts to establish the isolated identity and were recorded at a species level (Smith and Black, 1990; Than *et al*. 2008; Bourret *et al*., 2013).

3.2.4 Pathogenicity tests

The pathogenicity tests were done by following the methods described by Lewis-Ivey *et al*. (2004) and Than *et al*. (2008), with some modifications. Fresh tomato fruits were collected from the Pietermaritzburg Fresh Market Produce, Mkondeni, SA. Non-infected fruits were first sorted and disinfected with 1% sodium hypochlorite for 3 min, and washed three times with distilled water. They were gently blotted dry on sterilized tissue paper. Wounded/drop and non-wound/drop inoculation methods were used, as described by Than *et al*., 2008. For the wound inoculation methods, tomato fruits were pinpricked on the stem end area to a 1 mm depth and 5 µl of conidial suspension of each isolate at a concentration of 10^4 conidia ml^{-1} was pipetted on the wounded area of the fruits. For the non-wounded inoculation methods, 5 µl of the conidial suspension was pipetted in the stem end area without wounding the fruit. Control fruit was inoculated with 5 µl of distilled water. Inoculated fruits were placed in trays in a plastic container in order to maintain at least 90% relative humidity and initiate disease development. After 2 days, the plastic containers were removed and the fruits were incubated at room temperature. The experiment was repeated twice. Each experiment consisted of three replicates with 15 tomato fruits per replicate. The effect of wound and non-wound inoculation methods on disease incidence was observed after 3, 5, 7 and 10 days post-inoculation (dpi). Sample fruits were taken for wounding analysis using environmental scanning electron microscopy at 5 dpi. For pathogenicity, a disease index on a scale of 0 to 3^+ was established where 0, 1, 2, 3 and 3^+ correspond to none, slight, moderate, strong and extremely pathogenic, respectively. Fruits which were placed in Category 2, 3 and 3^+ were not marketable due to disease severity and physical damage. At 10 dpi, conidia from diseased tomato fruits were aseptically transferred on to PDA plates and were incubated at 25°C for 3 to 5 days. Microscopic tests were conducted using the resultant cultures for the colony and morphological characteristics of the spores to validate Koch's postulate.

3.2.5 Scanning electron microscopy (SEM) studies on the effect of wounding and pathogen development

The effect of wounding on disease development was studied using scanning electron microscopy following methods described by Capdeville *et al*. (2007), with some modifications. Sample tissues (3 mm x 3 mm) of each pathogen were cut from the wound and non-wound inoculated fruits. The with some modifications tissue material was fixed in 3% VV^{-1} glutaraldehyde for 3 hours and then washed in 0.05M sodium cacodylate buffer and dehydrated in a series of ethanol (10 min each in 30%, 50%, 70%, 80%, 90%, and 3 × 10 min in 100% ethanol) in a fume hood.

The specimens, mounted on SEM stubs, were then transferred into a critical point dryer basket under 100% ethanol and were dried in a Quorem K850 (Quorum Technologies Ltd., Ashford, Kent, UK) critical point dryer. All the specimens were coated with gold in a gold-palladium sputter coater (Quorum Q150R ES) (Quorum Technologies Ltd., Ashford, Kent, UK) and examined with a ZEISS, EVO LS 15 scanning electron microscope (Carl Zeiss SMT Ltd., Cambridge) operating at 5 kV.

3.2.6 Identification using molecular techniques and phylogenetic analysis

Fresh agar plate cultures of the most pathogenic *Colletotrichum* and *Galactomyces* isolates were sent to Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa) for DNA extraction, PCR and sequencing. Genomic DNA was extracted from the cultures using the ZR Fungal/Bacterial DNA Kit™ (Zymo Research, USA). The internal transcribed spacer (ITS) target region was amplified using OneTaq® Quick-Load® 2X Master Mix (NEB, UK) with the primers presented in Table 1.

Table 1: Primers used for PCR of *Colletotrichum and Galactomyces* ITS regions

Name of Primer	Target	Sequence (5’ to 3’)	Reference
ITS1	Small Sub-Unit	TCCGTAGGTGAACCTGCGG	White *et al*, 1990
ITS4	Large Sub-Unit	TCCTCCGCTTATTGATATGC	White *et al*, 1990

The PCR products were run on a gel extracted with the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, USA). The extracted fragments were sequenced in the forward and reverse direction using the BrilliantDye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000) (Nimagen, The Netherlands) and purified using a ZR-96 DNA Sequencing Clean-up Kit™, (Zymo Research, USA). Every sample of the purified fragments was analysed on an ABI 3500xl Genetic Analyzer (Applied Biosystems, ThermoFisher Scientific).

The .ab1 files generated by the ABI 3500XL Genetic Analyzer were converted into fasta files and analysed visually on MEGA X (Kumar *et al*., 2018) to generate the consensus sequences of the ITS target region of each isolate. The identity of the organisms under study was then determined using MolecularID on the MycoBank database and the nucleotide megablast online program of the National Center for Biotechnology Information nucleotide Basic Local Alignment Search Tool (BLASTn). Various matches/hits from the BLASTn results were then selected for the phylogenetic analysis. The evolutionary history was inferred using the Maximum Likelihood method and the best model fitting the data set with a 1000 bootstrap replications. Initial trees for the heuristic search were obtained automatically by applying the Maximum Parsimony method. The evolutionary model and the phylogenetic analyses were conducted in MEGA X (Kumar et al., 2018).

3.3 Results

3.3.1 Isolation of anthracnose and sour rot causing agents of tomato

A total of 55 isolates were recovered from symptomatic tomato fruits with typical characteristics of anthracnose and sour rot. *Colletotrichum* species dominated, with 60% of the total isolates. Based on the colour of the culture, mycelial abundance and spore masses on PDA plates, *Colletotrichum* isolates were classified into two groups: (i) white colonies, with very scarce mycelial growth and prolific production of conidia; and (ii) white colonies turning grey as the culture aged, with abundant mycelia and sparse production of conidia (Table 3.1). Of the 33 *Colletotrichum* isolates, 25 isolates were classified under Group-1 (75.8%) and 8 isolates were classified under Group-2 (24.2%). Out of the total fungal isolates, 22 (40%) were *Galactomyces*, and based on the colour of the colonies on PDA plates and spore shape, they were all classified as one group because all the isolates showed similar cultural characteristics and spore shape.

Table 3.1: Cultural and morphological characteristics of *Colletotrichum* and *Galactomyces* strains isolated from infected tomato fruits

Group	Isolate name	Culture colour	Conidial shape	Morphogroup
1	C1-5, 8, 11, 13-17, 19-23, 27, 31-37	White	Cylindrical	*Colletotrichum gloeosporioides*
2	C7, 18, 24-26, 28, 30, 40	White to light gray	Fusiform	*Colletotrichum acutatum*
Galactomyces	G1-18, 23-25, 29	Cream, yeast-like	Rectangular	*Galactomyces candidum*

3.3.2 Cultural and morphological characteristics

Based on the culture colour and the microscopic conidial shape, *Colletotrichum* isolates were divided into 2 morpho-group. All *Galactomyces* isolates developed the same cultural and morphological characteristics.

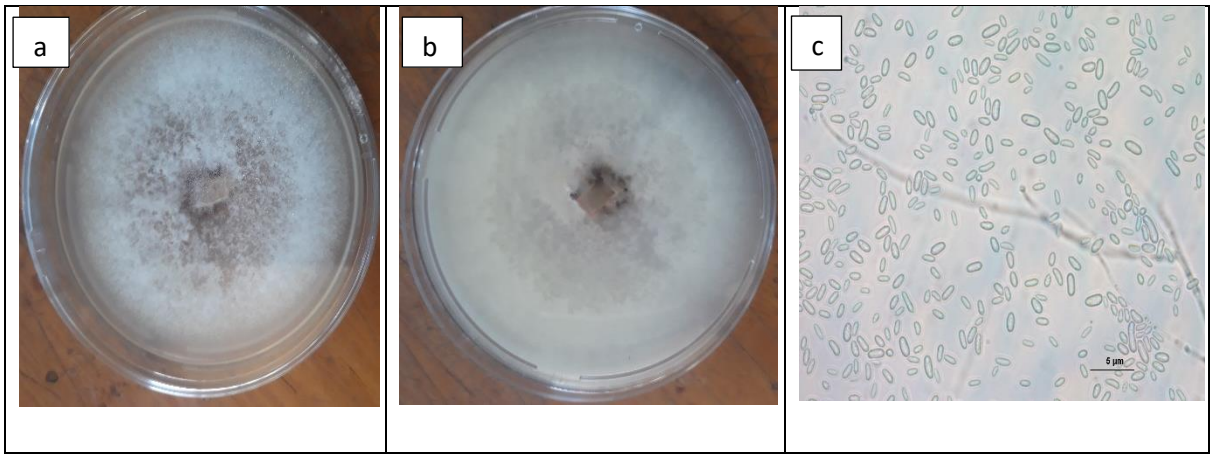


Figure 3.1: Cultural and morphological characteristics of *Colletotrichum gloeosporioides*: (a) View of the top of culture; (b) view of the bottom culture; (c) conidia under light microscopy (x400)

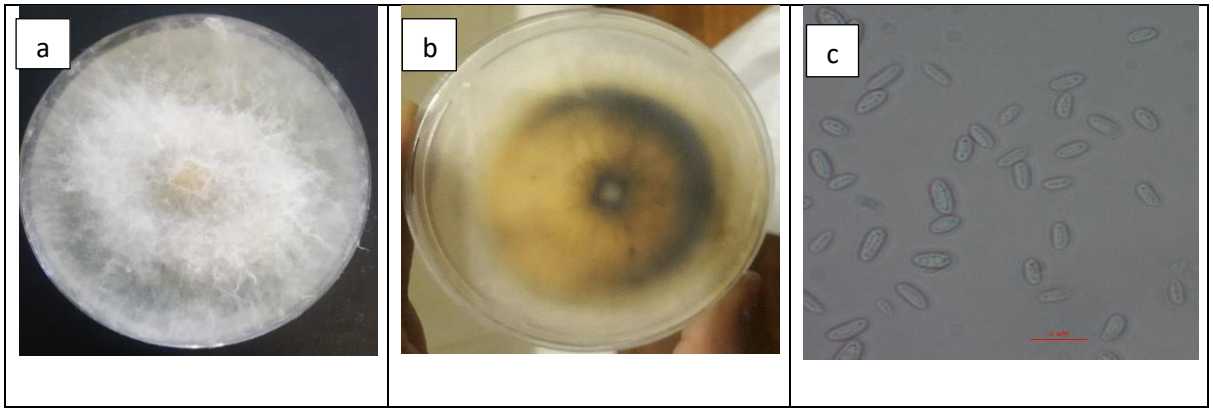


Figure 3.2: Cultural and morphological characteristics of *Colletotrichum acutatum*: (a) View of the top of culture; (b) view of the bottom culture; (c) conidia under light microscopy (x1000)

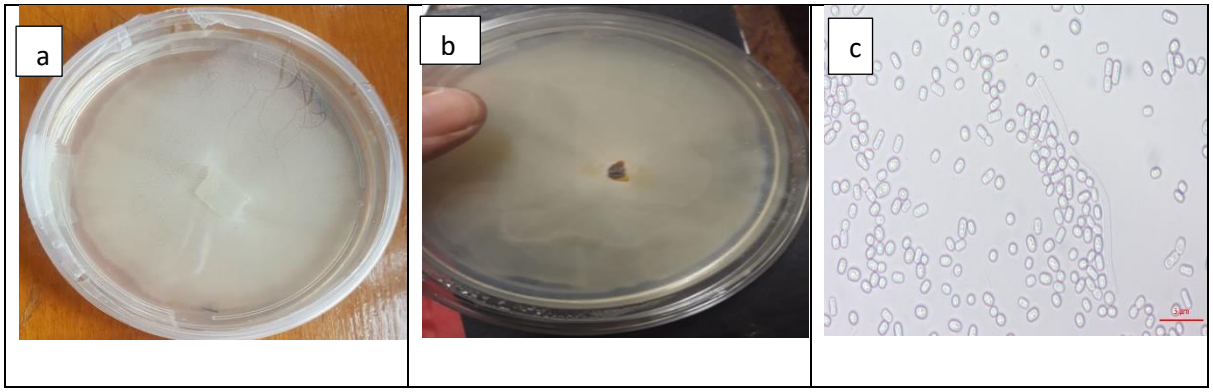


Figure 3.3: Cultural and morphological characteristics of *Galactomyces*, isolate G29: (a) View of the top of culture; (b) view of the bottom culture; (c) arthrospores under light microscopy (x1000)

3.3.3 Pathogenicity tests

About 66.7% of the *Colletotrichum* isolates were very pathogenic, while the remaining isolates were less pathogenic. When the isolates of *Galactomyces* were wound-inoculated, they were very pathogenic. When they were not wound-inoculated, they showed no pathogenicity. All wound-inoculated isolates caused anthracnose and sour rot lesions on infected tomato fruits, and the consequent lesions developed similar characteristics as naturally infected fruits. Fruits inoculated with *Colletotrichum* spore suspensions developed sunken lesions, with white to grey-coloured acervuli arranged concentrically. Fruits also developed a water-soaked appearance directly beneath the skin at the point of inoculation (Figure 3.4a). Fruits that were non-wound inoculated with *Colletotrichum* developed similar disease characteristics as wound-inoculated fruits. Tomato fruits wound-inoculated with *Galactomyces* spore suspension developed thick lesions with white, soft, creamy, yeast-like colonies (Figure 3.4b). Non-wound *Galactomyces* inoculated tomato fruits developed no symptoms to slight disease symptoms at

all stages (Table 2). Control fruit which was inoculated with distilled water did not develop any disease lesions (Figure 3.4c). Most fruits developed disease symptoms on Day 3 while others showed delayed disease incidence (Table 2). Among the isolated strains, C24 and C37A from the *Colletotrichum* isolates and G18, G23 and G29 from *Galactomyces* isolates were extremely pathogenic (Table 1). Microscopic identification of the conidia sub-cultured from *Colletotrichum* and *Galactomyces* infected fruits developed the same characteristics as those conidia used to inoculate the fruits, thereby validating the Koch's postulate.

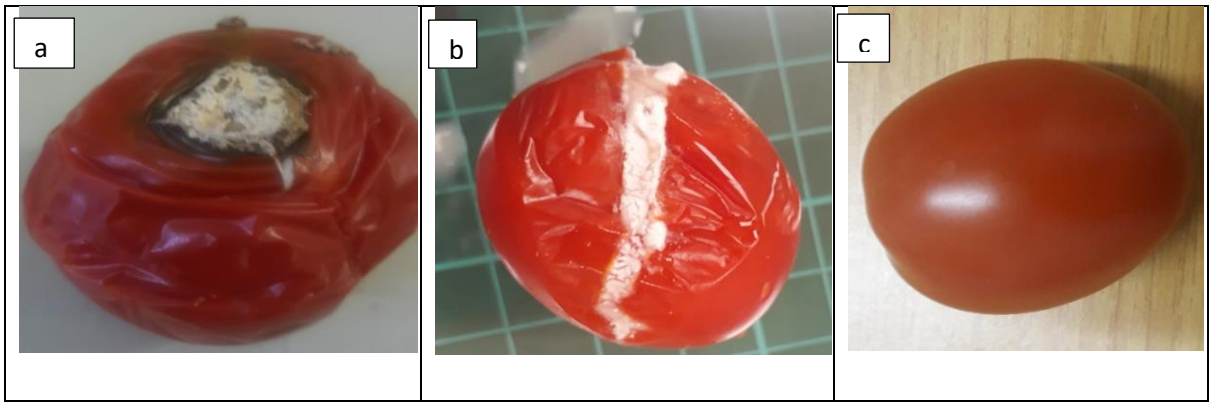
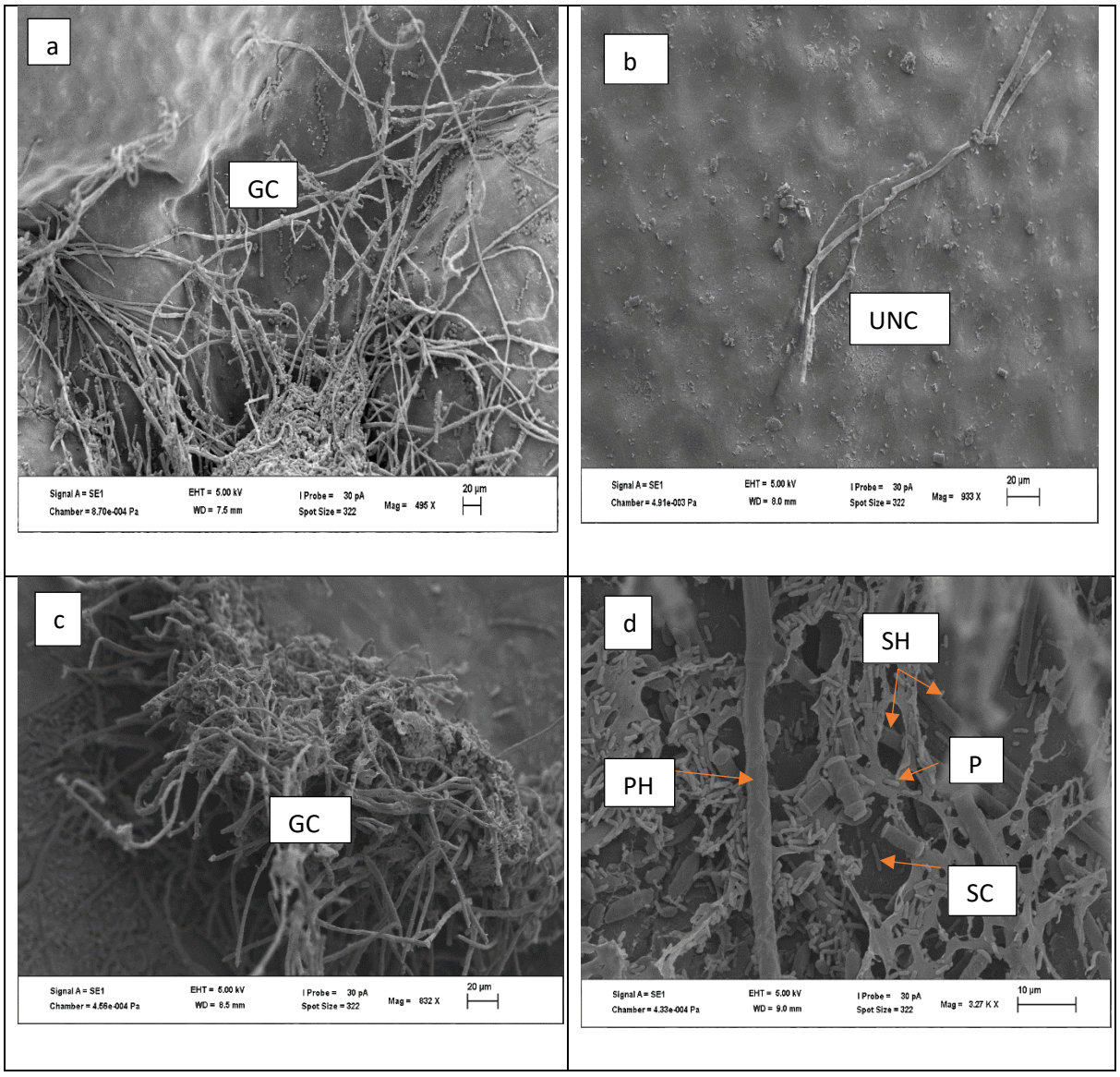


Figure 3.4: Pathogenicity test: (a) *Colletotrichum*; (b) *Galactomyces*; (c) control fruit inoculated with distilled water

All wound-inoculated fruits developed disease symptoms. Non-wound inoculated fruits with *Colletotrichum* isolates developed similar symptoms to the wound-inoculated ones, while non-wound *Galactomyces* inoculated fruits developed none to slight disease symptoms (Table 3.2). These results were supported by electron microscopy scanning studies that showed that infection by *Galactomyces* on non-wound inoculated fruits could occur only if a natural opening was present because there was no fungal colonization and proliferation of *Galactomyces* on the surface of the fruit (Figure 3.5b). Fruit wound-inoculated with *Galactomyces* developed conidial germination and proliferation on the wound site (Figure 3.5a). Wounding is not significant for *Colletotrichum* isolates because both wound- and non-wound inoculated fruits showed disease development. Scanning electron microscopy observations showed conidial germination and proliferation on wound-inoculated fruits with *Colletotrichum* (Figure 3.5c). *Colletotrichum* penetration, primary hyphae growth and secondary hyphae formation, conidial germination on the surface and secondary conidial

formation in the intramural area beneath the cuticle of the non-wound inoculated fruits was also shown (Figure 3.5d).



P: penetration; PH: primary hyphae; SH: secondary hyphae; GC: germinated conidia; UNC: ungerminated conidia

Figure 3.5: Effect of wound- and non-wound inoculation methods on fruits after 5 days of inoculation: scanning electron microscopy of: (a) wound-inoculated fruits with *Galactomyces*; (b) non-wound inoculated fruits with *Galactomyces*; (c) wound-inoculated fruits with *Colletotrichum*; (d) non-wound inoculated fruits with *Colletotrichum*

Table 2: Disease incidence and severity of lesions caused by isolates applied to healthy fresh tomato fruits

Group	Isolate	Disease incidence								Pathogenicity	
		Wound inoculation				Non-wound inoculation				Wound inoculation	Non-wound inoculation
		Day 3	Day 5	Day 7	Day 10	Day 3	Day 5	Day 7	Day 10		
Colletotrichum	C1-2	No	Yes	Yes	Yes	No	No	Yes	Yes	2	1
	C3	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	3	3
	C4	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	3	3
	C5	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	3	3
	C7-8	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	3	3
	C11	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	3	3
	C13	Yes	Yes	Yes	Yes	No	No	Yes	Yes	2	1
	C14	No	Yes	Yes	Yes	No	No	Yes	Yes	1	1
	C15-16	No	Yes	Yes	Yes	No	No	Yes	Yes	1	1
	C17	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	2	2
	C18-22	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	3	2
	C23	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	2	2
	C24	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	3^+	3^+
	C25	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	3	3
	C26	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	3	3
	C27	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	2	1
	C28	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	1	1
	C30	Yes	Yes	Yes	Yes	No	No	Yes	Yes	1	1
	C31-36	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	3	3
	C37A	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	3^+	3^+
	C40	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	3	3
Galactomyces	G1-11, 16, 24-25	Yes	Yes	Yes	Yes	No	No	No	No	3	0
	G12-15, 17	Yes	Yes	Yes	Yes	No	No	No	No	3	1
	G18, 23, 29	Yes	Yes	Yes	Yes	No	No	No	No	3^+	0

0: None pathogenic; 1: slightly pathogenic; 2: moderate; 3: strong; 3^+: extremely pathogenic

3.3.4 Molecular identification and phylogenetic analysis

The *Colletotrichum* isolate C37A and the *Galactomyces* isolate G29 were selected for molecular and phylogenetic analyses. The sequences of ITS region were 575bp and 375bp for the isolates C37A and G29, respectively. The data set for the analysis of isolate C37A consisted of 20 sequences. *C. boninense* CBS128547 (Accession number: JQ005159.1) and CBS123755 (Accession number: JQ005153.1) were used to root the tree. Kimura 2-parameter model with a discrete Gamma distribution (+G = 0.0500) was identified as the best fit evolutionary model. The selected tree had the highest log likelihood (-1099.80). Isolate C37A was grouped with other *C. gloeosporioides* isolates from different geographical regions, excluding previously SA isolates (Figure 3.6.). Phylogenetic analysis of the isolate G29 involved 19 nucleotide sequences including *Galactomyces pseudocandidus* culture CBS:10073 (accession number: KY107742.1) and isolate Soil55 (accession number: MG707673.1) that were selected as the outgroup. Tamura 3-parameter was selected as the best evolutionary model. The tree with the highest log likelihood (-1318.54) was selected (Figure 3.7.). The isolate G29 formed a single lineage within the *Galactomyces candidum* group (Figure 3.7.).

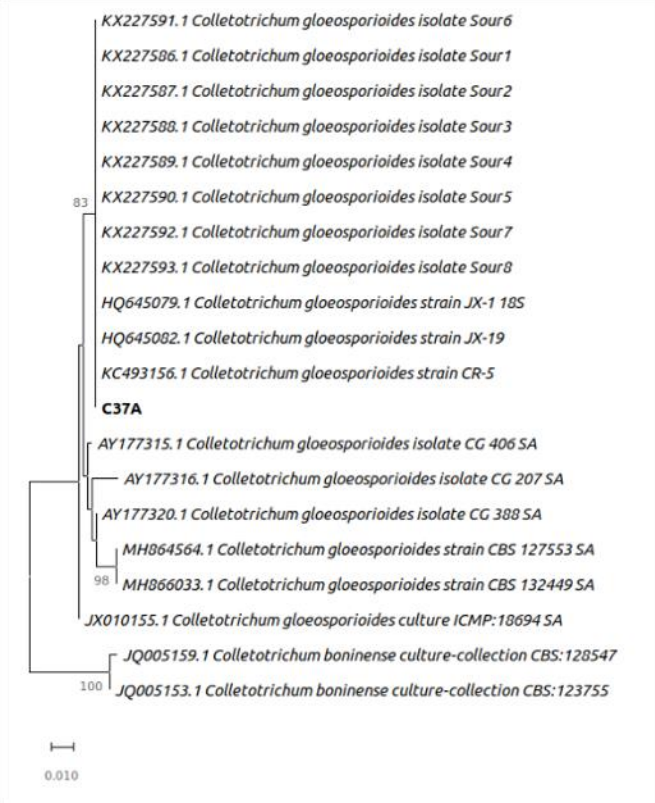


Figure 3.6: Phylogenetic tree showing the relationship of isolate C37A using maximum likelihood. The bootstrap values are shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

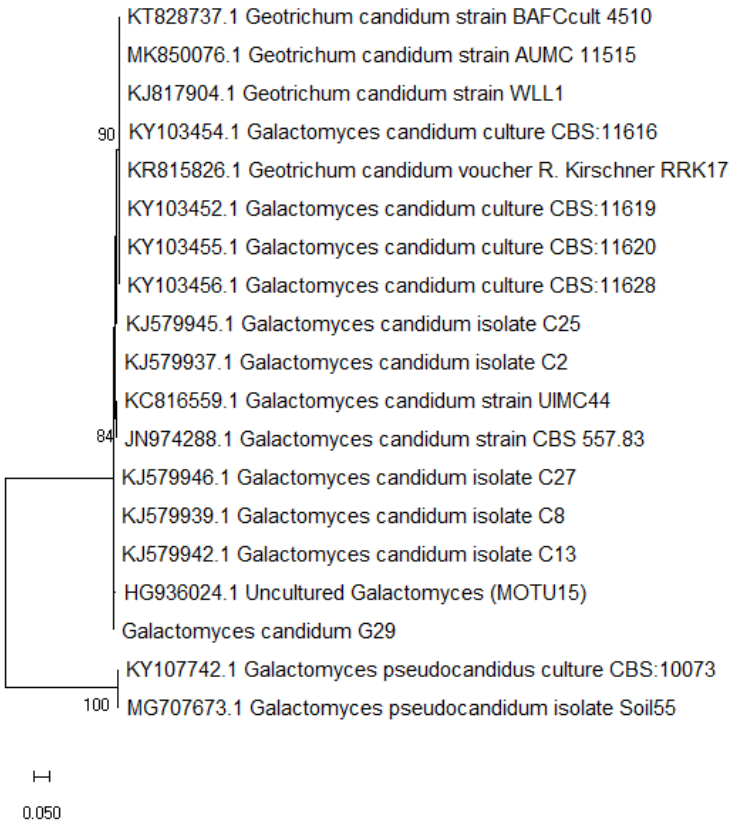


Figure 3.7: Phylogenetic tree showing the relationship of the isolate G29 using maximum likelihood. The bootstrap values are shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

3.4 Discussion

The main objectives of this study were to isolate and identify anthracnose and sour rot causing agents of tomato fruits using morphological and molecular analysis and their pathogenicity.

A total of 33 *Colletotrichum* isolates were recovered from symptomatic tomato fruits. Some colonies of the *Colletotrichum* isolates developed white growth on PDA without reverse colouration, while others developed white growth, turning grey as the culture aged. Morphological tests of the first set with white growth developed cylindrical conidial shape while the others with the grey mycelial colour had conidia with a fusiform shape. These results were consistent with

those of Smith and Black (1990) and Than *et al* (2008). Based on the cultural and morphological tests, the fungal isolates causing anthracnose of tomato fruits were identified as *Colletotrichum gloeosporioides* and *Colletotrichum acutatum*.

Most of the *Colletotrichum* strains (64%) were pathogenic, while some were not very pathogenic. Among the isolated strains, C24 and C37A expressed extreme pathogenicity. The pathogenicity differences among isolates could be due to the host range or host specificity and variability of the isolates (Than *et al*., 2008; Muñoz *et al*., 2009). Despite the difference in their pathogenicity, all *Colletotrichum* isolates caused anthracnose lesions on both wound- and non-wound inoculated fruits. Infection by *Colletotrichum* species is either by colonizing subcuticular tissues intramurally or being established intracellularly (Than *et al*., 2008). Wounding may accelerate passive infection of pathogenic microorganisms. However, *Colletotrichum* species produce special needle-like pressing organs called appressoria, which enable the fungi to adhere to fruit surfaces and to penetrate the epidermis of non-wounded fruits directly (Peres *et al*., 2005). Following penetration, the pathogens colonize the intramural region beneath the cuticle of the fruit. At this stage, the pathogen invades in a necrotrophic manner and spreads rapidly throughout the tissues, resulting in complete destruction of the fruit (O′Connell *et al*., 1985). In this study, non-wound inoculated fruits developed large lesions on fruits similar to those that developed on the wound-inoculated fruits. Scanning electron microscopy observations showed *Colletotrichum* penetration directly through the cuticle and epidermal cell walls, and complete colonization of the intramural region beneath the cuticle of the fruit by secondary hyphae. After entering the intramural region, the primary hyphae branched and established infection producing secondary hyphae. Secondary hyphae then grew extensively inter- and intracellularly by passing through the epidermal layer of the fruit, and then forming secondary conidia (Figure 3.5d). *Colletotrichum* establishment on fruit tissue is aided via virulence effectors, which are host-induced (Cannon *et al*., 2012). *Colletotrichum* species are also known to use other strategies such as alteration of the pH of fruits locally and ammonia accumulation in order to cause infection and to enhance their pathogenicity on fruits (Barad *et al*., 2017).

Although both wound and non-wound inoculated fruits developed anthracnose lesions, wound-inoculated fruits generally produced more severe lesions than non-wound-inoculated fruits. The results agreed with those of Nguyen *et al*. (2010), who recorded a 100% infection of wounded

green berries by *Colletotrichum* species. Injured fruit surfaces and wound sites are rich in nutrients (Spadaro and Droby, 2016). This generally increases the susceptibility of fruits to infection and lesion formation due to the readily availability of nutrients for pathogen germination resulting in more infection of fruit and vegetables (Lubba *et al.*, 2006). The success of pathogen colonization and pathogenicity depends on the pathogen's ability to retrieve nutrients from the host (Crouch and Beirn, 2009).

Although all *Colletotrichum* isolates were found to cause anthracnose, some isolates were slow to cause disease. It took 3 to 5 days for some isolates to initiate disease but they produced significant lesions on fruits on the 7th day. Delays in disease development could be due to the initial biotrophic life strategy adopted by the pathogens. In the early stage of infection, pathogens may proliferate in the host plant by suppressing the programed cell death (PCD) and host defence, causing minimal damage or delayed disease expression, presumably involving dormancy inside the fruits. However, at the later stage, this delayed initiation of disease development is followed by development of full anthracnose lesions once the pathogens have undergone their own physiological transition to the necrotrophic phase (Crouch and Beirn, 2009; Xie *et al*., 2010). Switching from the biotrophic phase to the necrotrophic phase enables fungal pathogens to evade the plant immune system initially and allow complete pathogenicity as the host becomes more susceptible (Vargas *et al*., 2012). The switching process is due to a lack of nutrients for pathogen development in the initial stages of fruit development (Crouch and Beirn, 2009).

Both the cultural and morphological tests for *Galactomyces* were consistent with those described by Bourret *et al*. (2013). All *Galactomyces* isolates grew rapidly on PDA plates, with a cream (not shiny) colour and fruity odour characteristics without reverse colouration (Figure 3.4a and b). Morphological observations of the shape of the conidia showed them to be cylindrical to rectangular shaped arthrospores with a septate hypha (Figure 3.4c).

All of the *Galactomyces* strains were very pathogenic when wound-inoculated, with Strains G18, G23 and G29 being the most pathogenic (Table 3.2). It took only 3 days for all *Galactomyces* isolates to initiate disease and develop sour rot on fruits. The disease progress was very rapid and caused rotting inside the fruit and eventually, the whole fruit rotted in less than 5 days. Fruits inoculated with *Galactomyces* spore suspensions developed thick lesions with white, soft, creamy yeast-like colonies. The skin of the tomato fruits cracked open, releasing a white, cheesy water-

soaked juice with a sour, fruity odour (Figure 3.4b). Control fruit that were inoculated with distilled water did not develop any lesions (Figure 3.4c).

Galactomyces infection is usually due to wounds of fruit postharvest, or cracks caused by excessively humid weather (McKay *et al*., 2012; Yaghmour *et al*., 2012). Wounding markedly accelerated disease development in tomato fruits. Similar to our finding, wounding of peach and nectarine significantly increased disease incidence and severity of sour rot (Yaghmour *et al*., 2012). This was supported by the scanning electron microscopy studies, which showed *Galactomyces* proliferation on wound sites (Figure 3.5a). Fruits used for this study were pink to pink-red fruits at the mature stage. Fruits were sorted for physiological or any mechanical damage. However, some non-wound inoculated fruits showed slight disease development. Disease development on non-wounded fruits could have been due to the presence of natural openings on the fruit. Scanning electron microscopy showed non-wound inoculated fruits without any sign of *Galactomyces* germination or infection and disease development (ungerminated conidia adhered to the surface as they were not removed during sample preparation for SEM analysis) (Figure 3.5b). *Galactomyces* is strictly a wound pathogen (McKay *et al*., 2012). Microwounds infected with as few as 20 spores have been reported to develop sour rot (Yaghmour *et al*., 2012).

Among the 55 isolates of *Colletotrichum* and *Galactomyces*, the most pathogenic strains from each group, were sent to Inqaba Biotech for sequencing, in order to confirm their identities using molecular methods. The use of curated databases for identification of fungi and fungal-like organisms has become a common practice. The identities of the isolates sent for sequencing of their ITS region were the same from both, the curated and GenBank databases. The isolate C37A is, therefore, an isolate of *Colletotrichum gloeosporioides* (Penz.). The fact that the dendrogram put this isolate in a group different to the strains of *Colletotrichum gloeosporioides* previously isolated in South Africa is an indication of its unique character. The isolate G29 belongs to *Galactomyces candidum* (Link.). This is in agreement with some of the studies previously conducted on the *Galactomyces* complex, which described it to include *Galactomyces candidum*, *Galactomyces pseudocandidus* and *Galactomyces europaeum* (de Hoog and Smith, 2004).

In the study, a combination of standard cultural and morphological techniques, as well as molecular tools were used to establish the identity of the current pathogenic isolates. Identification of these pathogens by cultural and morphological method alone was not sufficient because there

are no definitive morpho-taxonomic characters (López-Moral *et al*., 2017), and because the conidial and colony characteristics of fungal species have overlapping ranges (Alsohaili and Bani-Hasan, 2018). Morphological variation is accepted only for isolates within species (Sutton, 1992). Molecular identification techniques provide outstanding possibilities to correctly identify and characterize pathogenic isolates on or within their hosts and in the environment (Gherbawy and Voigt, 2010).

3.5 Conclusion

Both *Colletotrichum* and *Galactomyces* species were isolated from symptomatic fruits collected from different shops, indicating that both fungal pathogens are widely distributed on tomato fruit in KwaZulu-Natal. Although traditional methods could be used to identify these pathogens, they should be complemented with molecular techniques to overcome morphological limitations. Despite their pathogenicity, all wound and non-wound *Colletotrichum* inoculated tomato fruits developed anthracnose while *Galactomyces* isolates failed to cause sour rot. Wounding increases fruit susceptibility to fungal infection. There is therefore a need to roll out effective and sustainable control strategies.

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Chapter 4: Isolation, identification and characterization of biocontrol agents for the control of anthracnose and sour rot of tomato

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Abstract

Anthracnose and sour rot are major fungal pathogens associated with tomato crop losses after harvest. The objectives of the present study were to isolate and screen yeast strains for antagonistic behaviour to selected isolates of *Colletotrichum gloeosporioides* and *Galactomyces candidum*, and to study their potential as commercial biocontrol agents. A total of 148 yeast isolates recovered from the surface of tomato fruits were screened for antifungal activity *in vitro* using the dual culture assay. Isolates with the strongest inhibitory activity were screened for efficacy on healthy tomato fruits. The antagonism of the best 10 yeast isolates was then tested *in vivo*. The identities of the most effective yeasts were determined using molecular analysis of their sequences of the internal transcribed spacer regions. The interactions between the antagonist yeasts, the effect of the delay time for pathogen inoculation after yeast treatment, as well as the mechanism of control were studied. Our results showed that only 25 isolates had strong antifungal activity against *C. gloeosporioides* and *G. candidum*. Out of these 25 isolates, 4 were excluded for being phytotoxic to the fruits. The isolates Y108, Y121 and Y124 showed strong antagonistic efficacy against both pathogens with no detrimental effect on the fruit *in vivo*. Competition for nutrients, attachment to fungal hyphae and production of an extracellular matrix were among the probable modes of action of the antagonist yeasts in this study. Molecular studies identified all these three isolates as *Meyerozyma guilliermondii* (Wick) Kurtzman, previously known as *Pichia guilliermondii*, and with an asexual stage called *Candida guilliermondii*. The best isolates of *Meyerozyma guilliermondii*, especially isolate Y108, may be effective as biocontrol agents against *C. gloeosporioides* and *G. candidum* and could provide sustainable alternative to the use of chemical pesticides.

Keywords: Biocontrol agents; Meyerozyma guilliermondii; Colletotrichum gloeosporioides; Galactomyces candidum; Tomato fruits; Postharvest

4.1 Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most widely grown and extensively consumed vegetables worldwide (Sandoval *et al*., 2015). They are climacteric fruits, which may decay due to their rapid ripening process and susceptibility to abiotic and biotic stresses (Auret, 2007). Postharvest diseases, especially those caused by fungal pathogens, cause significant economic losses (Klein and Kupper, 2018). Anthracnose and sour rot caused by *Colletotrichum* and *Galactomyces* species, respectively, are major fungal pathogens associated with tomato crop losses after harvest (Wolf-Hall, 2010).

Postharvest losses are traditionally controlled by the application of synthetic fungicides pre-harvest and the rinsing of fresh tomato fruits with chlorinated water, postharvest. These applications are associated with the accumulation of chemical residues, as well as the production of secondary effects on fruit qualities (Weston and Barth, 1997; Sibomana *et al*., 2016). In addition, the loss of the effectiveness of the conventional fungicides due to the appearance of resistant strains has increased the search for low cost, non-chemical approaches for the control of postharvest diseases (Weston and Barth, 1997; Liu *et al*., 2013; Bhattacharjee and Dey, 2014; Sibomana *et al*., 2016). Moreover, the registration of new fungicide products has become slow and expensive, requiring many tests (Auret, 2007).

Since 2000, the use of microbial antagonists has emerged as an important component of postharvest disease control and an alternative to synthetic fungicides for reducing decay losses in harvested commodities (Sharma *et al*., 2009). Naturally occurring yeast antagonists isolated from fruit surfaces or artificially introduced antagonists have been reported to control postharvest diseases for fruits and vegetables (Pal and Gardener, 2006; Sharma *et al*., 2009; Spadaro and Droby, 2016). Examples are the use of yeasts such as *Candida oleophila* (Montrocher), *Candida azyma* (Yarrow) Meyer and *Debaryomyces hansenii* (Zopf) Lodder and Kreger-van Rij to control *Galactomyces* and *Colletotrichum* on fruits and vegetables (Wilson and Wisniewski, 1989; Gamagae *et al*., 2003; Ferraz *et al*., 2016).

Yeasts have been of a particular interest in biocontrol for a number of reasons, which include simple nutritional requirements; the ease of adaption to the fruit microenvironment; their abilities to colonize wound sites after extended periods under dry conditions; and their survival under a

wide range of environmental conditions. Yeasts are easy to grow and rarely produce toxic metabolites, thus making their mass production relatively inexpensive (Sharma *et al*., 2009; Stocco *et al*., 2019).

The modes of action by which yeast exert biocontrol activity against target pathogens have been linked with their ability to compete with fungal pathogens for nutrients and space, to adhere to host and pathogen tissues, to produce antibiotics and lytic enzymes, and to induce host resistance and parasitism (Droby *et al*., 2002; Ren *et al*., 2012; Xu *et al*., 2013; Ferraz *et al*., 2016; Aguirre-Güitrón *et al*., 2019). Therefore, the aims of the current study were to isolate and screen yeast cells for the control of *Galactomyces* and *Colletotrichum* and to investigate the possible mechanisms of biocontrol by observing interactions between the antagonistic yeasts and the fungal pathogens in tomato fruit wounds, using scanning electron microscopy (SEM).

4.2 Materials and methods

4.2.1 Fungal inoculum

Two previously isolated and identified fungal pathogens, *Colletotrichum gloeosporioides* (Penz.) and *Galactomyces candidum* (Link.), were subcultured on freshly prepared PDA plates and were incubated at 25℃ for 10 days. To re-activate the conidia and verify their virulence, the pathogens were wound inoculated into tomato fruit. After symptom development and sporulation, conidia from diseased fruit were transferred onto PDA plates to create fresh colonies. After 10 days of incubation at 25℃, a conidial suspension of each pathogen was prepared following methods as described in Section 3.2.2. The conidial concentration was determined using a haemocytometer and adjusted to 10^5 conidia ml^{-1} using distilled water.

4.2.2 Fruit material used for isolation of potential yeast antagonists

Fresh tomato fruits were collected from the Pietermaritzburg Fresh Market Produce, Mkondeni, SA. Selected healthy fruits with a uniform size and no physical damage were either immediately processed or stored at 4℃ until needed. Tomato fruit were first washed in running tap water to remove any debris adhered to the surface, then surface sterilized using 1% sodium hypochlorite (NaOCl) for 1 min, and finally washed in sterile distilled water three times and air-dried prior to use.

4.2.3 Isolation and maintenance of potential yeast antagonists

Epiphytic yeasts were isolated from the surfaces of tomato fruits following the method described by Chanchaichaovivat *et al.*, (2007) with some modifications. Up to three fruits were immersed into 30 ml of sterile distilled water in sterile plastic bags and massaged gently for 5 minutes. Serial dilutions of the suspension in the plastic bags were prepared and 100 µl of the 10^{-4} dilution was spread plated onto a yeast dextrose calcium agar (YDCA). Culture plates were incubated at 28℃ for 4 days and distinctive colonies were isolated, based on their different visual characteristics. To obtain pure cultures, all distinct colonies were three-way streaked onto YDCA plates and incubated at 28℃ for 4 days. Colony morphology of all pure cultures was visually checked, and the vegetative cells were examined under a light microscope to confirm the presence of yeast isolates. Isolates were transferred into double sterilized distilled water in McCartney bottles and stored at 4℃ for medium term storage and current use. For long term storage, the cultures were maintained in glycerol stock (30% glycerol into double sterilized distilled water) in microfuge tubes at -80℃.

4.2.4 Yeast cell suspension preparation

To prepare the yeast cell suspension, fresh cultures of each yeast isolates were first prepared by growing the isolates on YDCA plates for 4 days at 28℃. Subsequently, yeast cell suspensions were prepared by applying a loop full of yeast cells into Erlenmeyer flasks containing 250 ml of yeast peptone and dextrose (YPD) medium. The cultures were then incubated at 28℃ on a shaker at 160 rpm for 48 hours. Following incubation, the cells were concentrated by centrifugation at 5000 rpm for 10 min, then twice washed in deionized water and resuspended in double sterilized water. The cell concentration was determined using a Neubauer-improved (Hirschmann, Germany) haemocytometer and adjusted to the desired concentration using distilled water.

4.2.5 In vitro screening for antifungal activity of yeast isolates

Dual culture techniques were performed to screen the antifungal activity of the yeast isolates *in vitro* following methods described by Passari *et al*. (2016). An agar plug (4 x 4 mm) with growing mycelium of each pathogen was placed (upside down) at the centre of PDA plates and the yeast isolates were streaked on the opposite sides of the plate, 1.5 cm away from the fungal block. Plates without the endophytic yeasts served as controls. All plates were incubated at 25℃ until the fungi

on the control plate reached a radius of ≥3.5 cm. The criteria made for antifungal activity was in terms of the distance (zone of inhibition) between the edge of the fungal colony and the yeast smear. Once the fungal growth on the control plates reached a radius of ≥3 cm, each plate was assessed for antimicrobial activity based on zones of inhibition on the dual culture plates. The experiment was conducted in triplicates and was repeated on MEA plates. A rating pattern of 1 to 5 similar to those described by Bell *et al*. (1982) was used to rate the degree of antagonism of each yeast isolate towards each pathogen:

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

2 = Pathogen grows around the plug

3 = Pathogen grows a bit to the yeast side

4 = Pathogen grows to the yeast side but does not touch the yeast

5 = Pathogen overgrows the yeast

According to Bell *et al*. (1982), a biocontrol isolate is considered to be antagonistic towards a fungal pathogen if the mean score rate is ≤2 but is not highly antagonistic if the mean score is ≥3.

4.2.6 In vivo screening for yeast phytotoxicity

The yeast strains that had a mean score rating of 1 and 2 in the *in vitro* assays were selected for the screening of the yeast isolates' phytotoxicity *in vivo*. Fresh tomato fruits collected from the Pietermaritzburg Fresh Market Produce, Mkondeni, SA were first sorted and surface sterilized, as described in Section 4.2.2. The tomato fruits were pinpricked with a sterile needle at the stem end area to a 1 mm depth and they were dipped in a yeast suspension at a concentration of 1×10^5 cells ml^{-1}. Control plates were wounded and dipped in sterilized distilled water. The fruits were then air-dried and stored in enclosed plastic containers in order to maintain a high humidity at room temperature. After 10 days, the phytotoxicity of the yeast isolates was determined based on any alterations on the tomato fruits. The experiment was conducted twice. Each experiment consisted of three replicates with 5 tomato fruits per replicate. A yeast isolate that caused any alteration or visual decay on the tomato fruits was considered to be phytotoxic.

4.2.7 In vivo antagonistic activity of selected yeast isolates

Screening for potential yeast antagonists for biocontrol efficacy was done following the methods described by Dan *et al*. (2003), with some modifications. Fresh tomato fruits collected from the Pietermaritzburg Fresh Market Produce, Mkondeni, SA were first sorted and surface sterilized, as described in Section 4.2.2. Each tomato fruit was pinpricked with a sterile needle at the stem end area to a 1 mm depth. Ten yeast isolates that had high inhibitory ratings in the *in vitro* dual culture assay, without any phytotoxic effect on fruits, were selected for this assay.

Fruits were dipped in a yeast suspension at a concentration of 1 x 10^6 cells ml^{-1} and then, after 24 hours, 5 µl of conidial suspension (1 x 10^5 conidia ml^{-1}) of a pathogen was inoculated at the wound site of each fruit. Wounded fruits inoculated with each pathogen served as a negative control. Wounded fruits treated with sterilized distilled water served as a positive control. The fruits were then air-dried and stored in enclosed plastic containers in order to maintain at least 90% relative humidity at room temperature. After 2 days, the plastic containers were removed, and fruits were left under the same conditions. The disease incidence expressed as the percentage of infected fruits was recorded 10 days after treatment. The experiment was conducted twice. Each experiment consisted of three replicates with 5 tomato fruits per replicate. The yeast isolate with the highest fungal growth inhibition compared to inoculated control fruits were considered yeast antagonists and were used for further studies.

The quality of the fruits in terms of colour change and firmness were also examined 15 days after treatment. Quality parameters in terms of colour change and firmness were examined compared to the untreated water inoculated control fruits.

4.2.8 Molecular identification and phylogenetic analysis of selected yeast isolates

Fresh agar plate cultures of the most antagonistic isolates (Y108, Y121 and Y124) were sent to Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa) for DNA extraction, PCR and sequencing using the same primers as described in Section 3.2.6.

The identity of the organisms under study was then determined using MolecularID on the MycoBank database and the nucleotide megablast online program of the National Center for Biotechnology Information nucleotide Basic Local Alignment Search Tool (BLASTn). Various

hits from the BLASTn results were then selected for the phylogenetic analysis. The evolutionary history was inferred using the Maximum Likelihood method and the best model fitting the data set with 1000 bootstrap replications. Initial trees for the heuristic search were obtained automatically by applying the Maximum Parsimony method. The evolutionary model and the phylogenetic analyses were conducted in MEGA X (Kumar *et al*., 2018).

4.2.9 Delay time for yeast proliferation before pathogen inoculation

For this experiment, all the identified yeast isolates including Y54 and a commercial yeast B13 were used. Fresh tomato fruits collected from Pietermaritzburg Fresh Market Produce, Mkondeni, SA were first sorted, and surface sterilized as described in Section 4.2.2. Each tomato fruits were pinpricked with a sterile needle at the stem end area to a 1 mm depth. Wounded fruits were first dipped in the prepared yeast suspensions at a concentration of 1 x 10^6 cells ml^{-1} then inoculated with 15 µl of conidial suspension at a concentration of 1 x 10^5 conidia ml^{-1}. The pathogen inoculation was performed at five different times (0, 4, 8, 12 and 24 hours) after wounded fruits were treated with the antagonistic yeast isolates. Control fruits inoculated with sterilized distilled water and the pathogen served as a positive and negative control, respectively. After air drying, all the sample fruits were placed in trays enclosed in plastic containers in order to maintain at least 90% of relative humidity and initiate yeast and fungal activity at room temperature. After 2 days, the plastic containers were removed, and fruits were left under the same condition. The disease incidence expressed as the percentage of infected fruits was recorded 10 days after treatment.

4.2.10 Scanning electron microscopy studies for the interaction between antagonist yeast isolates, the pathogens conidia, and the surface of tomato fruit

Sample fruit were taken from the above experiment in Section 4.2.9 and the interaction between the antagonistic yeast isolates, the pathogens and the surface of tomato fruit was studied under a scanning electron microscope (Carl Zeiss SMT Ltd., Cambridge) following the procedures as described in Chapter 3 Section 3.2.5.

4.2.11 Statistical analysis

All the experiments were done using a randomized complete blocks design and the results were analysed using the SAS 2002 version 9.00. The mean comparison was done using Duncan's

multiple range test at a 0.05 level of significance. The effect of treatments was also analysed using a two-way analysis of variance (ANOVA).

4.3 Results

4.3.1 Isolation and in vitro selection for antifungal activity of yeast isolate

A total of 148 endophytic yeasts were recovered from the surface of fresh, healthy tomato fruits. The antifungal activity of all the 148 yeast isolates against the previously isolated fungal pathogens, *C. gloeosporioides* and *G. candidum*, was assessed *in vitro* on dual culture plates on PDA medium. The yeast isolates were evaluated for having an inhibitory activity towards the pathogen, resulting in a zone of inhibition between the edge of the fungal plug and the yeast smear. The evaluation of the results was based on the scale presented in Figure 4.1. Most yeast isolates had a mean rating of 5 because they showed no inhibitory activity towards *C. gloeosporioides* and *G. candidum* (Table 1). However, a mean rating of 1 was observed in 8.78% of the PDA plates inoculated with *C. gloeosporioides*, with 13 yeast isolates showing very strong inhibitory activity, with no mycelial growth was observed around the fungal plugs. A mean rating of 2 was observed in 6.67% of the PDA plates inoculated with *C. gloeosporioides*, with 10 yeast isolates showing strong inhibitory activity against *C. gloeosporioides* where mycelial growth was observed only on the fungal plugs but did not grow further towards the yeast smear. Among the 148 yeast isolates, 125 isolates (84.46%) were not highly antagonistic to *C. gloeosporioides*, with a mean rating of ≥3 (Table 4.1).

Similar results were also recorded for antagonistic activity screening the 148 yeast isolates against *G. candidum*. A mean rating of 1 and 2 was observed with 7.43% and 5.41% of the plates that were inoculated with *G. candidum*. Other isolates were not highly antagonistic against *G. candidum*, with mean ratings of 3 and 4 was observed on 12.16% and 22.30% of the PDA plates showing medium to poor inhibition, respectively. A mean score rating of 5 was observed in 52.70% of cases, with these isolates showing no antagonistic activity against *G. candidum* (Table 4.1).

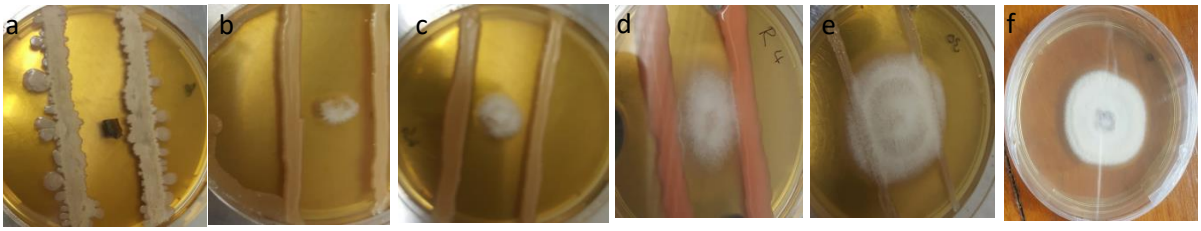


Figure 4.1: Rating scale of the antagonistic activity of yeast isolates in dual culture tests *in vitro* rated 1 to 5: (a) 1 where the pathogen does not grow at all; (b) 2 where the pathogen grows only on the fungal plug; (c) 3 where the pathogen grows towards the yeast but there is a zone of inhibition of 1 to 2 cm between the yeast and the pathogen; (d) 4 where the pathogen grows towards the yeast, but does not touch the yeast, resulting in a zone of inhibition up to 0.5cm between the yeast and the pathogen; (e) 5 where the pathogen overgrows the yeast with no zone of inhibition, and developing a similar colony diameter as the control plate; (f) the pathogen alone as a control plate.

Table 4.1: *In vitro* inhibitory effects of yeast isolates against *C. gloeosporioides* and *G. candidum*

Mean score rating	Yeast isolates against	
	C. gloeosporioides	*G. candidum*
1	8.78% (Y-6, 21, 27, 37, 83, 84, 85, 87, 108, 117, 119, 121, 126)	7.43% (Y-15, 21, 80, 83, 85, 87, 108, 117, 121, 125, 128
2	6.76% (Y-54, 80, 86, 88, 89, 97, 100, 124, 125, 130)	5.41% (Y-54, 86, 97, 100, 119, 124, 126, 130
3	11.49%	12.16%
4	23.65%	22.30%
5	49.32%	52.70%

4.3.2: In vivo screening for yeast phytotoxicity

Of the total 148 yeast isolates, only 25 showed a high inhibitory activity with a mean rating of ≤ 2 against both pathogens in dual culture assays. The phytotoxicity of these isolates was examined *in vivo*. Four isolates namely, Y15, Y37, Y89 and Y117, caused damaging effects on tomato fruits (Figure 4.2 and Table 4.2) compared to the control fruits.

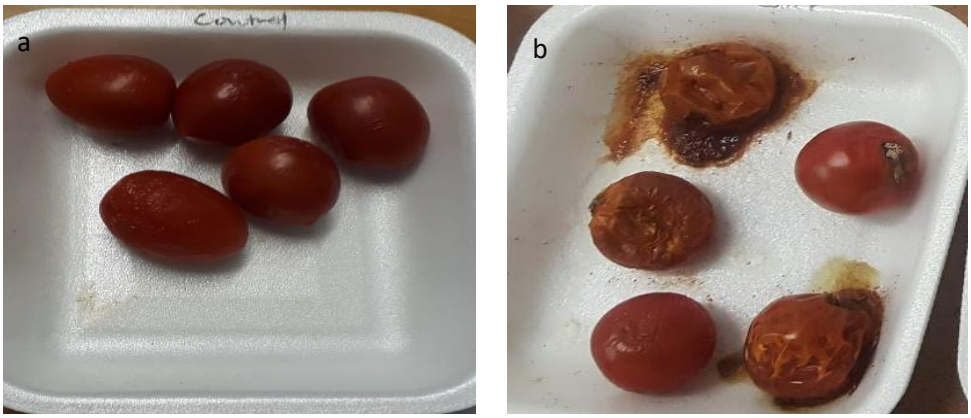


Figure 4.2: Effect of phytotoxic yeast isolates on tomato fruits: (a) untreated control fruit; (b) yeast treated fruit

Table 4.2: Phytotoxicity of yeast on the tomato fruits

Yeast isolate	Phytotoxicity
Y6	Negative
Y15	Positive
Y21	Negative
Y27	Negative
Y37	Positive
Y54	Negative
Y80	Negative
Y83	Negative
Y84	Negative
Y85	Negative
Y86	Negative
Y87	Negative
Y88	Negative
Y89	Positive
Y97	Negative
Y100	Negative
Y108	Negative
Y117	Positive
Y119	Negative
Y121	Negative
Y124	Negative
Y125	Negative
Y126	Negative
Y128	Negative
Y130	Negative

4.3.3 Antagonistic efficacy of yeast isolates to control C. gloeosporioides and G. candidum in vivo

A total of ten yeast isolates were non-phytotoxic to tomato fruits and inhibited both pathogens with a mean score rating of 1 and 2 in the in vitro dual-culture assay. These were selected for *in vivo* studies. Wounded fruits were first dipped in a yeast suspension and subsequently were inoculated with the same concentration of the pathogen conidial suspension. The antagonistic efficacy of the yeast isolates was recorded after 10 days, as presented in Table 4.3. Y108 showed the strongest suppressive effect against *C. gloeosporioides*, with no disease incidence recorded on fruits after 10 days. Meanwhile, Y85 and Y121 caused substantial reductions in disease incidence, reducing anthracnose by 6.67 and 13.13%, respectively. Y97 and Y130 caused little reduction in anthracnose incidence, with anthracnose incidence of 60.00 and 66.67%, respectively. For activity against sour rot, Y108, Y121 and Y124 produced the strongest suppressive effect with disease incidences being 6.67%. Y97 and Y130 were ineffective, with disease incidence being 73.33% (Table 4.3).

The quality of the fruits in terms of colour and firmness was also examined 15 days after yeast treatment (Table 4.4). Most fruits showed colour changes in a similar pattern to the positive control fruits (fruits inoculated with sterilized distilled water). The firmness of all the fruits also decreased after 10 days. However, fruits treated with Y108, Y121 and Y124 showed better quality than the other treated and non-treated fruits.

Table 4.3: Antagonistic efficacy of yeast isolates to control *C. gloeosporioides* and *G. candidum* *in vivo*

Antagonists	Mean % of disease incidence			
	C. gloeosporioides	Top 3 rank	*G. candidum*	Top 3 rank
Inoculated	93.33±11.5 [a]		100 ± 0.0 [a]	
Non-inoculated	13.13 ± 11.5 [e]		13.33 ± 11.5 [e]	
Y21	26.67 ± 11.5 [de]		6.67 ± 11.5 [e]	1
Y54	26.67 ± 11.5 [de]		20 ± 0.0 [de]	3
Y83	33.33 ± 11.5[cd]		60 ± 20.0 [bc]	
Y85	6.67 ± 11.5 [de]	2	40 ± 20.0 [cd]	
Y86	26.67 ± 11.5 [de]		13.33 ± 11.5 [e]	2
Y97	60.00 ± 20.0 [bc]		73.33 ± 23.1 [b]	
Y108	0.00 ± 0.0 [e]	1	6.67 ± 11.5 [e]	1
Y121	6.67 ± 11.5 [de]	2	6.67 ± 11.5 [e]	1
Y124	13.13 ± 11.5 [de]	3	6.67 ± 11.5 [e]	1
Y130	66.67 ± 11.5 [ab]		73.33 ± 11.5 [b]	
P value	0.0001		0.0001	
CV%	59.63		39.27	

The mean percentage of disease incidence of fruits inoculated with fungal pathogens after antagonistic yeast treatments. Values of each column followed by the same letter are not significantly different according to Duncan's multiple range comparison tests ($P<0.05$).

Table 4.4 The effect of antagonistic yeasts on the quality of fruits in terms of colour change and firmness after 10 days of treatment

Antagonists	Quality parameters			
	C. gloeosporioides		*G. candidum*	
	Colour change	Firmness	Colour change	Firmness
Starting fruit material	Pink	Firm	Pink	Firm
Inoculated	Very red	Very soft	Very red	soft
Non-inoculated	Red	Soft	Red	Soft
Y21	Very red	Very soft	Very red	Very soft
Y54	Very red	Very soft	Very red	Very soft
Y83	Very red	Very soft	Very red	Very soft
Y85	Very red	Very soft	Very red	Very soft
Y86	Very red	Very soft	Very red	Very soft
Y97	Very red	Very soft	Very red	Very soft
Y108	Very red	Soft	Very red	Soft
Y121	Red	Soft	Red	Firmer
Y124	Red	Soft	Red	Firmer
Y130	Red	Soft	Red	Firmer

4.3.4 Molecular identification

The consensus sequences of the ITS region of the three yeast isolates, Y108, Y121 and Y124, were 610 bp long. They all matched the ITS of *Meyerozyma guilliermondii* isolates, with probability values of 0.00 and 100% sequence identity when analysed on GenBank and MycoBank. The dataset for the phylogenetic analysis comprised 18 nucleotide sequences including the sequence of *Candida athensensis* CBS:9840, accession number KY101949, which was used as the outgroup. The Tamura-Nei model was identified as the best fit for our analysis. The tree with the highest log likelihood (-965.40) was obtained automatically by applying the Maximum Parsimony method. The isolates Y124 and 108 clustered together, while the isolate Y121 was in a unique lineage (Figure 4.4).

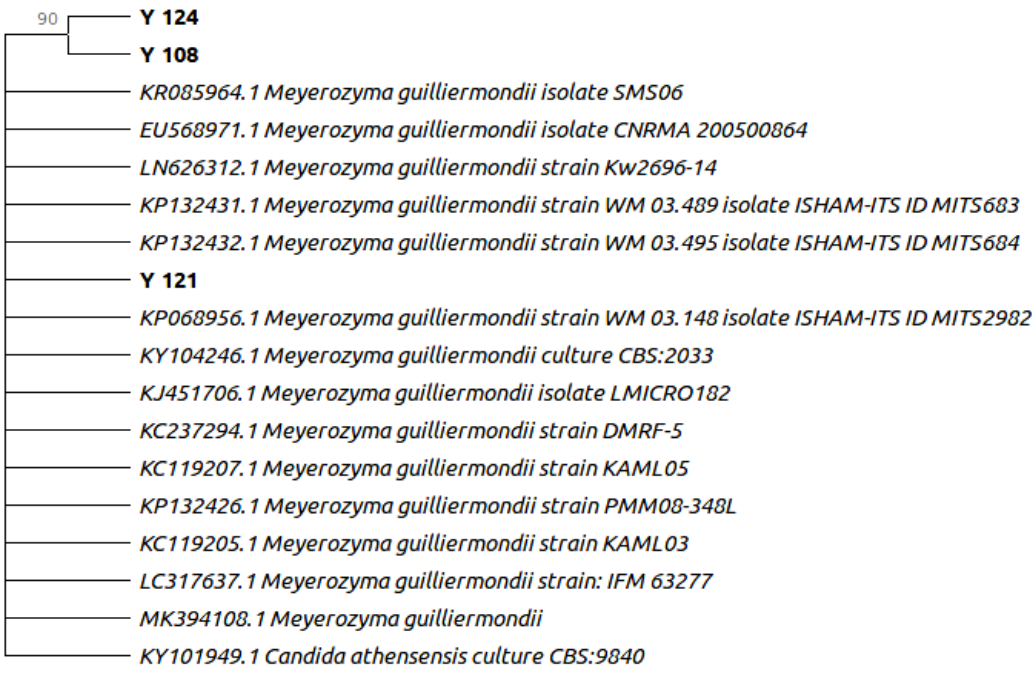


Figure 4.4: Phylogenetic tree showing the relationship of isolate Y108, Y121 and Y124 using maximum likelihood. The bootstrap values are shown next to the branches.

4.3.5 Delay time

As shown in Table 4.5, the efficacy of the tested yeast isolates on the control of disease incidence caused by *C. gloeosporioides* in tomato fruits positively correlated with the increase in delay time of pathogen inoculation. All the yeast isolates, including a previously isolated yeast, B13, caused a significant reduction (P = 0.0001) in anthracnose incidence compared to the pathogen inoculated control fruits. The efficacy of the isolates increased with increasing the delay between the inoculation of the yeast and pathogen inoculation from 0 to 24 hrs (Table 4.4). The strongest antagonistic efficacy was displayed by Y108 at all the delay times, followed by Y121 and B13 and Y124. Y54 had the lowest efficacy among the tested yeast isolates (Table 4.5).

This was true for the tomato fruits that were inoculated by *G. candidum*. All the tested yeast strains were significantly effective (P = 0.0001) in reducing sour rot incidence caused by *G. candidum*. The efficacy of each isolate tested against *G. candidum* also increased with increases in the delay between yeast and pathogen inoculations from 0 to 24 hours. The most antagonistic performance

was by Y108 and Y121 followed by Y124. Yeast B13 also showed a similar level of antagonistic activity *against G. candidum*. Y54 performed poorly (Table 4.5).

Table 4.5: Effect of pathogen inoculation delay time on disease development on fruits inoculated with *C. gloeosporioides*

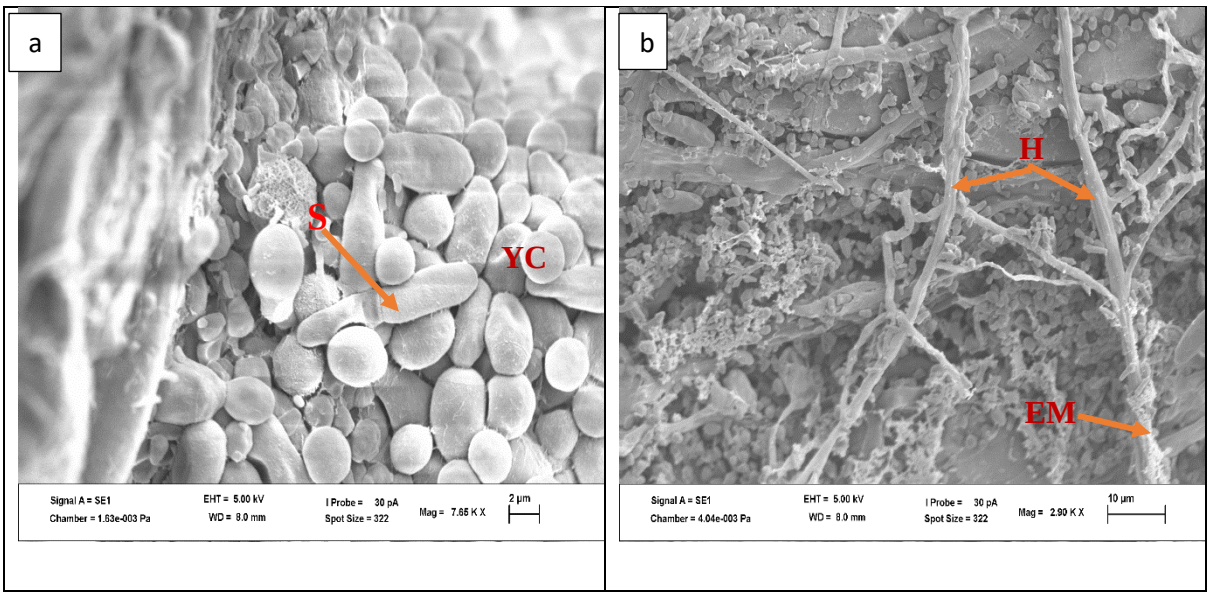
Antagonist	Mean % of disease incidence				
	Immediate	4 hr	8 hr	12 hr	24 hr
Y54[b]	50 ± 7.2	37.5 ± 0.0	33.3 ± 7.2	33.3 ± 7.2	29.2 ± 7.2
Y108[d]	16.7 ± 7.2	12.5 ± 0.0	8.3 ± 7.2	4.2 ± 7.2	4.2 ± 7.2
Y121[c]	25 ± 0.0	16.7 ±7.2	12.5 ± 0.0	8.3 ± 7.2	8.3 ± 7.2
Y124[c]	29.2 ± 7.2	25 ± 0.0	16.7 ± 7.2	16.7 ± 7.2	12.5 ± 0.0
B13 [c]	29.2 ± 7.2	25 ± 0.0	12.5 ± 12.5	12.5 ± 12.5	8.3 ± 7.2
Un-inoculated[d]			8.3 ± 7.2		
Inoculated[a]			87.5 ± 12.5		
P value Yeast			0.0001		
Time			0.0001		
Y*T			0.996		
CV%			32		

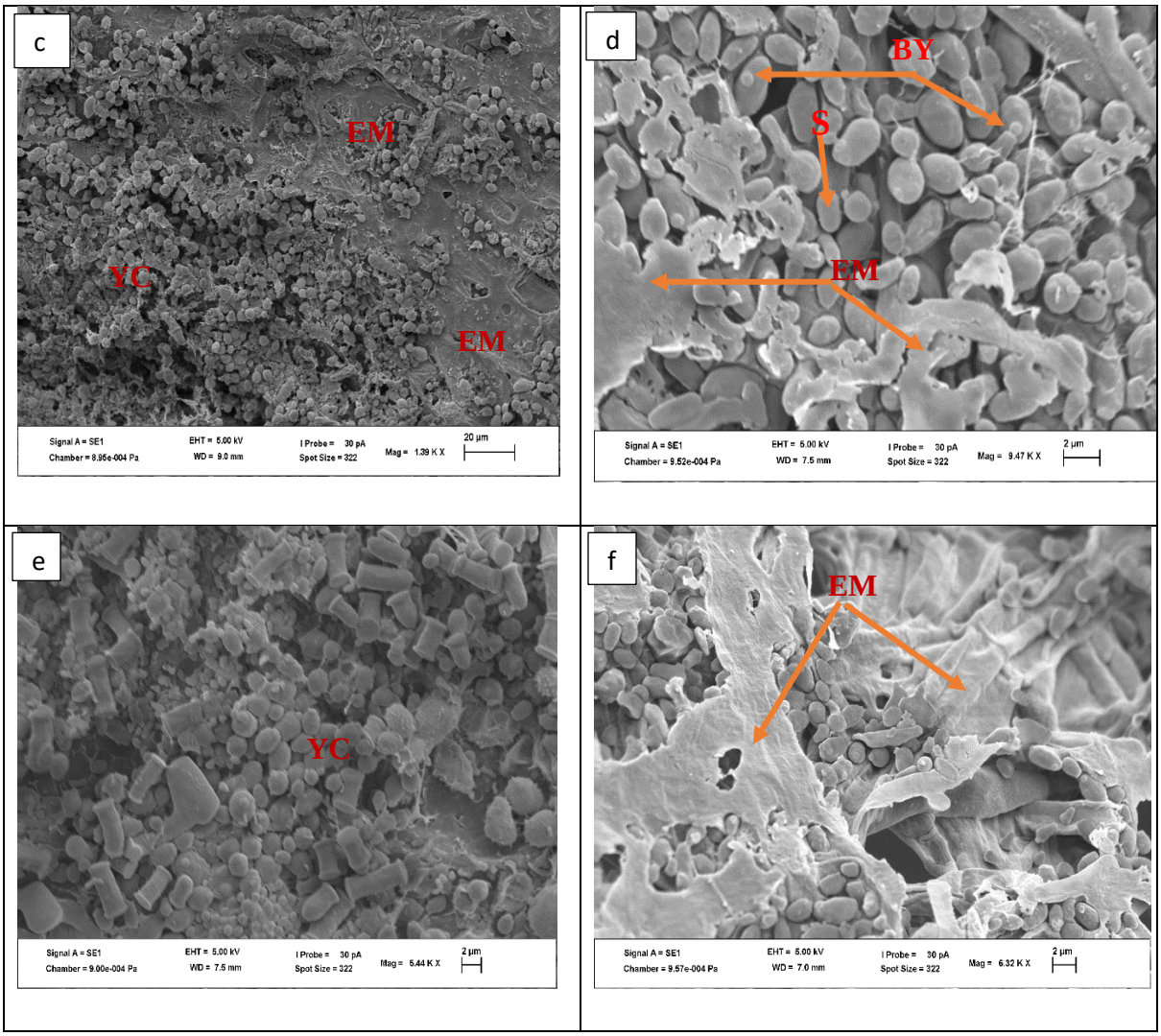
Table 4.6: Effect of pathogen inoculation delay time on disease development on fruits inoculated with *G. candidum*

Antagonist	Mean % of disease incidence				
	Immediate	4 hr	8 hr	12 hr	24 hr
Y54[b]	50 ± 0.0	45.8 ± 7.2	41.2 ± 7.2	33.3 ± 7.2	29.2 ± 7.2
Y108[c]	20.8 ± 7.2	16.7 ± 7.2	16.7 ± 7.2	8.3 ± 7.2	8.3 ± 7.2
Y121[c]	25.0 ± 0.0	20.8 ± 7.2	16.7 ± 0.0	12.5 ± 0.0	8.3 ± 7.2
Y124[c]	29.2 ± 7.2	25.0 ± 0.0	20.8 ± 7.2	16.7 ± 7.2	12.5 ± 7.2
B13[c]	37.5 ± 0.0	29.2 ± 7.2	16.7 ± 7.2	12.5 ± 0.0	12.5 ± 0.0
Un-inoculated[d]			8.3 ± 7.2		
Inoculated[a]			91.7 ± 12.5		
P value Yeast			0.0001		
Time			0.0001		
Y*T			0.676		
CV%			24.5		

4.3.6 Scanning electron microscopy (SEM) studies on interactions between antagonist yeasts, tomato and pathogen conidia

Wounds inoculated by both pathogens at the same time as yeast treatment showed some conidia of *C. gloeosporioides* and *G. candidum*. Yeast cells were seen attached to the *C. gloeosporioides* conidia, while in the case of *G. candidum*, the conidia were surrounded by yeast cells. With a four hour delay time for *C. gloeosporioides* inoculation showed fungal hyphae encircled with extracellular matrix with no conidial germination. While the wound site of fruits inoculated with *G. candidum* 4 hours after yeast treatment showed a total coverage of the wound site by yeast cells covered with extracellular matrix with fragmented hyphae on the top of yeast cells with no conidia germinating. The extracellular matrix was seen to cover most of the yeast cells for fruits inoculated with the pathogens 24 hours after yeast treatment.





BY: budding yeast; EM: extracellular matrix; H: fungal hyphae; S: fungal spore; YC: yeast cell

Figure 4.5 Scanning electron micrographs of tomato fruit wound sites treated with yeast antagonist Y108: (a) wounds inoculated with *C. gloeosporioides* at the same time of yeast treatment; (b) wound inoculated with *C. gloeosporioides* 4 hours after antagonist yeast treatment; (c) wound inoculated with *C. gloeosporioides* 24 hours after antagonist yeast treatment; (d) wounds inoculated with *G. candidum* at the same time of yeast treatment; (e) wound inoculated with *G. candidum* 4 hours after antagonist yeast treatment; (f) wound inoculated with *G. candidum* 24 hours after antagonist yeast treatment.

4.4 Discussion

Biological control of postharvest diseases using antagonistic yeast isolates may provide an alternative to the use of synthetic fungicides (Pal and Gardener, 2006; Sharma *et al*., 2009; Spadaro and Droby, 2016). The development of biocontrol agents involves the isolation and selection of potential antagonists that have desirable characteristics for commercial production (Droby *et al*., 2009). In this study, a total of 148 yeast isolates were isolated from the surface of tomato fruits and examined for their inhibitory effects against *C. gloeosporioides* and *G. candidum in vitro* in dual culture assays. The antagonistic activity of some yeast isolates was recognized by a zone of inhibition between the fungal plug and the yeast smear. 25 out of the 148 yeast isolates were scored with a mean rating of ≤ 2, reflecting a strong inhibitory activity against the pathogens. The inhibition zones in the dual culture assays could have been due to the production of inhibitory compounds such as antibiotics, antifungal metabolites or siderophores by the yeast isolates as biological control mechanisms. The difference in the size of the inhibition zone would reflect the concentration and rate of diffusion of the compounds from each isolate (Vargas *et al*., 2012). However, the production of these inhibitory compounds in culture plates may not always be efficient on fruits *in vivo*. Moreover, *in vitro* screening has been reported to favour antagonists that produce antibiotics when challenged by pathogens thereby biasing the selection process for potential antagonists (Wilson and Wisniewski, 1989). That is the reason why screening processes on the actual fruits are also recommended to evaluate potential antagonists. Unlike *in vitro* screening, *in vivo* screening provides information regarding the ability of a potential antagonist to survive on the host, its phytotoxicity towards the host and allows for the recognition of other biocontrol mechanisms against pathogens besides antibiosis (Wilson and Wisniewski, 1989; Ahima *et al*., 2019).

The 25 yeast isolates with the most inhibitory effects against the fungal pathogens were subjected to secondary screening for yeast phytotoxicity *in vivo* on healthy tomato fruits. One of the requirements of an antagonist yeast to be used as a biocontrol agent is that it does not express any phytotoxic effect to the host plant (Sharma *et al*., 2009). Fruits treated with Y15, Y37, Y89 and Y117 developed a soft consistency, with visible tissue damage, which was not the case for the untreated control fruits. These isolates were therefore considered phytotoxic and were discarded. The phytotoxic effect of these yeast isolates on tomato fruits could be due to the ability of these

isolates to develop to mycelial/pseudohyphal forms, which would enable them to invade the plant tissue, penetrate and cause necrosis. Vargas *et al*. (2012) reported the phytotoxicity of some yeast isolates on table grapes.

In order to select the best potential candidates, ten yeast isolates with strong inhibitory activity and no phytotoxic effect on fruits were selected to evaluate their ability to control postharvest anthracnose and sour rot of tomato fruits. The yeasts reduced disease incidence of treated fruits significantly (P = 0.0001), compared to the inoculated control fruits. Growth of the fungal pathogens was differentially inhibited by the ten yeast isolates. Y108, Y121, Y124 showed the highest antagonistic activity against both pathogens.

The effect of the antagonist yeast isolates on the quality of the tomato fruits was also evaluated 15 days after treatment. The results showed that the yeast treatments had no effect on the quality parameters. The colour of the tomato fruits changed from pink to red at the same rate as the uninoculated control fruits, as did the firmness of the fruits, which also decreased progressively. These changes could have been due to the normal ripening process of the fruits. Once harvested, the life of the fruit depends on the reserves, which once exhausted, cause the fruit to undergo accelerated ripening and ageing, with a subsequent deterioration (Wills *et al*., 2007; Pinheiro *et al*., 2013). Similar results have been reported by Zhang *et al*. (2007) and Aguirre-Güitrón *et al*. (2019). Aguirre-Güitrón *et al*. (2019) reported that the application of an antagonist isolate of *Meyerozyma caribbica* (Vaughan-Mart., Kurtzman, Mey. and O'Neill) Kurtzman and M. Suzuki, either fresh or a powder formulation, significantly controlled postharvest anthracnose of mango fruits, without affecting the quality parameters of the fruits during the storage. Similarly, Zhang *et al*. (2007) reported that the firmness of peach fruit treated with an antagonist *Cryptococcus laurentii* (Kuff.) Skinner, was not lower than those of the control fruits. However, Tian *et al*. (2018) reported a significant delay in both colour development and the softening of mango fruits treated with an antagonist *Metschnikowia pulcherrima* (Kamienski). This implies that inhibition of the ripening and senescence of the fruits was not a component of the mode of action of the yeasts in control of postharvest anthracnose and sour rot in this study.

Although the application of best 10 antagonist yeasts had no effect on the general quality parameters of the tomato fruits, wound healing was observed on most of the fruits treated with the antagonist yeast isolates (data not shown). This could be due to the induction of host resistance by

the antagonist yeasts or due to the defense mechanisms triggered by the host. Activation of a number of plant defense enzymes has been demonstrated in tomato fruits treated by *Candida guilliermondii*, which induced plant disease resistance (Zhao *et al.*, 2008). However, damaged fruits are reported to release damage-associated molecular patterns (DAMPs) in order to trigger secondary defense mechanisms and to activate wound healing processes. In cases where the pathogen is unable to manipulate the fruit defence mechanisms, the fruit typically respond by triggering a strong oxidative burst followed by the synthesis of phytoalexins and pathogenesis-related proteins (Spadaro and Droby, 2016). However, this was not the case in this study as our pathogens were able to cause significant damage on the inoculated control fruits. Therefore, wound healing may have been associated with the induction of host resistance and time healing by the yeast isolates.

The large ribosomal subunit (LSU) and the ITS region are widely used DNA markers in yeast taxonomy. The ITS region was used in this study to determine the taxonomic identities of the best isolates, Y108, Y121 and Y124. The consensus sequences of the ITS identified these isolates as members of *Meyerozyma guilliermondii* (Wick) Kurtzman, a yeast species readily found in natural environments and on fruits. *Meyerozyma guilliermondii* was firstly described as *Endomycopsis guilliermondii*. Its taxonomy was subsequently divided into the genus *Pichia* and *Candida*, which is *Pichia guilliermondii* with an asexual stage called *Candida guilliermondii*. However, in 2010 it was finally moved to the *Meyerozyma* genus based on molecular analysis (Kurtzman and Suzuki, 2010). There has been a growing interest in *M. guilliermondii* for its use in a variety of biotechnological applications such as vitamin production and postharvest disease control (Corte *et al*., 2015).

In this study, we found that the biocontrol efficacy of the yeast isolates was affected by time for development of preventative action. The sooner the application of the yeast treatments, the better the biocontrol efficacy of the antagonist yeasts. This agrees with previous studies conducted by Tian *et al*. (2018). When yeast treatments were applied 24 hours prior to pathogen inoculation, the antagonists had enough time to multiply, colonize and take control of the wound site, and were more effective in reducing disease incidence than when the same yeasts were applied at the same time as the pathogen inoculation. The yeast treatment and delay time for pathogen inoculation had a significant influence ($P = 0.0001$) on the disease incidence on tomato fruits. This was true for all

the yeasts used in our study, hence there was no significant interaction between the yeast treatments and delay times for pathogen inoculation.

The efficacy of a previously isolated yeast B13 (a strain of *Candida fermentati*) was also evaluated *in vivo*. B13 decreased the disease incidence for both pathogens but was less effective than *M. guilliermondii* isolate Y108. According to Pretscher *et al*. (2018), yeasts isolated from the original environment of the fungal pathogens are usually more active in terms of antagonistic activity (Wilson and Wisniewski, 1989; Pretscher *et al*., 2018).

Although the underlying mechanisms of the biological control activity of the antagonist yeast *M. guilliermondii* isolates Y108 are not clear, the scanning electron microscopy results showed that competition for nutrient and space with the pathogens appeared to be a mode of action inhibiting the development of *C. gloeosporioides* and *G. candidum*. In the treatments where the yeast and the pathogens were applied at the same time, the conidia of *C. gloeosporioides* and *G. candidum* could be seen among the yeast cells. However, no conidia were seen on the samples taken from the later sampling times. There was some mycelial growth seen on the fruits inoculated by *C. gloeosporioides*. However, there was no conidial germination and the presence of an extracellular matrix on the hyphae of *C. gloeosporioides* could have inhibited the conidial germination and the proliferation of the pathogen. Yeast cells were also seen attached to the empty hyphae of *C. gloeosporioides*. The wound site of fruits inoculated by the pathogen in the later sampling times was completely covered by the yeast cells, and an extracellular matrix was seen to cover most yeast cells. In the case of *G. candidum*, broken pieces of fungal hyphae without any sign of conidial germination were seen among the yeast cells on fruits inoculated with the pathogen at the later sample times.

Multiple mechanisms were displayed for the control of anthracnose and sour rot as evidenced by the antagonist yeast isolate *M. guilliermondii* Y108, which showed competition for nutrient, space and attachment, and the production of an extracellular matrix that appeared to affect the hyphal integrity of the pathogens. All the other isolates examined showed similar results. Attachment and production of an extracellular matrix by antagonist yeasts that affect pathogen hyphae have been reported by Wisniewski *et al*. (1991). Similar results have also been reported by Mekbib *et al*. (2011), who reported the production of an extracellular matrix by antagonistic yeast strains of *Cryptococcus laurentii* and *Candida sake* as a mode of action against green mold in oranges.

Attachment and the production of an extracellular matrix were reported to facilitate rapid colonization of the wound site by biocontrol yeasts (Janisiewicz, 1988; Zhao *et al*., 2008; Spadaro and Droby, 2015). The matrix may also lyse the fungal hyphae, thereby increasing the availability of simple carbon sources which may stimulate yeast growth (Zhang *et al*., 2010).

Strains of *M. guilliermondii* have been known for their killer activity for more than 20 years. Their use as a biocontrol agent was first reported in 1991 (Wisniewski *et al*., 1991). They have been studied for their biocontrol efficacy and have shown biocontrol potential against postharvest diseases such as anthracnose, blue mold, gray mold and green mold (Droby *et al*., 1997; Zahavi *et al*., 2000; Liu *et al*., 2010; Lahlali *et al*., 2011; Zhang *et al*., 2011). Similar to our results, multiple modes of action have been reported in the biocontrol of these fungal pathogens such as: attachment to the fungal hyphae (Nantawanit *et al*., 2010), competition for nutrients (Chanchaichaovivat *et al*., 2008) and matrix produced cell wall degrading enzymatic activity (Zhang *et al*., 2011). Strains of *M. guilliermondii* have also been studied against *G. candidum* causing sour rot in red tomato fruits where a direct attachment of the antagonist yeasts to the fungal hyphae and competition for nutrients were reported to be the main modes of action to inhibit the growth of the fungal pathogen (Robledo-Leal *et al*., 2016).

Activation of a number of plant defense enzymes has been demonstrated in tomato fruits treated by *M. guilliermondii* which induced the plant disease resistance (Zhao *et al*., 2008).

4.5 Conclusion

The results demonstrated that *M. guilliermondii* isolate Y108 has potential as a biocontrol agent to control *C. gloeosporioides* and *G. candidum*. It represents a sustainable alternative to the use of synthetic pesticides and should be exploited in that regards to control postharvest diseases of tomato fruits on a large scale.

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Chapter 5: Control of postharvest diseases of tomato caused by *Colletotrichum gloeosporioides* and *Galactomyces candidum* using rapid hot water treatments

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Abstract

The accelerated ripening and senescence of fruit during storage result in increased fruit susceptibility to postharvest diseases, reducing the shelf-life and increasing consumer rejection of many fruits including tomato. The objective of this study was to evaluate rapid hot water treatments (rHWTs) for the control of anthracnose and sour rot of tomato, and improvement of postharvest quality. Tomato fruit were wounded, inoculated with *Colletotrichum gloeosporioides* or *Galactomyces candidum* and were left to dry. After 24 hours, the fruit were dipped in hot water at 20, 44, 47, 50, 53, 56, 59, 62, 65, 68, 71 and 80°C for periods of 10, 20 and 30 seconds to determine the optimal temperature x time combinations for control of disease incidence and improved postharvest quality after 10 days of storage at room temperature. Shorter exposure times were also tested at the best working temperature x time regimes to control both pathogens. The effects of heat treatments on the fungal pathogens and on the tomato fruit were also studied using the scanning electron microscopy. Heat damage was observed on fruit treated at 62°C for 20 s or longer, after which damage occurred such as peeling, scalding, cracking and aging. Damage occurred either at the time of treatment or developed after 10 days of storage at 25°C. rHWTs at 56 x 20s, 59°C x 10s and 62°C x 10s significantly reduced disease incidence while maintaining fruit quality. Moreover, treatments 56°C x 15 s and 62°C x 8s were even more effective. Heat treatments caused the melting of the wax platelets of the fruit, sealing the cracks present on control fruit. It is unlikely that rHWT has a direct control action on the fungi because the pathogens continued to infect fruit even with high temperatures and long exposure times, i.e., with more heat exposure. Hence, induction of host resistance is the most likely mode of action of HWTs in this study. rHWTs have the capacity to control of *C. gloeosporioides* and *G. candidum*, and to maintain postharvest quality of tomato fruit, and could be used to manage postharvest diseases of tomato fruit on a commercial level.

Keywords: *Postharvest, Tomato, Colletotrichum gloeosporioides, Galactomyces candidum, rapid hot water treatments.*

5.1 Introduction

The accelerated ripening and senescence of the fruit during storage results in increased susceptibility to postharvest diseases, and a reduced shelf-life, which increases consumer rejection and postharvest losses (Toor and Savage, 2006; Pinheiro *et al.*, 2013). Pre-storage heat treatments have been used as an alternative to synthetic fungicides to control postharvest diseases of various harvested commodities (Palou, 2009; Jemric *et al*., 2011; Huan *et al*., 2017; Wang *et al*., 2017). Hot water treatment (HWT) is the most important and popular postharvest heat treatment because it is relatively effective, simple, cheap, easy to apply, controls a range of pathogens on many crops, causes no physiological damage, extends shelf-life, and can be combined with other disease control methods (Geysen *et al*., 2005; Palou, 2013). Water is the most efficient medium for delivering thermal energy to the fruit surface (Geysen *et al*., 2005; Pareek, 2017).

Postharvest HWTs have been investigated for the control of a range of postharvest diseases in citrus (Palou, 2009), nectarine (Jemric *et al*., 2011), orange (Yan *et al*., 2016), peach (Huan *et al*., 2017) and many other fruit and vegetable crops. Abd-El-Kareem and Saied (2015) applied HWT of lemon fruit at 60^0C for 10s, which reduced both the severity and disease incidence of *Galactomyces citri-aurantii* by more than 80%. Li *et al*. (2013) applied HWT of 54^0C for 4 min to papaya fruit, which inhibited *Colletotrichum gloeosporioides,* reducing anthracnose by 66.6% and extending shelf-life quality. The effect of HWTs on the control of postharvest decay on tomato fruit has also been studied. Boonkorn (2016) showed that pre-storage HWT for 40℃ at 10 min reduced decay in tomato fruit. Animashaun (2015) treated tomato fruit at 50℃ for 30 min, which inhibited *Alternaria alternata*.

The temperature values and the treatment time are the most critical factors for an effective and successful outcome of HWTs (Fallik, 2004). Temperature values ranging between 34.5 and 63.0℃, and exposure times from 10 s to 120 s have been tested on tomato fruit. Combinations of higher temperatures with shorter exposure times have been consistently more effective (Tuan *et al*., 2004; Strano *et al*., 2014; Wang *et al*., 2017).

Most studies on tomato fruit have focused on the effect of HWT on the abiotic factors as they impact on ripening and fruit quality rather than on the biotic factors, and have been done on immature green fruit rather than pink or red fruit (McDonald *et al*., 1999; Baloch *et al*., 2006;

Luengwilai *et al*., 2012; Boonkorn, 2016; Imahori *et al*., 2016; Loayza *et al*., 2016; Wei *et al*, 2016;). There is little information on the effect of HWTs on pink or red tomato fruit, which are most preferred by consumers.

The objectives of this study were:

1. To identify a narrow range of temperature x exposure time combinations of rHWT that do not cause heat damage to tomato fruit, and ideally, to enhance their quality traits and shelf-life;
2. To test the selected range of temperature x time combinations for most effective rHWT combinations to control *Colletotrichum gloeosporioides* and *Galactomyces candidum*;
3. To investigate the mechanisms of decay control using scanning electron microscopy (SEM).

5.2 Materials and Methods

5.2.1 Tomato fruit used in this study

Tomato fruit at the stage of turning pink / red were purchased from the Pietermaritzburg Fresh Market Produce, Mkondeni, South Africa. Healthy fruit with a uniform size and no visual physical damage were selected. They were processed immediately or stored at 4°C until needed. Fruit were first washed in running tap water to remove any debris adhered to the surface, then surface sterilized using 1% sodium hypochlorite (NaOCl) for 1 min, then rinsed three times in sterile distilled water, and air-dried prior to use.

5.2.2 Determination of combinations of temperature x exposure time that are not harmful to healthy tomato fruit

The abiotic effect of rHWT was evaluated on the tomato fruit. Tap water was heated to the test temperatures in an insulated 100L water bath, which has a temperature control system with an accuracy of 0.1°C.

The tested temperature regimes were 20, 44, 47, 50, 53, 56, 59, 62, 65, 68, 71 and 80°C (±0.1°C). For each temperature, the tomato fruit were exposed for a period of 10, 20 and 30 seconds. Each treatment was applied to five tomato fruit as a single replicate. Tomato fruit were placed in plastic

mesh sacks to immerse them in the water bath. After treatment, fruit were air-dried, placed on open polystyrene plates and stored at 25°C. Non-dipped tomato fruit were used as the control. After 10 days of storage, the fruit were assessed for quality and heat damage based on visual observations. Heat damage, fruit quality and decay were the parameters used to assess the abiotic impact of the HWTs. Fruit with peeling, cracking, an abnormal appearance or shriveling of the skin were defined as heat-damaged. Fruit that changed colour, softened or decayed were recorded as low-quality fruit.

5.2.3 HWT and C. gloeosporioides and G. candidum

5.2.3.1. Fungal inoculum preparation

Two previously isolated and identified fungal pathogens, *C. gloeosporioides* and *G. candidum*, were subcultured on freshly prepared PDA plates and they were incubated at 25℃ for 10 days. To re-activate the conidia and verify their virulence, the pathogens were wound inoculated into tomato fruit. After symptom development and sporulation, conidia from diseased fruit were transferred onto PDA plates to create fresh colonies. After 10 days of incubation at 25℃, a conidial suspension of each pathogen was prepared following methods as described in Section 3.2.2. The conidial concentration was determined using a haemocytometer and adjusted to 10^5 conidia ml^{-1} using distilled water.

5.2.3.2. Efficacy of HWTs for the control of *C. gloeosporioides* and *G. candidum* infection on tomato fruit

The tomato fruit were pricked with a sterile needle at the stem end area to a 1 mm depth and the holes were inoculated with 10 µl of conidial suspension (10^5 conidia ml^{-1}) of *C. gloeosporioides*. The inoculated fruit were then air-dried and stored in enclosed plastic containers in order to maintain at least 90% relative humidity at room temperature. After 24 hours, the fruit were immersed in a hot water bath for the test temperature x time combinations of 44 - 80°C x 10 - 30s, as described in Section 5.2.2. Untreated inoculated and uninoculated tomato fruit were used as control fruit. The experiment was conducted twice, and each experiment consisted of three replicates with 7 tomato fruit per replicate. Treated fruit were air-dried, placed in open polystyrene plates and stored at 25°C. The disease incidence was recorded 10 days after treatment, expressed

as the percentage of infected fruit. The same procedure was used to determine the efficacy of HWTs for the control of *G. candidum* infection on tomato fruit.

5.2.3.3. Determination of shorter times at the best working temperatures

The best temperature x exposure times for the control of both the fungal pathogens were determined from the above experiment. These temperature x time combinations were tested together with additional shorter exposure times, and both the lowest and highest temperature regimes in order to link these results with the previous results. Therefore, temperature regimes of 20, 56, 59, 62 and 80°C (±0.1°C) and exposure times of 8, 10, 15, 20 and 30 seconds were used in this experiment. The same procedures were used as described in Section 5.2.4. Inoculated but untreated, and uninoculated, untreated tomato fruit were used as control fruit. The experiment was conducted once, and the experiment consisted of three replicates with 15 tomato fruit per replicate. Treated fruit were air-dried, placed in open polystyrene plates and stored at 25°C. The disease incidence was expressed as the percentage of infected fruit at 20 days after treatment. The same procedure was used to optimize HWTs for the control of *G. candidum* infection on tomato fruit.

5.2.4 Scanning electron microscopy studies for the effect of HWTs on C. gloeosporioides and G. candidum conidia, and the surface of tomato fruit

Sample fruit were taken from the above experiment in Section 5.2.5 and the effect of HWTs on the control of *C. gloeosporioides* and *G. candidum* infection on tomato fruit was studied under the scanning electron microscope (Carl Zeiss SMT Ltd., Cambridge) following the procedures as described in Chapter 3 Section 3.2.5.

5.2.5 Statistical analysis

All replicated experiments were conducted using a randomized complete blocks design, and the results were analysed using R version 3.6.2. The results were analysed using a two-way analysis of variance (ANOVA). The treatment means comparison was done using Duncan's multiple range test at a 0.05 level of significance.

5.3 Results

5.3.1 Determination of the temperatures and exposure times combinations that are not harmful to healthy tomato fruit

The temperature regimes whereby no heat damages were observed on the skin of tomato fruit were 20^0C, and from 44°C to 59°C at all exposure times (Table 5.1). No skin damage was recorded on fruit treated at 62°C for 10s, while those subjected to more than 10s showed some heat damage. For all excessive treatments, damage was observed such as peeling, cracking, scalding and shrinkage (premature ageing), either immediately after the HWT, or after 10 days of storage. HWT above 62°C resulted in skin peeling, cracking, scalding or shrinkage of tomato fruit at all the exposure times, with reduced fruit quality compared to the untreated control fruit (Table 5.1 and Figure 5.1). The fruit were less firm, their colour changed rapidly, and more decay occurred when compared with fruit treated at lower temperatures. The fruit immersed at 20ºC showed no skin damage. However, they showed similar quality deterioration as the untreated control fruit in terms of colour change, firmness and decay.

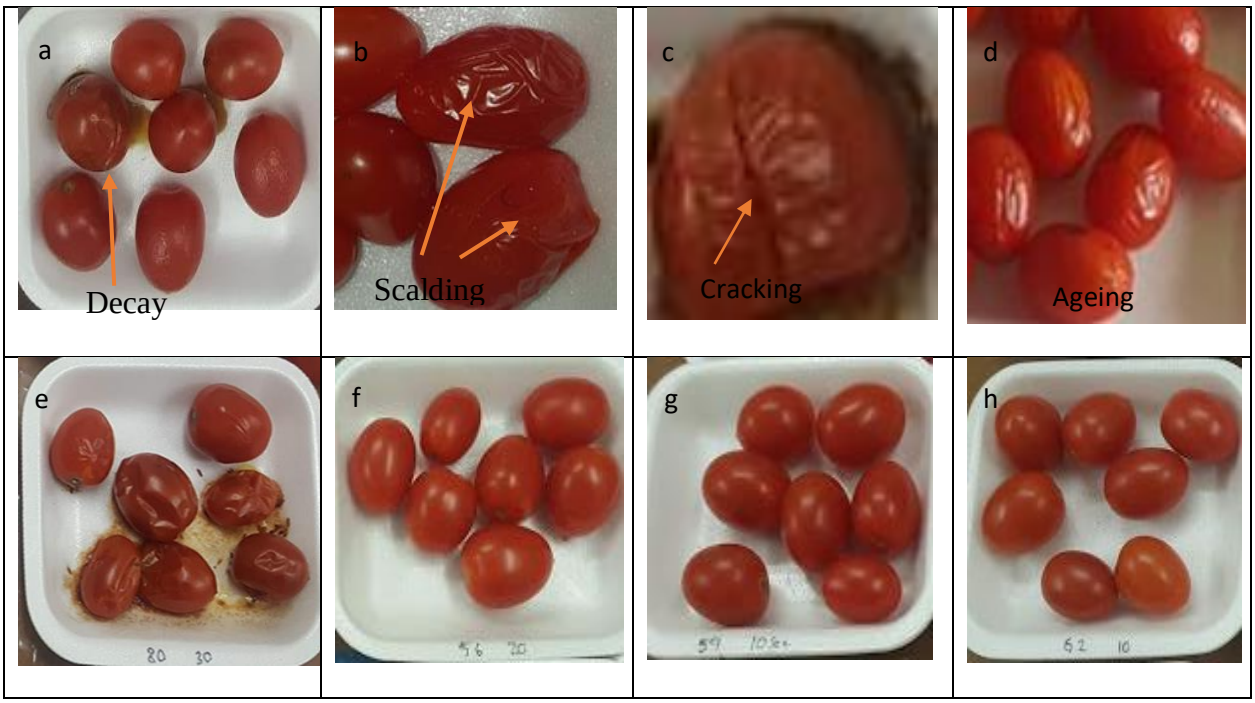


Figure 5.1: Fruit quality issues after HWT with various temperature x exposure time combinations: (a) control: not heat treated; (b, c and d) heat damage of fruit treated at 80°C immediately after treatment; (e, f, g and h) tomato treated at (e) 80°C x 30s, (f) 56°C x 20s, (g) 59°C x 10s, (h) 62°C x 10s. All photographs at 10 days post-treatment at 25°C.

Table 5.1: Effect of HWT at different temperature and exposure time on the overall fruit quality

Temp (°C)	Time	Heat damage				Fruit quality		Decay
		Peeling	Cracking	Scalding	Shrinkage (ageing)	Colour change	Softening	
	10s	-	-	-	-	+++	++	-
20	20s	-	-	-	-	+++	++	++
	30s	-	-	-	-	+++	++	++
	10s	-	-	-	-	++	+	+
44	20s	-	-	-	-	++	+	+
	30s	-	-	-	-	++	+	+
	10s	-	-	-	-	++	+	+
47	20s	-	-	-	-	++	+	+
	30s	-	-	-	-	++	+	+
	10s	-	-	-	-	+	+	+
50	20s	-	-	-	-	+	+	+
	30s	-	-	-	-	+	-	+
	10s	-	-	-	-	+	-	+
53	20s	-	-	-	-	+	-	+
	30s	-	-	-	-	+	-	+
	10s	-	-	-	-	-	-	-
56	20s	-	-	-	-	-	-	-
	30s	-	-	-	-	-	-	-
	10s	-	-	-	-	-	-	-
59	20s	-	-	-	-	-	-	-
	30s	-	-	-	-	-	-	-
	10s	-	-	-	-	-	-	-
62	20s	-	-	-	+	+	+	-
	30s	-	-	-	+	+	+	-
	10s	-	-	-	+	+	+	++
65	20s	-	-	-	+	+	+	+++
	30s	-	-	-	+	++	++	+++
	10s	+	-	-	+	++	++	+++
68	20s	+	-	-	+	+++	++++	+++
	30s	+	-	-	+	+++	++++	+++
	10s	+	-	+	+	+++	++++	++++
71	20s	+	-	+	++	+++	++++	++++
	30s	+	-	++	++	+++	++++	++++
	10s	+	+	++	+++	+++	++++	++++
80	20s	+	+	++	+++	+++	+++++	++++
	30s	+	+	++	+++	+++	++++	++++
Untreated	NA	-	-	-	-	+++	++	+

5.3.2 Efficacy of HWTs for the control of C. gloeosporioides and G. candidum infection on tomato fruit

HWTs significantly ($p<0.001$) reduced the incidence of infection by both pathogens with an increase in temperature and exposure times up to 62℃ (Table 5.2 and 5.3). Treatments above 62℃ and more than 10s increased disease incidence. The treatment at 59℃ x 10s produced the best control against *C. gloeosporioides*, with a disease incidence of 11.1%, followed by 62℃ x 10s treatment (15.6%) and 56℃ x 20s (20.0%) compared to the high level of the inoculated control fruit (91.1%), 10 days after treatment (Table 5.2). Fruit treated with the optimum temperature x time combinations had an overall better quality than the uninoculated, non-treated control fruit.

Table 5. 2: Effect of HWT on control of anthracnose caused by *C. gloeosporioides* on tomato fruit after 10 days of storage at 25°C

Exposure temperature (°C)	% *C. gloeosporioides* incidence (Mean ± SE) Exposure time (Seconds)		
	10	20	30
20	60.0±3.8 fgh	57.8±2.2 ghi	57.8±2.2 ghi
44	55.6±2.2 hi	53.3±3.8 hi	53.3±3.8 hi
47	55.6±2.2 hi	53.3±3.8 hi	53.3±0.0 hi
50	53.3±3.8 hi	53.3±0.0 hi	51.1±0.0 hi
53	48.9±2.2 hijk	46.7±0.0 hijkl	37.8±2.2 jklmn
56	28.9±4.4 mnop	20.0±0.0 opq	35.6±2.2 klmn
59	11.1±4.4 q	24.4±4.4 nopq	33.3±3.8 lmno
62	15.6±2.2 pq	28.9±2.2 mnop	37.8±2.2 jklmn
65	42.2±2.2 ijklm	71.1±5.9 efg	84.4±2.2 bcde
68	77.8±4.4 cde	80.0±3.8 cde	91.1±2.2 abc
71	88.9±2.2 abc	86.7±0.0 abcd	73.3±23.4 def
80	100.0±0.0a	97.8±2.2 ab	100.0±0.0 a
Inoculated		91.1±5.9 abc	
Un-inoculated		15.6±2.2 pq	
P (T)		0.001	
P (t)		0.01	
P (T x t)		0.001	
CV%		14.3	

Similarly, disease incidence of *G. candidum* was significant affected ($p<0.001$) by the best temperature treatment x exposure times. The treatment at 59℃ x 10s produced the best control against *C. gloeosporioides*, with a disease incidence of 13.3%, followed by 62℃ x 10s treatment

(17.7%) and 56℃ x 20s (24.4%), compared to inoculated control fruit (100%) after 10 days of treatment (Table 5.3). Although the disease incidences of both pathogens were reduced at the same temperature and time combinations, *C. gloeosporioides* showed more sensitivity than *G. candidum* as the percentage of disease incidence of sour rot recorded was higher than that of anthracnose (Table 5.3 and 5.4).

Table 5.3: Effect of HWT on control of sour rot caused by *G. candidum* on tomato fruit after 10 days of storage at 25°C

Exposure temperature (°C)	% *G. candidum* incidence (Mean ± SE) Exposure time (Seconds)		
	10	20	30
20	62.2±5.9 de	62.2±5.9 de	60.0±3.8 de
44	66.7±3.8 d	62.2±2.2 de	60.0±3.8 de
47	64.4±2.2 de	60.0±3.8 de	53.3±3.8 efg
50	55.6±5.9 def	53.3±3.8 efg	48.9±2.2 fgh
53	48.9±2.2 fgh	44.4±2.2 ghi	37.8±2.2 ij
56	31.1±2.2 jk	24.4±2.2 klm	40.0±2.2 hij
59	13.3±0.0 n	26.7±0.0 kl	37.8±0.0 ij
62	17.7±4.4 lmn	31.1±2.2 jk	44.4±2.2 ghi
65	55.6±2.2 def	77.8±2.2 c	88.9±2.2 ab
68	88.9±5.9 ab	86.7±7.7 bc	95.6±4.4 ab
71	95.6±2.2 ab	95.6±2.2 ab	97.8±2.2 a
80	97.8±2.2 a	100±0.0 a	100±0.0 a
Inoculated		100±0.0 a	
Un-inoculated		15.6±2.2 mn	
P (T)		0.001	
P (t)		0.001	
P (T x t)		0.001	
CV%		9.6	

5.3.3 Determination of shorter time and exposure times at the best working temperatures

To precisely identify the best temperature x time combinations of HWTs for the control of *C. gloeosporioides* and *G. candidum* infection on tomato fruit, the previously best temperatures, as well as the highest and lowest temperatures, were tested in combination with times of 8, 10, 15, 20 and 30 seconds. However, in this experiment, disease incidence was assessed 20 days after treatment. This increased the overall disease incidence relative to a storage time of 10 days post-treatment (Table 5.2, 5.3, 5.4 and 5.5). Temperatures of 56, 59 and 62^{0}C were still effective against

both pathogens. However, for these temperatures, 15, 10 and 8 seconds were specifically the best exposure times for pathogen control as well as maintaining the quality of tomato fruit (Table 5.4 and 5.5). As with the previous results, *C. gloeosporioides* was more sensitive to the HWT, and developed less disease than *G. candidum*.

Table 5.4: Effect of exposure times on the control of anthracnose of tomato fruit immersed at best working temperature regimes after

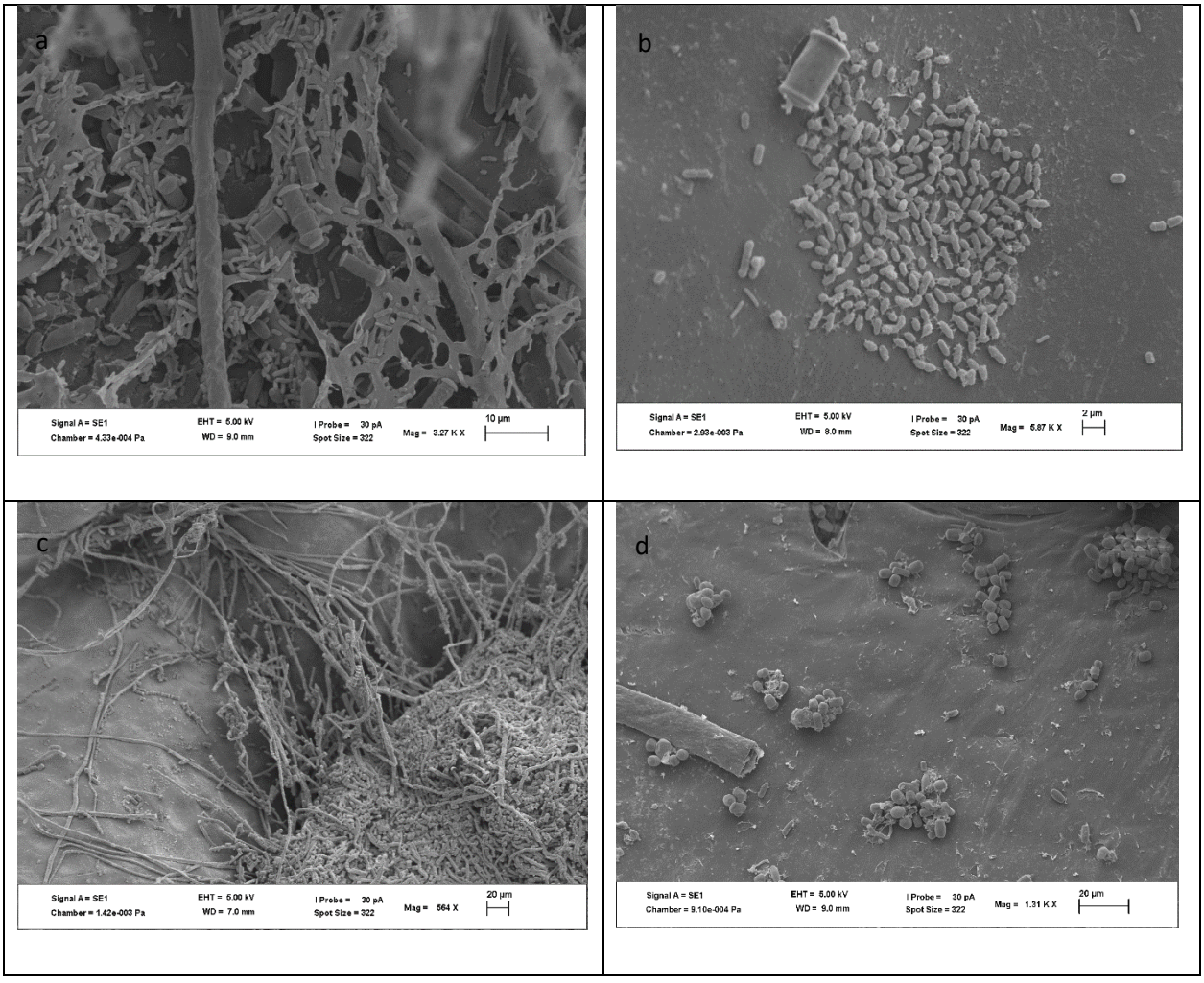
Exposure temperature (°C)	**% *C. gloeosporioides* incidence (Mean ± SE) Exposure time (Seconds)**				
	8	**10**	**15**	**20**	**30**
20	60.0±3.8 de	60.0±0.0 de	62.2±5.9 de	60.0±3.8 de	60.0±2.2 de
56	57.8±2.2 de	35.6±2.2 gh	20.0±3.8 jk	42.2±2.2 fg	64.4±2.2 d
59	24.4±2.2 ij	15.6±2.2 k	28.9±2.2 hi	37.8±2.2 fg	55.6±4.4 e
62	17.8±2.2 jk	22.2±2.2 ijk	40.0±3.8 fg	44.4±2.2 f	75.6±2.2 c
80	95.6±2.2 ab	97.8±2.2 ab	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a
Inoculated			91.1±2.2 b		
Untreated			17.7±2.2 jk		
P value Temp			0.001		
time			0.001		
T x t			0.001		
CV%			8.5		

Table 5.5: Effect of exposure times on the control of sour rot of tomato fruit immersed at best working temperature regimes

Exposure temperature (°C)	% *G. candidum* incidence (Mean ± SE) Exposure time (Seconds)				
	8	10	15	20	30
20	55.6±2.2 efg	55.6±2.2 efg	53.3±3.8 efg	57.8±2.2 def	60.0±0.0 de
56	64.4±2.2 d	44.4±2.2 hij	24.4±2.2 no	42.2±5.9 ijk	60.0±3.8 de
59	35.6±2.2 kl	17.8±2.2 o	33.3±3.8 lm	37.8±2.2 jkl	48.9±2.2 ghi
62	20.0±3.8 no	26.7±3.8 mn	44.4±5.9 hij	51.1±2.2 fgh	75.6±
80	91.1±2.2 b	95.6±2.2 ab	95.6±2.2 ab	100±0.0 a	100±0.0 a
Inoculated			88.9±2.2 b		
Un-inoculated			20.0±0.0 no		
P value Temp			0.001		
time			0.001		
T x t			0.001		
CV%			9.4		

5.3.4 Scanning electron microscopy

It was observed under SEM that the number of conidia was reduced but some were still present. Control fruit had many conidia that germinated and grew. In contrast, on the surface of fruit treated with rHWT there were many conidia present but they did not germinate, and there was no mycelial growth. The epicuticular surfaces of rHWT treated fruit were smooth and the cracks between wax layers were covered and sealed.



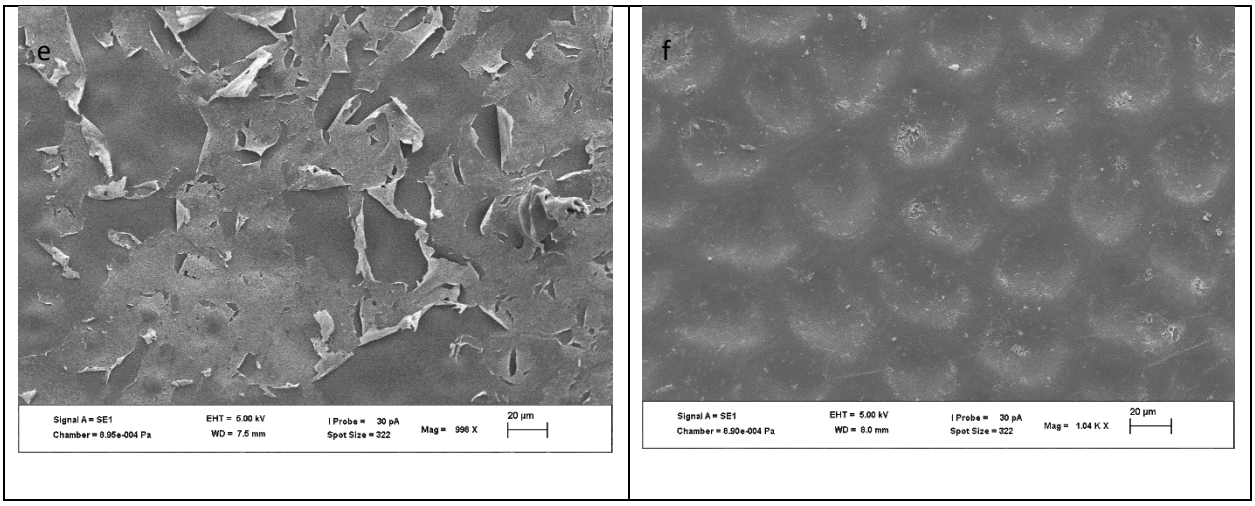


Figure 5.2: SEM images of tomato fruit surfaces (a) inoculated by *C. gloeosporioides* but not rHWT treated; (b) inoculated by *C. gloeosporioides* and treated at 59°C x 10s; (c) inoculated by *G. candidum* but not rHWT treated; (d) inoculated by *G. candidum* and treated at 59°C x 10s; (e) uninoculated and untreated control fruit; (f) uninoculated but rHWT treated at 59°C x 10s.

5.4 Discussion

Postharvest diseases, especially those caused by fungal pathogens, cause significant economic losses. They are the major factors limiting the prolonged storage life of fresh harvested fruit and vegetable. Synthetic fungicides have been used to reduce postharvest losses; however, the presence of chemical residues and production of secondary effects on fruit qualities have limited their use, and many are now effectively banned for use on fruit and vegetables (Weston and Barth, 1997; Sibomana *et al*., 2016). Development of resistant strains of pathogens, and increased public concerns over human health and environmental risks are other constraints that have affected the continued use of these synthetic fungicides (Liu *et al*., 2013; Sibomana *et al*., 2016). Postharvest heat treatments have been shown to be one of the most promising technologies available in controlling postharvest decay in many fruit and vegetables, while maintaining fruit quality (Lurie and Pedreschi, 2014; Spadoni *et al*., 2015; Siddiqua *et al*., 2018).

The first part of this study was to test temperature x time combinations in a previously determined region of activity, to determine which are not harmful to tomato fruit. The temperatures that did not damage tomato fruit were observed at 44 to 59°C at 10s to 30s, as well as 62°C x 10s.

Temperature x times combinations are critical for an effective and successful outcome for an rHWT of fresh produce (Fallik, 2004), to avoid damaging the sensitive tissues of the commodities (Tuan *et al*., 2004; Rodoni *et al*., 2016).

The life of fruit after harvest depends on nutrient reserves, which once exhausted, cause the fruit to undergo accelerated ripening and ageing (Wills *et al*., 2007). As a climacteric fruit, tomato ripening is accompanied by colour change, softening and other physiological changes, with a subsequent deterioration in quality traits (Xin *et al*., 2010; Pinheiro *et al*., 2013). However, appropriate HWTs have been reported to delay all ripening characteristics and to extend the shelf-life of many fruit and vegetables postharvest (Lurie and Pedreschi, 2014; Spadoni *et al*., 2014). In this study, fruit colour changes, and softening and decay of tomato fruit were delayed as temperature treatment increased. When subjected to rHWTs of 56℃ and 59℃ x 10, 20 or 30s, as well as 62℃ x 10s, the colour of tomato fruit remained at the stage of changing colour from pink to red, and the fruit remained firm. Fruit treated at less than 56℃ changed colour to red more rapidly, and became softer than the relatively higher temperature treated fruit. However, their colour changes were more delayed, and the fruit were firmer than the untreated control fruit. The delay of colour change and softening of heat-treated tomato fruit would be due to the inhibition of the synthesis of lycopene, ethylene and cell wall degrading enzymes. Heat treatments have been reported to cause a reversible stress that interrupts normal metabolic activity, and to reduce or inhibit the development of physiological disorders, contributing to the delay of ripening and senescence and thereby maintaining the overall quality of the produce during storage and minimizing the risk of postharvest decay (Perini *et al*., 2017).

According to Zhang *et al*. (2017), cell wall degrading enzymes and ethylene production are commonly the most disrupted activities following heat treatment of fruit and vegetables. Fruit softening is one of the most direct characteristics of ripening, and is a major determinant of the storage life and marketable quality of many fresh produce. Excessive softening increases fruit susceptibility to decay and reduces shelf-life, which increases consumer rejection and postharvest losses (Tadesse *et al*., 2012; Liu *et al*., 2013; Pinheiro *et al*., 2013; Mama *et al*., 2016). According to Paniagua *et al*. (2014), fruit softening is associated to the modification of cell wall components due to cell wall degrading enzymes such as the polygalacturonase, pectin methylesterase, pectate lyase, β-galactosidase, cellulase, which are generally encoded by ripening-related genes. Heat

treatments reduce the activity of cell wall degrading enzymes and consequently delay the modification of cell wall components (Lurie, 1998). It has been reported that heat treatments at 50°C for 10 min decreased the levels of polygalacturonase enzyme, which is strongly correlated with the pulp softening of banana fruit (Chopsri *et al*., 2018). A delay in the increase of water-soluble pectin in association with decreased total pectin was observed in hot water treated (50°C x 4 min) papaya fruit, which partially accounted for the inhibition of fruit softening (Li *et al*., 2013). Pectin plays an important role in the firmness of fruit, and its degradation leads to the disassembly of the cellulose and hemicellulose network, and accelerates the rate of fruit softening (Xin *et al*., 2010).

Besides softening, other characteristics of ripening include enhanced colour change; increased ethylene production; increased sugar/acid ratio; increased rate of respiration (Li *et al*., 2013; Pinheiro *et al*., 2013; Toor and Savage, 2006). The colour of tomato fruit changes from mature green to pink, light-red and red as the fruit ripens (Gierson and Kader, 1986). In this study, some HWTs delayed or inhibited normal colour changes. Animashuan (2015) reported that HWTs of 40 and 50°C for 10 min controlled tomato colour development. Similar results were also reported on tomato fruit by Mama *et al*. (2016). Lycopene is the dominant carotenoid that imparts the red colour to tomato fruit (Animashuan, 2015).

Temperatures above 62°C dramatically decreased fruit quality. Extremely high-temperature treatments are reported to cause excessive membrane damage, leading to impaired physiological functioning such as the loss of pigments, flesh softening, and the development of internal cavities, as well as scalding, shrivelling and failure to soften, and an increased susceptibility to decay (Sivakumar and Fallik, 2013; Pareek, 2017).

Anthracnose and sour rot are major postharvest diseases of tomato fruit. In this study, rHWTs at 56°C x 20 seconds, and 59 and 62°C x 10 seconds were effective in reducing disease incidence of both anthracnose and sour rot. However, lower exposure times of 15 and 8 seconds at 56 and 62°C, respectively, were more effective. The effect of heat treatments on the reduction of postharvest diseases has been reported to be a combination of an indirect effect on the host fruit and a direct effect on the pathogen (Palou, 2013). However, the HWT at 80°C x 30 seconds delivered the maximum heat load into the fruit and the inoculated pathogens. Yet disease incidence for both *C. gloeosporioides* and *G. candidum* were 100%, which was more than the inoculated controls

(91.1 and 88.9%, respectively). This indicates that the pathogens were not killed by the heat directly, and that the triggering of host resistance responses appears to be the mode of action of HWT against these pathogens. Similar results were achieved with HWT on citrus for the control of *P. digitatum* by Abraha and Laing (2010). Again, at high temperatures, heat alone did not kill the pathogen, whereas at the optimum temperature x time combinations host resistance controlled the pathogen.

The indirect effect of heat treatment is based on constitutive and induced defence mechanisms against pathogens and pests in fruit. HWTs prevent decay by inducing host defense responses against the pathogen. Induced defence mechanism involves complex interactions, which trigger physiological and pathological responses such as the production of antimicrobial chemical compounds and pathogenesis-related proteins (Liu *et al*., 2012; Khademi *et al*., 2013; Chen *et al*., 2015; Pareek, 2017). The involvement of heat stress on the induction of resistance responses has been reported in apple (Maxin *et al*., 2012; Spadoni *et al*., 2015), banana (Wang *et al*., 2012) and peach (Spadoni *et al*., 2014). Apart from the nature and characteristics of heat treatment, this induction is affected by the genotype and physiological condition of the fruit being treated (Palou, 2013).

HWT has been reported to cause the melting of the wax platelets of the fruit, sealing the stomata, thereby providing a mechanical barrier against postharvest pathogens as part of a constitutive defense mechanism (Lu *et al*., 2007). In this study, the epicuticular surface of untreated fruit showed a number of cracks under SEM, whereas those of the heat-treated fruit were very smooth (Figure 5.2e-f). Cracks on untreated fruit were reported to deepen and become wider during long term storage (Roy *et al*., 1999).

Under the SEM there was a reduction in the number of conidia and the occurrence of mycelial growth of both pathogens (Figure 5.2a-d). Although a number of conidia of each pathogen were seen on the surface of the heat-treated fruit, their activity was inhibited as they did not germinate, and no penetration or damaged was observed on the epicuticular surface near the conidia. The inhibitory effects of HWTs on spore germination, germ tube elongation, and host penetration has been reported in many fruit (Liu *et al*., 2012; Chen *et al*., 2015). Spore encapsulation and mummification by molten wax has been reported to inactivate early-germinated conidia (Schirra *et al.,* 2000).

The overall quality of fresh produce treated with optimized HWTs is usually significantly better than untreated produce, despite the less than optimum temperature x time combinations used, usually at relatively low temperatures with long exposure times. In recent studies, rapid HWTs (rHWTs) with higher temperatures x shorter times have been found to be more effective for the control of postharvest diseases of orange (Strano *et al*., 2014), mango (Wang *et al*., 2017) and citrus fruit (Laing and Basdew, 2018). To date, this study appears to be the first to apply rHWT to tomato fruit to control postharvest diseases. The period or duration for fruit immersion is an obstacle in packhouses where a large volume of products needs to be processed quickly (Strano *et al*., 2014). The speed of rHWTs reduce the hot water exposure time to as little as 8s, making this technology feasible for giant packhouses processing large volumes of fruit. The levels of control of multiple pathogens are equivalent those offered by synthetic fungicides, and fruit quality and shelf-life are enhanced substantially.

5.5 Conclusion

Rapid HWT treatments were optimized for the control of two postharvest diseases on tomato. The best treatments also enhanced fruit quality and extended shelf-life. This technology could be tested in a commercial packhouse to evaluate its potential for implementation at a large scale. In the long term, the technology of rHWT could be applied to all fresh produce to control pathogens and to extend shelf-life, the equivalent of pasteurization of milk, beer and other spoilable liquids.

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Chapter 6: Integration of rapid hot water treatments with yeast biocontrol agents for the control of postharvest anthracnose and sour rot of tomato fruit

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Abstract

The effect of rapid hot water treatments (rHWTs) at 56°C for 15s, 59°C for 10s or 62°C for 8s, the antagonist yeasts *Meyerozyma guilliermondii* isolates Y108, Y121 and Y124, and a previously isolated yeast B13 (strain of *Candida fermentati*), were investigated alone or in combination, for the control of anthracnose and sour rot caused by two pathogens of tomato, *Colletotrichum gloeosporioides* and *Galactomyces candidum,* and for their effect on the postharvest quality of tomato fruit. The rHWTs and the yeast isolates, as stand-alone treatments, significantly reduced disease incidence caused by both pathogens. The combination of rHWTs and yeasts were more effective than individual treatments. Fruit treated with rHWT at 62°C for 8s and yeast Y108 had the lowest disease incidence. Fruit subjected to heat treatments only or in combination with selected yeasts, had a better overall quality than fruit treated with yeasts alone, and the control fruit. As a routine packhouse treatment of tomato fruit, the application of an rHWTs at 62°C for 8s (or 56°C for 15s, 59°C for 10s) combined with a yeast biocontrol agent (such as *Meyerozyma guilliermondii* isolate Y108) would provide a safe, effective control option against *C. gloeosporioides* and *G. candidum*, and would improve postharvest fruit quality.

Key words: *Integration, rapid hot water treatments, biocontrol agent, postharvest, anthracnose, sour rot, tomato.*

6.1 Introduction

Postharvest diseases of fruit crops, especially those caused by fungal pathogens, may cause significant economic losses (Klein and Kupper, 2018). Tomato fruit are perishable fruit with a short shelf-life and are prone to considerable losses after harvest due to mechanical injuries, physiological disorders, senescence (Pinheiro *et al*., 2013), and losses of up to 50% due to postharvest pathogens (Lydia, 2015). *Colletotrichum* and *Galactomyces* are fungal pathogens associated with tomato crops, resulting losses in the quality and marketability of the fruit (Wolf-Hall, 2010; Pinheiro *et al*., 2013).

Synthetic fungicides have been widely used to control postharvest losses; however, the development of resistant strains, consumer rejection of chemical residues in fruit, and the production of secondary effects on fruit quality have started to limit the continued use of these chemicals (Weston and Barth, 1997; Sibomana *et al*., 2016). Moreover, the registration of new fungicide products has become slow and expensive, with the result that few new fungicides have been released in the last 20 years (Auret, 2007). This has prompted a search for alternative approaches for the control of postharvest diseases and to maintain fruit quality in the global horticultural industry (Liu *et al*., 2013; Sibomana *et al*., 2016; Palou, 2018).

Since 2000, biocontrol agents have emerged as an important component of postharvest disease control, which may provide an alternative to synthetic fungicides for reducing decay losses in harvested commodities (Sharma *et al*., 2009). Among these, some naturally occurring strains of yeasts have been found to control a range of postharvest diseases of fruit and vegetables. They have great potential as commercial product because of their simple nutritional requirements, ease of mass production and adaptation to the fruit microenvironment. They have the ability to colonize wound sites after extended periods under dry conditions, survive under a wide range of environmental conditions and compete with pathogens on the surface of fruit without producing toxic compounds, in most cases (Pal and Gardener, 2006; Sharma *et al*., 2009; Spadaro and Droby, 2016; Stocco *et al*., 2019).

Postharvest heat treatments are also a promising technology for the control of postharvest diseases of various harvested commodities while maintaining fruit quality (Palou, 2009; Jemric *et al*., 2011; Spadoni *et al*.; 2015; Huan *et al*., 2017; Wang *et al*., 2017; Siddiqua *et al*., 2018). The absence of

chemical residues in/on fruit and the minimal impact of the heat treatments on the environment are important due to the growing need to implement non-polluting, antifungal treatments as an answer to regulatory and consumer demands (Palou, 2013**).**

Although both antagonist yeasts and heat treatments have been shown some level of success as stand-alone treatments, in many cases they have not provided a satisfactory level of postharvest disease control relative to equivalent treatments with synthetic fungicides (Zhao et al., 2010). The integration of different disease management strategies has been proposed as a way to deliver a satisfactory level of fruit disease control, allowing the growers to avoid applying synthetic chemical fungicides (Palou, 2009; Wisniewski, 2016). Therefore, the aim of the current study was to investigate the efficacy of combinations of a rapid hot water treatment (rHWT) with an antagonist yeast isolate to control *C. gloeosporioides* and *G. candidum* infection on tomato fruit.

6.2 Materials and Methods

6.2.1 Fruit used in this study

Fresh tomato fruit were purchased at the Pietermaritzburg Fresh Produce Market, Mkondeni, SA. Selected healthy fruit with a uniform size and no physical damage were either immediately processed or stored at 4℃ until needed. The fruit were first washed in running tap water to remove any debris adhered to the surface, then surface sterilized using 1% sodium hypochlorite (NaOCl) for 1 min, and finally washed in sterile distilled water three times and air-dried prior to use.

6.2.2 Fungal inoculum preparation

Two previously isolated and identified fungal pathogens, *Colletotrichum gloeosporioides* (Penz.) and *Galactomyces candidum* (Link.), were subcultured on freshly prepared PDA plates and they were incubated at 25℃ for 10 days. To re-activate the conidia and verify their virulence, the pathogens were wound inoculated into tomato fruit. After symptom development and sporulation, conidia from diseased fruit were transferred onto PDA plates to create fresh colonies. After 10 days of incubation at 25℃, a conidial suspension of each pathogen was prepared following methods as described in Section 3.2.2. The conidial concentration was determined using a Neubauer improved haemocytometer (Hirschmann, Germany) and adjusted to 10^5 conidia ml^{-1} using distilled water.

6.2.3 Yeast cell suspension preparation

To prepare the yeast cell suspension, fresh cultures of each yeast isolates were first prepared by growing the isolates on YDCA plates for 4 days at 28℃. Subsequently, yeast cell suspension were prepared a loop full of yeast cells put into Erlenmeyer flasks containing 250 ml of yeast peptone and dextrose (YPD) medium. The cultures were then incubated at 28℃ on a shaker at 160 rpm for 48 hours. Following incubation, the cells were concentrated by centrifugation at 5000 rpm for 10 min, then twice washed in deionized water and resuspended in double sterilized water. The cell concentration was determined using Neubauer improved haemocytometer (Hirschmann, Germany) and adjusted to the desired concentration using distilled water.

6.2.4 Efficacy of rHWT and antagonist yeast isolates to control C. gloeosporioides and G. candidum infection on tomato fruit as single treatments or in combination, tested in vivo

Three rHWTs (56℃ for 15 s; 59℃ for 10 s and 62℃ for 8s) and four antagonist yeast isolates (*Meyerozyma guilliermondii* isolate Y108, Y121 and Y124, and a previously isolated yeast B13, (a strain of *Candida fermentati*) were tested alone or in combination to control *C. gloeosporioides* and *G. candidum* infection on tomato fruit. Previously washed, surface sterilized and air-dried tomato fruit were pinpricked with a sterile needle at the stem end area to a depth of 1 mm and then inoculated with 15 µl of a conidial suspension (10^5 conidia ml^{-1}) of the fungal pathogens. The inoculated fruit were then air-dried and were stored in enclosed plastic containers in order to maintain at least 90% relative humidity at room temperature. After 24 hours, the inoculated fruit were subjected to twenty-one different treatments (including controls) as summarized in Table 6.1. The treatments used procedures described previously in Chapter 4, Section 4.3.3, and Chapter 5, Section 5.2.3.2, respectively, for the rHWT and antagonist yeasts. For the integrated applications, inoculated fruit were first treated with rHWT then dipped in the prepared yeast suspensions at a concentration of 1 x 10^6 cells ml^{-1}. After this, fruit were re-inoculated with 15 µl of the conidial suspension of the test pathogen (1 x 10^5 spores ml^{-1}). Uninoculated, non-treated fruit and inoculated but non-treated fruit were used as best-case and worst-case controls, respectively. The experiment was conducted three times, and each experiment consisted of four replicates with 15 tomato fruit per replicate. Treated fruit were air-dried, placed in open polystyrene plates and stored

at 25°C. Disease incidence fruit was recorded 14 days after treatment, expressed as the percentage of infected fruit.

The percentage increase in efficacy of both single treatments was then calculated as follows:

$$\text{Reduction in DI (\%)} = \frac{\text{DI of the control} - \text{DI of the treatment}}{\text{DI of the control}} \times 100$$

$$\text{Increase in efficacy (\%)} = \frac{\text{Reduction in DI of HWT + Yeast} - \text{Reduction in DI of HWT or Yeast}}{\text{Reduction in DI of HWT + Yeast}} \times 100$$

Table 6.1: Treatment design for screening the efficacy of antagonist yeast isolates and rHWTs, alone or in combination, for the control of *C. gloeosporioides* and *G. candidum* infection on tomato fruit

Treatments	**HWT**	**Biocontrol**
T1 (Un-inoculated)	-	-
T2 (Inoculated)	-	-
T3	56°C x 15s	-
T4	59°C x 10s	-
T5	62°C x 8s	-
T6	-	B13
T7	-	Y108
T8	-	Y121
T9	-	Y124
T10	56°C x 15s	B13
T11	56°C x 15s	Y108
T12	56°C x 15s	Y121
T13	56°C x 15s	Y124
T14	59°C x 10s	B13
T15	59°C x 10s	Y108
T16	59°C x 10s	Y121
T17	59°C x 10s	Y124
T18	62°C x 8s	B13
T19	62°C x 8s	Y108
T20	62°C x 8s	Y121
T21	62°C x 8s	Y124

6.2.5 Assessment of fruit quality

The fruit which were treated as in above Section 6.2.4 were assessed for quality parameters visually 14 days after treatment. The quality parameters examined were based on colour change and firmness as well as the external appearance of the fruit such as surface marks, blemishes or injuries which affected the edibility and/or marketability of the fruit.

6.2.6 Statistical analysis

All the experiments were done using a randomized complete blocks design and the results were analysed using Genstat version 18.2 (2020). The effects of the treatments were analysed using a two-way analysis of variance (ANOVA). The comparison of treatment means was done using the Bonferroni test at a 0.05 level of significance.

6.3 Results

6.3.1 Effect of rHWTs and yeast isolates, alone or in combination, for the control of C. gloeosporioides and G. candidum infections on tomato fruit

The effects of the heat treatments and the antagonist yeast isolates on disease incidence due to fungal pathogens are summarized in Table 6.2 and 6.3. Application of the rHWTs and yeast isolates to tomato fruit significantly reduced disease incidence caused by both fungal pathogens, *C. gloeosporioides* and *G. candidum* ($P < 0.05$). When applied alone, the antagonist yeast isolates had a stronger inhibitory effect on both pathogens than the rHWTs alone. However, better disease control resulted from the combination of rHWTs with the antagonist yeast isolates, although not significantly different from the application of antagonist yeasts alone. Relative efficacy of the combination treatments versus the single treatments against each of the pathogens are presented in Table 6.4 and 6.5. The combination of rHWTs and antagonist yeast isolates improved the performance of each single treatments, with a maximum increase in efficacy of 34.88% for rHWT and 18.60% for antagonist yeast isolates against *C. gloeosporioides* infections. Similarly, there was a maximum of 26.10% and 21.75% increase in efficacy of rHWTs and antagonist yeast isolates, respectively, against *G. candidum* infection in tomato fruit. A combination of rHWT at 62°C for 8s in combination with yeast isolate Y108 was the most effective treatment against both

C. gloeosporioides and *G. candidum* infections. The integration of both treatments also reduced the inconsistencies of single treatments in the control of both fungal pathogens.

Table 6.2: Effect of rHWTs and yeast isolates, alone or in combination, for the control of *C. gloeosporioides* infection on tomato fruit

Treatment	Mean % of disease incidence (*C. gloeosporioides*)	Standard error
(56°C x 15s) + B13	2.08[a]	2.08
(56°C x 15s) + Y108	2.08[a]	2.08
(59°C x 10s) + Y108	2.08[a]	2.08
(59°C x 10s) + Y121	2.08[a]	2.08
(62°C x 8s) + Y124	2.08[a]	2.08
(62°C x 8s) + Y108	2.08[a]	2.08
(56°C x 15s) + Y121	4.17[ab]	2.41
(59°C x 10s) + Y124	4.17[ab]	2.41
(62°C x 8s) + B13	4.17[ab]	2.41
(62°C x 8s) + Y121	4.17[ab]	2.41
(56°C x 15s) + Y124	4.17[ab]	4.17
(59°C x 10s) + B13	6.25[ab]	2.08
Y108	8.33[abc]	3.40
Y121	10.42[abc]	2.08
B13	12.50[abcd]	2.41
Un-inoculated	12.50[abcd]	2.41
Y124	18.75[bcde]	3.99
59°C x 10s	22.92[cde]	3.99
62°C x 8s	27.08[de]	2.08
56°C x 15s	33.33[e]	3.40
Inoculated	91.67[f]	3.40
CV%	41.5	
P value	0.001	

The mean percentage of disease incidence of fruit inoculated with fungal pathogens after antagonist yeast treatments. Values of each column followed by the same letter are not significantly different according to the Bonferroni comparison test ($P<0.05$).

Table 6.3: Effect of rHWTs and yeast isolate, alone or in combination, for the control of *G. candidum* infection on tomato fruit

Treatment	Mean % of disease incidence (*G. candidum*)	Standard error
(56°C x 15s) + Y108	2.08[a]	2.08
(59°C x 10s) + Y124	2.08[a]	2.08
(59°C x 10s) + Y108	2.08[a]	2.08
(62°C x 8s) + Y108	0.00[a]	0.00
(56°C x 15s) + Y121	4.15[ab]	2.40
(59°C x 10s) + B13	4.15[ab]	2.40
(59°C x 10s) + Y121	4.15[ab]	2.40
(62°C x 8s) + Y121	4.15[ab]	2.40
(62°C x 8s) + B13	4.18[ab]	4.18
(56°C x 15s) + Y124	6.23[ab]	2.08
(62°C x 8s) + Y124	6.23[ab]	2.08
(56°C x 15s) + B13	6.25[abc]	4.00
Y108	8.33[abcd]	3.41
Y121	12.50[abcde]	2.42
Un-inoculated	12.50[abcde]	2.42
B13	16.70[bcde]	0.00
59°C x 10s	20.85[cde]	2.40
62°C x 8s	22.93[de]	2.08
Y124	22.93[de]	2.08
56°C x 15s	27.10[e]	5.25
Inoculated	97.93[f]	2.08
CV%	38.7	
P value	0.001	

The mean percentage of disease incidence of fruit inoculated with fungal pathogens after antagonist yeast treatments. Values of each column followed by the same letter are not significantly different according to Bonferroni comparison test (P<0.05).

Table 6.4 Increase in efficacy of HWT or antagonist yeast treatments after integration of both methods for the control of *C. gloeosporioides* infection on tomato fruit

Treatments		Reduction in disease incidence (%)			Increase in efficacy (%)	
HWT	Yeast	HWT	Yeast	HWT + Yeast	HWT	Yeast
56°C x 15s	B13	63.64	86.36	97.73	34.88	11.63
	Y108		90.91	97.73	34.88	6.98
	Y121		88.63	95.45	33.33	7.15
	Y124		79.55	95.45	33.33	16.66
59°C x 10s	B13	75.00	86.36	93.18	19.51	7.32
	Y108		90.91	97.73	23.26	6.98
	Y121		88.63	97.73	23.26	9.31
	Y124		79.55	95.45	21.42	16.66
62°C x 8s	B13	70.46	86.36	95.45	26.18	9.52
	Y108		90.91	97.73	27.90	6.98
	Y121		88.63	95.45	26.18	7.15
	Y124		79.55	97.73	27.90	18.60

Table 6.5 Increase in efficacy of HWT or antagonist yeast treatments after integration of both methods for the control of *G. candidum* infection on tomato fruit

Treatments		Reduction in disease incidence (%)			Increase in efficacy (%)	
HWT	Yeast	HWT	Yeast	HWT + Yeast	HWT	Yeast
56°C x 15s	B13	72.33	82.95	93.61	22.73	11.39
	Y108		91.49	97.88	26.10	6.53
	Y121		87.23	95.76	24.47	8.91
	Y124		76.59	93.64	22.76	18.20
59°C x 10s	B13	78.71	82.95	95.76	17.80	13.38
	Y108		91.49	97.88	19.56	6.53
	Y121		87.23	95.76	17.80	8.91
	Y124		76.59	97.88	19.56	21.75
62°C x 8s	B13	76.59	82.95	95.57	19.86	13.20
	Y108		91.49	100	23.41	8.51
	Y121		87.23	95.76	20.02	8.91
	Y124		76.59	93.64	18.21	18.21

6.3.2 Fruit quality assessment

Fruit subjected to HWTs, either as a stand-alone treatment or integrated with the antagonist yeast isolates tended to be firmer than the fruit dipped in the cell suspension of the antagonist yeast isolates. Fruit dipped in the suspension of the antagonist yeast isolates had an occasional limited marketability, mostly due to their colour and softness. Fruit treated with the combined treatments

had excellent visual quality, with a firm texture, maintained their colour and had no issue of edibility or marketability.

Table 6.6: Quality assessment of tomato fruit after 14 days of storage at 25°C following various treatments.

Treatments	Colour	Firmness	Visual quality
Starting fruit material (Day 0)	Pink red	Very firm	Excellent
T1 (Un-inoculated)	Red	Soft	Fair (limit of marketability)
T2 (Inoculated)	Very red	Extremely soft	Severe disease, non-edible
T3 (56°C x 15s)	Pink red	Firm	Minor injury
T4 (59°C x 10s)	Pink red	Firm	Minor injury
T5 (62°C x 8s)	Pink red	Firm	Minor injury
T6 (B13)	Very red	Less firm	Minor injury (Occasional limit of marketability)
T7 (Y108)	Very red	Less firm	Minor injury (Occasional limit of marketability)
T8 (Y121)	Very red	Less firm	Minor injury (Occasional limit of marketability)
T9 (Y124)	Very red	Less firm	Minor injury (Occasional limit of marketability) (Occasional limit of marketability)
T10 (56°C x 15s + B13)	Pink red	Very firm	Excellent
T11 (56°C x 15s + Y108)	Pink red	Very firm	Excellent
T12 (56°C x 15s + Y121)	Pink red	Very firm	Excellent
T13 (56°C x 15s + Y124)	Pink red	Very firm	Excellent
T14 (59°C x 10s + B13)	Pink red	Very firm	Excellent
T15 (59°C x 10s + Y108)	Pink red	Very firm	Excellent
T16 (59°C x 10s + Y121)	Pink red	Very firm	Excellent
T17 (59°C x 10s + Y124)	Pink red	Very firm	Excellent
T18 (62°C x 8s + B13)	Pink red	Very firm	Excellent
T19 (62°C x 8s + Y108)	Pink red	Very firm	Excellent
T20 (62°C x 8s + Y121)	Pink red	Very firm	Excellent
T21 (62°C x 8s + Y124)	Pink red	Very firm	Excellent

6.4 Discussion

Finding non-chemical alternatives for the control of postharvest diseases during storage and the selling process has been of a focus of much research for more than three decades. This has prompted researchers to develop integrated control strategies by combining various alternatives, seeking to achieve comparable level of efficacy as synthetic fungicide (Singh and Sharma, 2018; Palou, 2019; Sinha *et al*., 2019). In this study, rHWTs were combined with yeast isolates to control *C. gloeosporioides* and *G. candidum* infections in tomato fruit, postharvest.

The treatments used in this study, i.e., rHWTs at 56°C for 15s; 59°C for 10s and 62°C for 8s, and the antagonist yeast of *M. guilliermondii* isolates Y108, Y121 and Y124, and a yeast isolate B13 (a strain of *Candida fermentati*) were selected because of the control that they demonstrated in previous studies as single treatments against *C. gloeosporioides* and *G. candidum* infection on tomato fruit. In this study, they were used in all possible single or two-way combinations, *in vivo*. All individual treatments significantly reduced the disease incidence of both *C. gloeosporioides* and *G. candidum* infections in tomato fruit ($P < 0.05$). Combination treatments were more effective than single treatments (Table 6.2 and 6.3).

The results further showed that the integration of both treatments improved decay control of both single treatments (Table 6.4 and 6.5). This is logical because one is a curative treatment and the other is a preventative treatment, and they have different modes of action and therefore they are able to expand on the level of control that either of them could possibly offer on their own. Zong *et al.* (2010) found that a combination of HWT with *Candida guilliermondii* or *Pichia membranaefaciens* provided superior control of *B. cinerea* in tomato fruit. In another study, the combination of a rHWT (53°C for 2 min) and an antagonist yeast isolate of *P. membranaefaciens* was more efficient than solo treatments against blue and green mold decay in artificially inoculated citrus fruit (Zhou *et al*., 2014). Synergistic effects have also been reported when an HWT (50°C for 20 min) was combined with a fungal antagonist, *Trichoderma harzianum* DGA01 (Alvindia and Acda, 2012). Similar studies have reported the beneficial combination of heat treatments and antagonist yeasts against *C. acutatum* in loquat fruit (Liu *et al*., 2010), *Penicillium expansum* in pear fruit (Zhang et al., 2008) and *Rhizopus stolonifer* in peach fruit (Zhang *et al*., 2007).

Postharvest HWTs have been investigated for the control of postharvest diseases of many fruit crops: tomato (Zong et al., 2010), citrus (Palou, 2009), nectarine (Jemric *et al*., 2011), orange (Yan *et al*., 2016) and peach (Huan *et al*., 2017), and many other commodities. Abd-El-Kareem and Saied (2015) showed that rHWT of lemon fruit at 60 for 10s reduced both the severity and disease incidence of *Galactomyces citri-aurantii* by more than 80%. An rHWT (54°C for 4 min) has been reported to inhibit *C. gloeosporioides* causing anthracnose in papaya (Li *et al*., 2013). rHWT induces host resistance mechanisms, which trigger physiological responses such as the production of antimicrobial chemical compounds, stabilization of membranes and production of pathogenesis-

related proteins, thereby reducing decay in fruit (Zamani *et al*., 2009; Maxin *et al*., 2012; Wang *et al*., 2012; Spadoni *et al*., 2015; Pareek, 2017).

Yeasts such as *Candida oleophila, C. azyma* and *Debaryomyces hansenii* have been used to control *Galactomyces* and *Colletotrichum* on fruit and vegetables (Wilson and Wisniewski, 1989; Gamagae *et al*., 2003; Ferraz *et al*., 2016). Their ability to control target pathogens has been linked with their ability to compete with fungal pathogens for nutrients and space, to adhere to host and pathogen tissues, to produce antibiotics and lytic enzymes, and to induce host resistance and parasitism (Droby *et al*., 2002; Ren *et al*., 2012; Ferraz *et al*., 2016; Aguirre-Güitrón *et al*., 2019).

Although both BCAs and HWTs provided significant levels of efficacy as stand-alone treatments, neither of them provided complete control of both anthracnose and sour rot infections. Several studies have also reported that the application of individual treatments does not provide a commercially acceptable level of decay control, with a persistence and broad-spectrum activity comparable to synthetic fungicides. The major drawback of using microbial antagonists as stand-alone treatments is their failure to control previously established infections (Talibi *et al*., 2014). On the other hand, HWT treatments are effective against prior infections but does not provide residual action, and are therefore unable to provide persistent protection of fruit from future decay (Schirra *et al*., 2000; Zamani *et al*., 2009; Pareek, 2017).

The integration of these two methods is a promising approach to overcome the drawbacks of individual treatments, to deliver effective disease control (Talibi *et al*., 2014; Palou, 2019). Heat treatments provide a curative treatment against latent or active infections by triggering a cascade of host resistance responses, which then produce antifungal compounds and pathogenesis-related proteins, which destroy the pathogens (Palou, 2013). It may also cause melting of the epicuticular wax of fruit, sealing cracks and thereby reducing pathogen penetration (Liu *et al*., 2012; Pareek, 2017). Subsequent application of an antagonist yeast may restrict incoming infections, and will provide a persistent, preventative treatment against postharvest pathogens (Zhang *et al*., 2017). Hence, the combination treatment provides a short-acting curative treatment via rHWT, and a durable preventative treatment in terms of the invisible coat of yeast on the surface of the fruit. The combination resolves the weakness of the treatments by themselves with complementary curative and preventative treatments. The research presented here showed that the best treatments

were reliable and worked against the two major postharvest pathogens of tomato, which will reduce the risks of applying the integrated treatment instead of applying a fungicide.

Maintaining the postharvest quality of many fruit and vegetables during marketing and storage is a major problem faced by producers worldwide (Sinha *et al*., 2019). The term quality is a combination of complex internal and external attributes such as colour, appearance, flavor, texture, nutritional characteristics and safety of the produce (Yahia *et al*., 2011). Initial consumer purchases depend on the external appearance, colour and firmness of fruit, while repeat purchases depend on the internal quality parameters of the fruit (Escribano and Mitchman, 2014). Fruit colour and softening, the most direct characteristics of ripening and major determinants of the storage life and marketable quality of many commodities, have been reported to increase as fruit ripen in storage. Excessive softening and ripening (colour change), increase fruit susceptibility to decay, and reduce shelf-life, which increases consumer rejection and postharvest losses (Tadesse *et al*., 2012; Liu *et al*., 2013; Pinheiro *et al*., 2013; Mama *et al*., 2016). They may also influence the value and consumer acceptance of the produce by affecting consumer perception of the sweetness or flavor, and may evoke emotional feelings (Yahia *et al*., 2011). Therefore, maintaining the colour, firmness and appearance of tomato fruit are critical in their postharvest life.

In this study, quality parameters such as the firmness, colour change and the overall external appearance of the fruit in terms of edibility and/or marketability were evaluated visually and by hand-feel after 14 days of room storage. Although yeast treatments alone provided for better disease control than the heat treatments alone, they had no effect on the quality parameters of the fruit during storage, and the fruit followed a similar postharvest profile as the untreated non-inoculated control fruit, confirming previous results (Chapter 4). The colour of the tomato fruit enhanced at the same rate as the uninoculated control fruit and the firmness of the fruit also decreased progressively. These changes could be due to the normal ripening process of the fruit (Wills *et al*., 2007; Pinheiro *et al*., 2013). Similar results have been reported by Zhang *et al*. (2007) and Aguirre-Güitrón *et al*. (2019). Aguirre-Güitrón *et al*. (2019) reported that the application of an antagonist isolate of *Meyerozyma caribbica*, either fresh or in a powder formulation, significantly controlled postharvest anthracnose of mango fruit, without affecting the quality parameters of the fruit during the storage. Similarly, Zhang *et al*. (2007) reported that the firmness of peach fruit treated with an antagonist *Cryptococcus laurentii* was not lower than those of the control fruit.

This implies that the inhibition of the ripening and senescence of the fruit was not a component of the mode of action of the yeasts in control of postharvest anthracnose and sour rot in this study. This improved quality of fruit treated with the combined treatments could be attributed mostly to the effect of rHWTs on the fruit. However, heat may partially disinfect fruit surfaces allowing successful establishment of antagonist yeasts at the wound site, which in turn may provide a residual effect to the fruit (Wszelaki *et al*., 2003). In a contrasting result, Tian *et al*. (2018) reported a significant delay in both colour development and the softening of mango fruit treated with an antagonist, *Metschnikowia pulcherrima*.

Fruit that received rHWT, either as a single treatment or in combination with the antagonist yeasts, tended to be firmer and their colour appeared to be more stable than those fruit that were dipped in the antagonist yeast suspension alone, or the control fruit. Fruit treated with rHWT in combination with an antagonist yeast had the best overall quality, with an excellent external appearance and without any skin damage. The positive effect of heat treatments on the overall quality of fruit has been reported in several studies. HWT has been reported to trigger several developmental and physiological responses, and to induce beneficial effects on fruit physiology by inhibit ripening and delaying senescence during storage (Perini *et al*., 2017). In general, HWT is reported to affect the colour, and to inhibit synthesis of lycopene, ethylene and cell wall degrading enzymes, which reduces the risk of postharvest decay while maintaining the overall quality of the produce (Talibi *et al*., 2014). In most studies on the combination of HWTs with biological control agents, the HWT have been applied at relatively low temperatures with long exposure times. Rapid HWTs that combine higher temperatures with much shorter exposure times have been found to be more effective in the control of postharvest diseases of orange (Strano *et al*., 2014), mango (Wang *et al*., 2017) and tomato fruit (Ziena and Laing, 2020, unpublished). Laing and Basdew (2018) reported on the efficacy of a combination of rHWT (64°C for 15s) in combination with the antagonist yeast B13 to control *P. digitatum* infections on lemon, and Navel and Valencia orange fruit. Levels of disease control matched the fungicide imazalil. To date, and to the best of our knowledge, this study is the first report of integration of rHWTs with antagonist yeasts to control postharvest diseases in tomato fruit.

6.5 Conclusion

The integration of rHWTs with antagonist yeast isolates of *M. guilliermondii* were effective against *C. gloeosporioides* or *G. candidum* infections on tomato fruit. They also provided for excellent postharvest quality that was superior to both individual treatments, and the control. Such combinations are compatible with existing facilities and postharvest handling procedures, and could be implemented on a commercial scale in existing tomato packhouses. They represent a viable alternative to the use of synthetic pesticides for the control of postharvest diseases of tomato fruit on a large scale.

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Chapter 7: Conclusions and Recommendations

7.1 Thesis Overview

Tomato (*Solanum lycopersicum* Mill.) is one of the most widely grown and extensively consumed vegetable worldwide (Sandoval *et al*., 2015). Anthracnose and sour rot caused by *Colletotrichum* and *Galactomyces* species, respectively, are the most important postharvest pathogens, causing significant losses of tomato fruit globally (Živković *et al*., 2010). Previously these diseases have been controlled using synthetic fungicides. However, with the mounting concern for human health and environmental risks, and the loss of pesticides to resistance, the search for non-chemical alternatives for the control of postharvest diseases during the storage and selling process has been of a focus of much research for more than three decades. Biocontrol agents have emerged as an important component of postharvest disease control, and may provide an alternative to synthetic fungicides (Sharma *et al*., 2009). Postharvest heat treatment is also a promising technology for the control of postharvest diseases of various commodities, while maintaining the crop quality (Palou, 2009; Spadoni *et al*., 2015; Siddiqua *et al*., 2018). However, in prior research, the application of either biocontrol or heat treatment alone has not provided an acceptable level of decay control and shelf-life extension, with a persistence and broad-spectrum activity comparable to that provided by fungicides. This study was undertaken to evaluate the efficacy of rHWTs and biocontrol agents, alone or in combination, for the control of the two major postharvest pathogens of tomato fruit.

The purpose of this overview is to review the specific objectives of this thesis and their outcomes, to identify future research needs, to recommend future research directions, and finally to make specific recommendations and highlight some of the potential impacts of this research to the tomato industry in South Africa.

7.2 Research objectives and respective outcomes

Objective 1: To isolate and identify primary pathogens of tomato causing anthracnose and sour rot after harvest, to test their pathogenicity and study the effect of wound and non-wound inoculation methods on infection process and disease development.

- ¶ The two primary pathogens of tomato in South Africa causing anthracnose and sour rot were identified as *Colletotrichum gloeosporioides* (Penz.) and *Galactomyces candidum* (Link.).
- ¶ Both pathogens caused complete decay of the fruit.
- ¶ All wound and non-wound *Colletotrichum* inoculated fruits developed anthracnose, while non-wound *Galactomyces* inoculated fruits failed to develop sour rot, indicating that *Galactomyces* is strictly a wound pathogen.

Objective 2: To isolate and screen yeast cells for the control of *Galactomyces* and *Colletotrichum in vitro* and *in vivo*; to investigate their effect on the postharvest quality of tomato fruit; to start investigating potential modes of action; and to identify the most effective biocontrol yeast strains.

- ¶ A total of 148 yeast isolates recovered from the surface of tomato fruits were screened for antifungal activity *in vitro* using a dual culture assay.
- ¶ 25 isolates had strong antifungal activity against *C. gloeosporioides* and *G. candidum in vitro.*
- ¶ Isolates Y108, Y121 and Y124 showed the strongest antagonistic efficacy *in vivo*.
- ¶ Molecular studies identified all these three isolates as *Meyerozyma guilliermondii* (Wick) Kurtzman, previously known as *Pichia guilliermondii*, and with an asexual stage called *Candida guilliermondii*.
- ¶ Competition for space and nutrients, attachment to fungal hyphae and production of an extracellular matrix were among the probable modes of action of the antagonist yeasts.
- ¶ The application of these strains of yeast had no effect on tomato fruit quality. However, other strains had a detrimental effect on fruit quality, which looked like early senescence of treated fruit, possibly mediated by ethylene release.

Objective 3: To evaluate the effects of rapid hot water treatments (rHWTs) on tomato quality, and for the control of anthracnose and sour rot of tomato, postharvest.

- ¶ Temperatures of 20°C, and 44 to 59°C were tested with exposure periods of 10, 20 and 30 and 60 seconds. All temperatures applied for 10s were safe for tomato fruits, extending shelf-life and slowing colour development.

- ¶ rHWTs at 56℃ x 15s, 59℃ x 10s and 62℃ x 8s were the most effective treatments, controlling disease incidence of both pathogens, and maintaining fruit quality.
- ¶ rHWTs caused the melting of the wax platelets of the fruit, sealing surface cracks present on control fruits. Induction of host defence, and inhibition of sporulation and mycelial growth are among the possible modes of action of rHWTs.

Objective 4: The investigate the effect of integrating rHWTs of 56°C for 15s, 59°C for 10s or 62°C for 8s, combined with the yeasts *Meyerozyma guilliermondii* isolates Y108, Y121 and Y124, and a previously isolated yeast, B13 (a strain of *Candida fermentati*), for the control of *Colletotrichum gloeosporioides* and *Galactomyces candidum,* and for their effect on the postharvest quality of tomato fruit, alone or in combination

- ¶ rHWTs at 56°C for 15s, 59°C for 10s or 62°C for 8s significantly reduced both pathogens and maintained good fruit quality and an enhanced shelf-life.
- ¶ The yeasts Y108, Y121, Y124 and B13 significantly reduced disease incidence of both pathogens but failed to maintain fruit quality.
- ¶ Although not significantly different from the application of yeasts alone, the integrated treatments provided the best control of disease incidence, and resulted in better fruit quality than the single treatments and the control fruits.

7.3 Recommendations for Future Research

- ¶ Anthracnose typically has a pre-harvest infectious phase which remains latent in infected fruit, until fruit ripen, usually in the postharvest situation. Control of this disease is difficult. Preharvest applications of antagonistic yeasts could provide control of this phase of the anthracnose disease cycle. Test pre-harvest applications of the best yeasts, evaluating their ability to survive on fruit surfaces prior to harvest, to colonize wounds and to exclude latent and wound-infecting pathogens.
- ¶ The production and formulation of yeasts for application to fruit postharvest is needed, to ensure that their performance as biocontrol agents is optimized.

- ¶ Molecular tools in genomics and proteomics could be used to understand the interactions of between heat treatments, antagonist yeasts, host tissue, pathogens, biotic and abiotic elicitors of defense mechanisms, and environmental effects.
- ¶ Integrated treatments could be studied on different tomato cultivars and other fruits and vegetables,
- ¶ Further assessment could be done on the feasibility of implemented the integrated package for treatment of tomato fruit on both small and large-scale farms.

Potential impact of this research

- ¶ If the technology is adopted, it will result in reduced losses of fresh tomatoes
- ¶ There would be reduced usage of fungicides for the postharvest treatment of tomato fruit, with a positive impact on the environment and the consumers.
- ¶ It would reduce the postharvest losses of tomato fruit, affecting the costs of production and risks faced by tomato farmers.
- ¶ These treatments could reduce the cost of fresh tomatoes to South African consumers, with a positive health impact.
- ¶ With improved shelf-life, the export of treated tomatoes would be more attractive, and could make the SA tomato fruit globally competitive.

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Appendixes

ITS SEQUENCING REPORT

Client's Name:	Luwam Ziena
Institution:	UKZN
Report Compiled By:	Inqaba Biotechnical Industries (Pty) Ltd

MATERIAL AND METHODS:

Genomic DNA was extracted from the cultures received using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, Catalogue No. D6005). The ITS target region was amplified using OneTaq® Quick-Load® 2X Master Mix (NEB, Catalogue No. M0486) with the primers presented in Table 1. The PCR products were run on a gel and gel extracted with the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Catalogue No. D4001). The extracted fragments were sequenced in the forward and reverse direction (Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000) and purified (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™, Catalogue No. D4050). The purified fragments were analysed on the ABI 3500xl Genetic Analyzer (Applied Biosystems, ThermoFisher Scientific) for each reaction for every sample, as listed in Section 1. CLC Bio Main Workbench v7.6 was used to analyse the .ab1 files generated by the ABI 3500XL Genetic Analyzer and results were obtained by a BLAST search (NCBI).

Table 1: ITS Primers sequences

Name of Primer	Target	Sequence (5' to 3')	Reference
ITS1	Small Sub-Unit	TCCGTAGGTGAACCTGCGG	White et al, 1990
ITS4	Large Sub-Unit	TCCTCCGCTTATTGATATGC	White et al, 1990

BLASTN 2.2.31+
Reference: Stephen F. Altschul, Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

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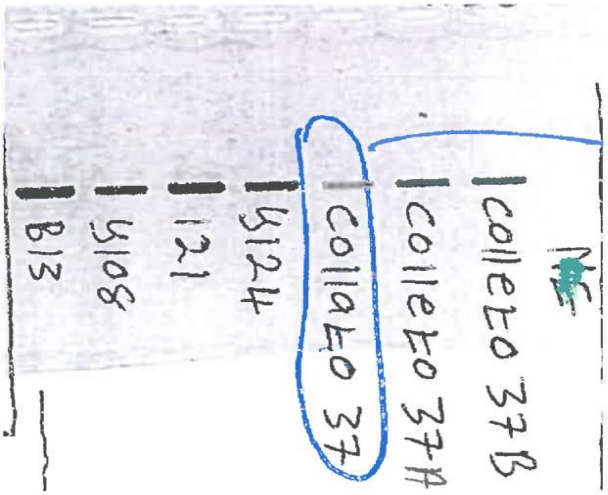
ITS SEQUENCING REPORT

SECTION 2: RESULTS

Figure 1. A photographic image of an agarose gel indicating the fragments generated by the ITS1/ITS4 PCR.



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ITS SEQUENCING REPORT

SECTION 2: RESULTS

BLAST: The BLAST results correspond to the similarity between the sequence queried and the biological sequences within the NCBI database.

Name of sample	121
Request ID	Y27XKKUX014
Predicted Organism	*Meyerozyma guilliermondii*
GenBank Accession	MK394108.1

Name of sample	B13
Request ID	Y27XV9V3014
Predicted Organism	*Candida railenensis*
GenBank Accession	FM178302.1

Name of sample	Colleto-37
Request ID	Y9D0FG93014
Predicted Organism	*Colletotrichum siamense, Colletotrichum gloeosporioides*
GenBank Accession	KP703372.1, KC010549.1

Name of sample	Colleto 37A
Request ID	Y27Y1RAA014
Predicted Organism	*Colletotrichum gloeosporioides*
GenBank Accession	KC493156.1

Name of sample	Colleto 37B
Request ID	Y27Z88M3016
Predicted Organism	*Colletotrichum gloeosporioides, Colletotrichum siamense*
GenBank Accession	MN220648.1, MK471371.1

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ITS SEQUENCING REPORT

SECTION 2: RESULTS

BLAST: The BLAST results correspond to the similarity between the sequence queried and the biological sequences within the NCBI database.

Name of sample	Y108
Request ID	Y27YBP9Y014
Predicted Organism	*Meyerozyma guilliermondii*
GenBank Accession	MK394108.1

Name of sample	Y124
Request ID	Y27YHV2B014
Predicted Organism	*Meyerozyma guilliermondii*
GenBank Accession	MK394108.1

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ITS SEQUENCING REPORT

SECTION 3: SUMMARY

Name of Sample	BLAST Prediction
121	*Meyerozyma guilliermondii*
Colleto 37	*Colletotrichum siamense, Colletotrichum gloeosporioides*
Colleto 37A	*Colletotrichum gloeosporioides*
Colleto 37B	*Colletotrichum gloeosporioides, Colletotrichum siamense*
Y108	*Meyerozyma guilliermondii*
Y124	*Meyerozyma guilliermondii*
B13	*Candida railenensis*

[redacted]

Dr Erika Viljoen
Genomics Scientist

2/12/19
Date

[redacted]

Dr Christiaan Labuschagne
Genomics Manager

2/12/19
Date

Acronyms

AD	Anno Domini
ANOVA	Analysis of Variance
APX	Ascorbate Peroxidase
BCA	Biocontrol Agent
BLASTn	Basic Local Alignment Search Tool
℃	Degree Celsius
CAT	Catalase
CHI	Chitinase
CI	Chilling Injuries
cm	Centimetre
CO_2	Carbon Dioxide
DAFF	Department of Agricultural and Forestry
DIM	Demethylation inhibitor
DNA	Deoxyribonucleic Acid
dpi	Days Post-Inoculation
FAO	Food and Agriculture Organizations
GNS	ß-1,3-glucanase
GR	Glutathione Reductase
HAT	Hot Air Treatment

HSPs	Heat-Shock Proteins
HWD	Hot Water Dips
HWRB	Hot Water Rinsing and Brushing
HWT	Hot Water Treatment
ITS	Internal Transcribed Spacer
LSU	large ribosomal subunit
M	Molar
MBC	Methyl benzimidazole carbamates
MEA	Malt Extract Agar
min	Minutes
ml	Millilitre
mm	Millimetre
NCBI	National Center for Biotechnology Information
ND	Not determined
OECD	Organisation for Economic Co-operation and Development
PAL	Phenylalanine Ammonia Lyase
PAMPs	Pathogen Associated Molecular Patterns
PCD	Programed Cell Death
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PHI	Post Harvest Innovation

POD	Peroxidase
PPO	Polyphenol Oxidase
QoI	Quinone Outside Inhibitors
RH	Relative Humidity
rHWTs	Rapid Hot Water Treatments
ROS	Reactive Oxygen Species
Rpm	Revolutions Per Minute
s	Seconds
SA	South Africa
SEM	Scanning Electron Microscopy
SOD	Superoxide Dismutase
SOPP	Sodium ortho-phenylphenate
UK	United Kingdom
US	United States
UV	Ultra Violet
VHT	Vapour Heat Treatment
YDCA	Yeast Dextrose Calcium Agar
YPD	Yeast Peptone and Dextrose
µl	Microlitre