Thyme oil and thyme oil hydrosol as alternative fungicides against *Phyllosticta citricarpa* (causative agent of citrus black spot)

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DEDICATION

This thesis is dedicated to my late father (John Mudau Sibiya), my late aunt (Carilota Khahleyani Sibiya), my late grandma (Rosita Mathonsi) and my late sister (Ntombi Margaret Magunga) who transitions on my birthday.

DECLARATION OF INDEPENDENT WORK

I, Bheki Thapelo Magunga, do hereby declare that this research project submitted to Central University of Technology, Free State, for the degree PHILOSOPHIAE DOCTOR: ENVIRONMENTAL HEALTH is my own work and has not been submitted before to any institution by myself or any other person in fulfilment of the requirements for the attainment of any qualification.

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Student signature Date

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SUMMARY

The South African citrus industry is one of the most important agricultural industries economically, as it contributes considerably to the country's Gross Domestic Product (GDP) (Dlikilili and Van Rooyen, 2018; Venter, 2018). The citrus sector in the country also contributes greatly to employment opportunities, particularly for disadvantaged communities, as it currently employs over 125 000 people across the country (Genis, 2018). Over the years and despite the Covid 19 pandemic, the number of citrus trees planted in hectares (ha) has continued to increase. This is due to the high vitamin C content of citrus fruits, the increase in investments and aggressive new plantings of soft citrus, lemons, and new varieties of oranges (Cramer and Chisoro-Dube, 2021). Citrus cultivation for commercial purposes is reported mainly in Limpopo, Western Cape, Mpumalanga, Eastern Cape, KwaZulu-Natal, and Northern Cape. Within the Southern African Development Community (SADC), countries such as Zimbabwe, Swaziland and Mozambique also produce citrus fruits in much smaller quantities (Department of Agriculture and Fisheries, 2017). Most citrus fruits produced in South Africa are exported to countries such as Europe and the United States of America (USA) because of far greater returns from the export market than the domestic market. However, the spread of citrus black spot (CBS) has resulted in fears of a possible ban on South African citrus exports to other countries (Kau *et al.,* 2018; Van Dyk and Maspero, 2004). Evidence that CBS affects the agricultural industry negatively regarding food production, security and trade is available.

CBS is a citrus disease caused by the fungus *Phyllosticta citricarpa,* which affects almost all commercial citrus species externally but does not cause internal decay (Yonow *et al.,* 2013; Roberts *et al.,* 2012; Fialho *et al.,* 2010). Almost all commercially sold citrus species are susceptible to *Phyllosticta citricarpa* infection, with lemons and Valencia oranges

known to be highly susceptible. CBS was first discovered, recorded and described by Benson in Australia and was observed on Valencia oranges over 120 years ago (Paul, 2006; Kotzé, 2000; Kiely, 1948;). *Phyllosticta citricarpa* produces sexual and asexual spores, with each stage producing different spores responsible for spreading CBS using various modes of dispersion (Truter, 2010). Synthetic fungicides are currently used to minimize the spread of CBS; however, their use has raised health and environmental pollution concerns, including resistance to pathogens (Du Plooy *et al.,* 2009). Hence, research into developing alternative agents for replacing synthetic fungicides for fungal disease control in agriculture continues, such as using essential oils (EOs) and their hydrosol. EOs are complex mixtures of secondary plant metabolites with relatively high vapour pressure and are poorly soluble in water.

The EOs reported in various studies have shown to exhibit antifungal properties by targeting structures responsible for the life cycle of fungal organisms such as ascospores and conidia *in vitro* and *in vivo* in different fresh produce. The overall aim of the study was to investigate the *in vivo* effect of thyme oil and thyme oil hydrosol against citrus fungal pathogen *Phyllosticta citricarpa* (Feyaerts *et al.,* 2018). Thyme oil characterization was done using gas chromatography-mass spectrometry GC-MS and GC × GC-TOFMS. Their effectiveness, together with that of hydrosol, was tested against *Phyllosticta citricarpa* (CBS causative agent) using methods such as broth microdilution assay (minimum inhibitory concentration (MIC) determination) and fungicidal or fungistatic activity. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were used to evaluate morphological changes that occurred due to EOs, while the inhibition of pathogen growth on leaves and fruits was also determined. The results of the study revealed that the pathogenic fungi *P. citricarpa* depend on increased mitochondrion activity, such as conidia, and these structures are sensitive to thyme oil used in the study. Furthermore, thyme oil hydrosol used in the study has indicated a moderate antifungal activity against this fungal pathogen.

CHAPTER ONE

A GENERAL INTRODUCTION TO THE CITRUS INDUSTRY IN SOUTH AFRICA

The Citrus Industry in South Africa

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1.1 The citrus industry in South Africa

The South African citrus industry is one of the most important agricultural industries that contribute economically to the country by increasing foreign currency earnings, thereby contributing considerably to the country's Gross Domestic Product (GDP) (Dlikilili and Van Rooyen, 2018; Venter, 2018). With a history dating back to the 1600s, when the first citrus fruit trees were planted in the Western Cape (Dlikilili, 2018), the citrus fruit industry has since grown and currently represents one of South Africa's most important agro-commodities by value and volume (Sinngu, 2014). Citrus fruits currently constitute one of the most important horticultural crops in South Africa. In terms of gross value, the industry is currently the third-largest horticultural industry in the country, preceded by the deciduous fruit and vegetable industries (Dlikilili and Van Rooyen, 2018; Sinngu, 2014). The diversity of growers characterizes this industry, varying from large and highly profitable commercial to small-scale producers (Dlikilili and Van Rooyen, 2018). Furthermore, the citrus industry has continued to experience growth as citrus fruits are grown in fifteen regions across the country, supplying numerous citrus varieties such as soft citrus, lemons, limes, oranges, and grapefruit (Dlikilili and Van Rooyen, 2019). As much as South Africa's unemployment rate has increased due to the Covid 19 pandemic, the citrus sector in the country has always presented good employment opportunities, particularly for disadvantaged communities (Van Lill and Bakker, 2022; Urquhart, 1999; Van Lill and Bakker, 2022). Currently, estimates indicate that the citrus industry in South Africa employs over 125 000 people across the country, or 14% of the agricultural job market, with large numbers of workers in the orchards and packing houses (Genis, 2018). However, these numbers could be higher if the unspecified number of people employed throughout the citrus supply chain services, such as transport, port handling and other related industries (Genis, 2018; Sinngu, 2014), is also included.

1.2 Major citrus fruits produced and production areas in South Africa

According to Genis (2018), about 72 731 hectares (ha) of citrus trees were planted across South Africa in 2017. However, the hectares have slightly increased to 77 676 ha planted citrus trees in South Africa, producing over 2.1 million tonnes of fruits (Philips, 2018). Furthermore, in 2019, 86,808 ha of citrus trees were planted in South Africa. This growth trend has continued as it was estimated to be around 95,200 ha in 2020. An estimated 98,700 ha was planted to citrus in South Africa in 2021 (Cramer and Chisoro-Dube, 2021). Although the Covid 19 pandemic has disrupted many industries worldwide, the citrus industry has grown despite the crisis. In particular, the demand for citrus fruits has increased during the pandemic because of health benefits such as the high vitamin C content in citrus fruits. Furthermore, the growth trend is forecast to continue in 2022, based on the significant investments and aggressive new plantings of soft citrus, lemons, and new varieties of oranges (Cramer and Chisoro-Dube, 2021). Within the Southern African Development Community (SADC), countries such as Zimbabwe, Swaziland and Mozambique also produce citrus fruits, although in much smaller quantities (Figure 1) (Department of Agriculture, Forestry and Fisheries, 2017). In South Africa, citrus cultivation for commercial purposes is reported mainly in Limpopo, Western Cape, Mpumalanga, Eastern Cape, KwaZulu-Natal and Northern Cape, while North-West, Gauteng, and Free State provinces do not produce citrus fruits (Morokolo, 2016). Figure 1 depicts the percentage breakdown of the major citrus-producing provinces. It is evident that the highest citrus fruit production takes place in Limpopo (Figure 1). It contributes 42% of the total area planted to citrus in South Africa, followed by the Eastern Cape at 26%, Western Cape at 17%, and Mpumalanga at 7%. A lower percentage (2%) is cultivated in the Northern Cape and KwaZulu-Natal, respectively (Figure 1).

The industry is spread across various climatic zones, from a warmer and sub-tropical climate in Mpumalanga, Limpopo, and KwaZulu-Natal provinces to Mediterranean climates in the Eastern and Western Cape (Genis, 2018; Sinngu, 2014;). This range of climatic conditions provides an ideal environment for growing a full range of citrus fruits. Mpumalanga, Limpopo, and KwaZulu-Natal climates are warmer and better suited to

cultivating grapefruit and Valencia oranges. On the other hand, the Western Cape and Eastern Cape are 'cooler' citrus-growing areas, and production is focused on Navel oranges, lemons, and soft citrus fruits (Sinngu, 2014). The area planted per citrus variety or group during 2016 is shown in Figure 2, where the most-planted citrus variety was Valencia at 38%. Limpopo contributed 60% of all Valencia oranges planted in 2016, followed by Navel oranges (22%) (Department of Agriculture, Forestry and Fisheries, 2017). The Eastern Cape contributed 40% of all Navel oranges planted in 2016. Soft citrus accounted for 16% of the total area planted for citrus products that year, followed by lemons and limes at 13%, and grapefruit at 11% (Department of Agriculture, Forestry and Fisheries, 2017).

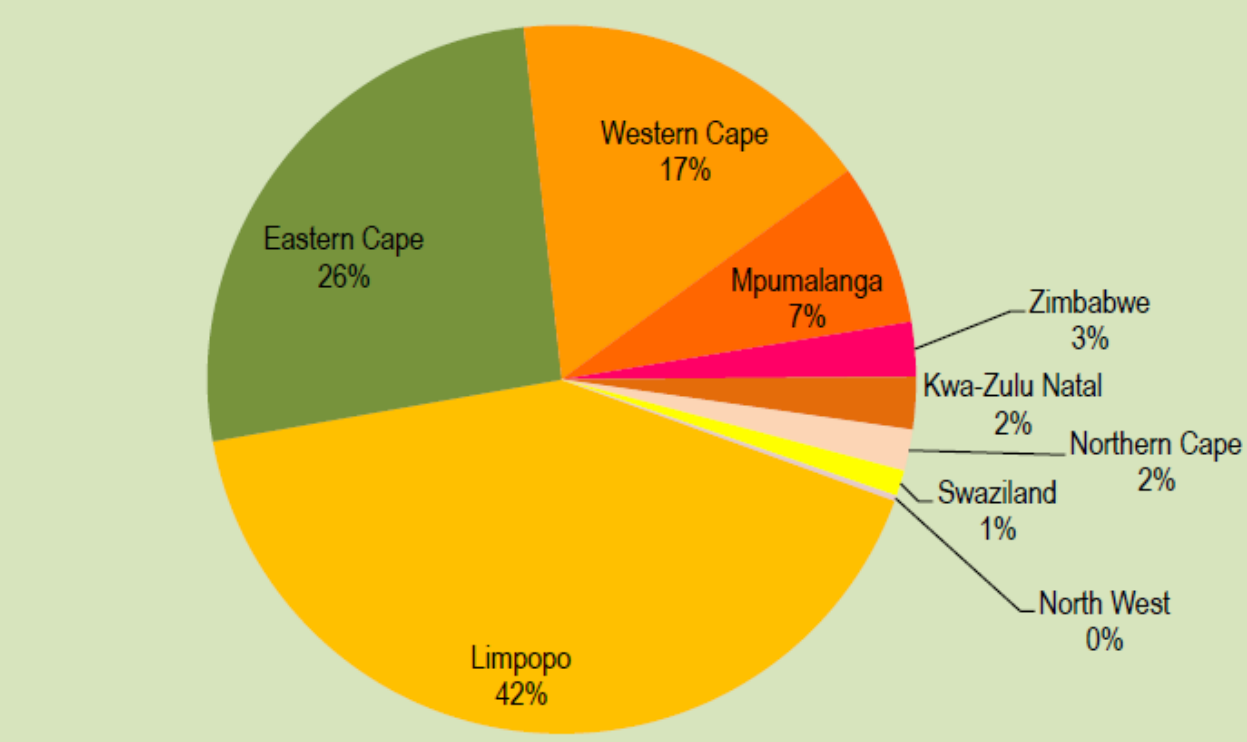

Figure 1: Citrus production areas in hectares in 2017 (cited from the Department of Agriculture, Forestry and Fisheries, 2017).

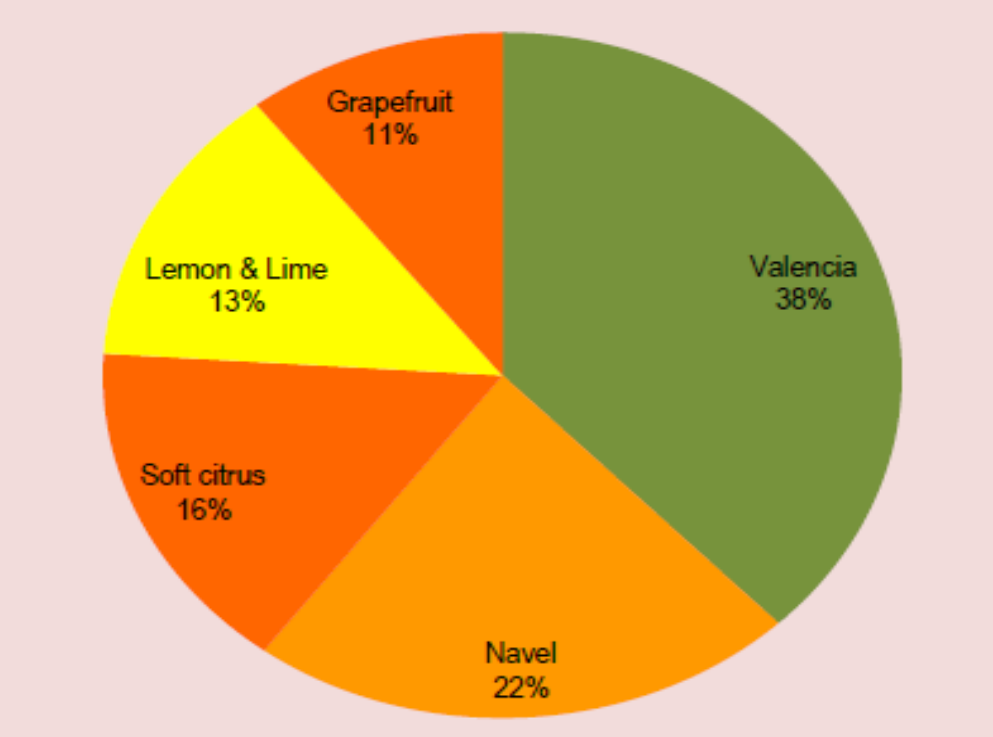

Figure 2: Area planted per variety group in hectares in 2017 (cited from the Department of Agriculture, Forestry and Fisheries, 2017).

1.3 Markets for the citrus industry

As South Africa produces more fruit than can be absorbed and consumed by the local market and the fruit is of high quality, these citrus fruits are then sold through different marketing channels, such as national fresh produce markets, informal markets (street hawkers and bakkie traders), directly to processors for juice making and dried fruit production (Van Dyk and Maspero, 2004). The fruits are also sold directly to wholesalers and retailers through signed contracts. A large percentage of fruit is exported to foreign countries through export agents (Kau *et al.,* 2018; Van Dyk and Maspero, 2004). Most citrus growers export as much of their crop as possible because of the greater returns from the export market than the domestic market (Genis, 2018; Urquhart, 1999). Furthermore, reports suggest that emerging black farmers who export citrus fruits are being established. It is recorded that 123 black farmers operate in South Africa; 10.7% have citrus orchards, and 42% of those farmers export their products (Kau *et al.,* 2018;

Van Dyk and Maspero, 2004). Furthermore, these initiatives assist in the South African context with transformative progress of the industry to support the establishment and growth of sustainable and profitable black citrus growers with market linkage to ensure food security, job, and wealth creation (Kau *et al.,* 2018; Genis, 2018). For example, The Alice Kat Citrus Project in the Kat River area of the Eastern Cape was an agricultural development project initiated by the former Ciskei homeland. Several farms previously owned by white farmers were bought by a parastatal and allocated to approximately 16 black farmers (Urquhart, 1999).

As indicated in the preceding subsection, citrus production in South Africa is mainly destined for the export market. The biggest contributor to the total volume of South African citrus exports is oranges, which have contributed more than 63% (1,064,089 tons) to total citrus product exports since 2016, and the numbers have continued to increase, as in 2019, the export figure was estimated to be around 1,186,426 tons (Table 1) (Department of Agriculture, Forestry and Fisheries, 2017; United States Department of Agriculture, 2020). Lemon and lime exports were second in 2016 at 14% (237,131 tons) and increased to about 350,245 tons in 2019. Grapefruit and soft citrus made up 12% (202,527 tons) and 11% (189,730 tons) of the exports, respectively, in 2016 (Department of Agriculture, Forestry and Fisheries, 2017) and increased to 258,423 tons and 295,606 tons, respectively, in 2019, (United States Department of Agriculture, 2020; Department of Agriculture, Forestry and Fisheries, 2017). The volumes of citrus products sold to export and processing markets increased between 2016 and 2019 (United States Department of Agriculture, 2020; Department of Agriculture, Forestry and Fisheries, 2017). Even though the South African citrus industry is very important in boosting the country's economy, the industry still faces huge obstacles. These include the rejection of citrus fruits by some markets, such as the USA, South Korea and the European Union (EU), citing safety problems resulting in stringent sanitary and phytosanitary (SPS) conditions due to the fungal disease citrus black spot (CBS).

Table 1: South Africa's citrus exports to the world in metric tons, from 2016-2019 (cited from Department of Agriculture, Forestry and Fisheries, 2017; United States Department of Agriculture, 2020).

Description	2016	2017	2018	2019
Oranges	1,064,089	1,170,813	1,278,935	1,186,426
Lemons & limes	237,131	299,323	315,197	350,245
Grapefruit	202,527	230,635	288,155	258,423
Soft citrus*	189,730	209,754	260,850	295,606
All	**1,693,477**	**1,910,525**	**2,143,137**	**2,090,701**

*** Includes mandarins (including tangerines and Satsumas), and clementines.**

1.4 Problems affecting the citrus industry

South Africa's citrus industry has received much media and policy attention due to concerns over increased fungal infection, namely CBS interceptions on the country's EU-bound shipments, leading to fears of a possible ban on South African citrus exports (Olivier, 2017). It is evident that CBS negatively affects the agricultural industry in terms of food production, security, and trade. As such, some trading partners were reluctant to import fresh fruit from production areas where CBS occurs; this has resulted in South Africa losing more than 45% of its export revenue due to postharvest infection of citrus fruit (80-90% losses), which was intended for the EU and Asian markets (Christie, 2016; Lesar, 2013; Montesinos-Herrero *et al.,* 2009). One of the main difficulties related to fungal pathogens is their progressive nature because they sometimes do not show during harvest or fruit grading in the pack house; symptoms can develop during storage or weeks after harvesting (Erasmus *et al.,* 2015). In exporting fruit, this window period matches the commercial shipping period and the fruit's arrival at the point of sale. The result is customer complaints at best and huge financial losses at worst (Erasmus *et al.,* 2015).

These losses affect profit margins, initial input costs, and lower retailer and consumer confidence in the marketplace (Erasmus *et al.,* 2015). The application of synthetic fungicides usually minimizes symptoms and yield losses caused by CBS infection during the fruit's susceptibility period; however, their use raises health and environmental pollution concerns, including resistance to pathogens (Arraiza *et al.,* 2018). Moreover, the treatment used currently against this pathogen has raised consumer concerns on issues such as food safety (Du Plooy *et al.,* 2009). The food safety concerns are due to the chemical residues left on food when synthetic fungicides are applied. Hence, research into developing alternative agents for replacing synthetic fungicides for fungal disease control in the agricultural sector continues (Christie, 2016; Du Plooy *et al.,* 2009). An alternative antifungal needs to (a) overcome resistance problems observed against established commercial products, (b) have acceptable levels of persistence in the environment, and (c) have market and technical advantages over synthetic fungicides (Arraiza *et al.,* 2018). Essential oils (EOs) and their hydrosol can represent a new class of CBS antifungals due to their antimicrobial effects (Arraiza *et al.,* 2018). In addition, the probabilities of creating new resistant strains by using EOs as fungicidal agents are less likely since they have different chemical constituents that work in synergy simultaneously (Arraiza *et al.,* 2018).

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CHAPTER TWO

A LITERATURE REVIEW

THYME OIL AND HYDROSOL, AN ALTERNATIVE APPROACH IN THE BIOLOGICAL CONTROL OF *PHYLLOSTICTA CITRICARPA*: A LITERATURE REVIEW

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2.1 General background

Citrus black spot (CBS) has become a serious, widespread problem for citrus production throughout the world (Frare *et al.,* 2018; Yonow *et al.,* 2013; Roberts *et al.,* 2012; Fialho *et al.,* 2010) and a quarantine disease for countries in the European Union (EU) and the United States of America (USA) (Baldassari *et al.,* 2009). CBS is a citrus disease caused by the fungus *Phyllosticta citricarpa,* which affects almost all commercial citrus species externally, but does not cause internal decay (Dewdney *et al.,* 2010; Timmer, 1999; Davies and Albrigo, 1994). The disease causes foliar and fruit blemishes affecting the rind of the fruit, causing cosmetic lesions (Paul *et al.,* 2005). Heavy infection near the pedicel of the developing fruit may lead to premature fruit drop. Losses may be substantial because affected fruits are no longer suited for the fresh fruit market (Baayen *et al.,* 2002). This impacts the economy worldwide, as it was estimated that losses due to CBS could amount to millions of dollars (Schreuder, 2017). Furthermore, fruit that is heavily infected with *P. citricarpa* is used for juice production, which yields a much lower income per ton produced, thereby directly decreasing its economic value on local and international markets (Schreuder, 2017). This fungal disease, with a high economic impact on citrus, was first discovered in Australia (Baldassari *et al.,* 2009).

2.2 Citrus black spot origin and spreading

CBS was first discovered, recorded and described by Benson in Australia, where it was observed on Valencia oranges over 120 years ago (Carstens *et al.,* 2017). The report indicated that CBS infection in citrus fruits resulted in significant losses in citrus production areas in Australia (Schreuder, 2017; Truter, 2010). Soon after, McAlpine described the asexual stage of the fungus in 1899 (Carstens *et al.,* 2017; McAlpine, 1899), and years later, in 1948, Kiely described the sexual stages of the fungus (Kiely, 1948).

In 1939, a severe CBS epidemic in New South Wales was reported; however, the first official report of the disease from citrus-growing areas outside Australia was only reported in China in 1920 (Carstens *et al.,* 2017; Lee, 1920). Later, reports of the disease were

noted in Argentina in 1928, South Africa in 1929 and Brazil in 1980 (Carstens *et al.,* 2017). In 1945 the citrus production areas in the Northern provinces (Mpumalanga, Limpopo) of South Africa were badly affected by CBS, where the disease was reportedly introduced through bud wood imported from Australia. This developed into an epidemic crisis, leading to 90% of fruit produced in unsprayed orchards being rejected in the export market, resulting in huge financial losses (Schreuder, 2017; Baayen *et al.,* 2002). Later, the disease was observed in other provinces such as KwaZulu-Natal and the Eastern Cape (Hlatshwayo, 2016). Research also suggests the pathogen was found in Florida in the USA in 2010 (Carstens *et al.,* 2017). This is due to the global expansion of citrus production and the movement of propagation material that results in the introduction of this fungal pathogen into major citrus production areas around the world, especially in countries or regions with a hot, wet, humid summer rainfall climate (Carstens *et al.,* 2017).

On record, some of the countries known to be affected by the pathogen include African countries (Kenya, Mozambique, Nigeria, South Africa, Zimbabwe, and Swaziland), Asian countries (China, Hong Kong, Indonesia, Japan, Philippines, and Taiwan), Oceania (Eastern Australia) and South American countries (Argentina, Brazil, Peru, and Uruguay) and North America (USA) (Martínez-Minaya *et al.,* 2015; Baldassari *et al.,* 2009; Baayen *et al.,* 2002). However, the disease is known to be absent and has never been reported in regions or countries with Mediterranean winter rainfall climates, even within the same country. For example, in South Africa, Australia, and China, the disease has only been reported in citrus production areas with summer rainfall (Carstens *et al.,* 2017; Carstens *et al.,* 2012).

Previously, CBS was thought to be present in countries such as New Zealand, Egypt, Uruguay, India, and Singapore, but it was later discovered that it was *Guignardia mangiferae* and not *P. citricarpa* (Hlatshwayo, 2016). *Guignardia mangiferae* does not cause any CBS symptoms, although it has been isolated from symptomatic and asymptomatic citrus fruit rind and leaves (Meyer *et al.,* 2006). This harmless endophyte can also be isolated from other wide ranges of hosts. For example, it has been isolated in Australia on the leaves of various native species growing near citrus groves (e.g., *Telopea speciosissima*) and on garden plants (Baayen *et al.,* 2002). In the South African

Western Cape, the fungus is commonly present in indigenous plants, while *P. citricarpa* is mostly found on citrus fruits only (Wulandari *et al.,* 2009; Baayen *et al.,* 2001).

2.3 Citrus black spot host range

Almost all commercially sold citrus species are susceptible to *Phyllosticta citricarpa* infection, with lemons and Valencia oranges known to be highly susceptible. In most cases, lemons and Valencia oranges are normally the first to be observed with *P. citricarpa* infection when an outbreak of this fungus occurs (Paul, 2006; Kotzé, 2000; Kiely, 1948). The largest citrus producer in the USA was recently affected by CBS, and it was first detected on Valencia oranges during a multi-pest survey (Zavala *et al.,* 2014; Er *et al.,* 2013; Schubert *et al.,* 2012;). The disease may also cause significant losses on grapefruit and limes and has been reported to occur on citron, pomelos, and mandarins as well, particularly under favourable environmental conditions such as hot, wet, humid summer rainfall climate areas that are suitable for citrus production (Carstens *et al.,* 2017; Paul *et al.,* 2005). However, rough lemons were observed to be tolerant to CBS infection, even though this tolerance is still unexplained (Hlatshwayo, 2016). Moreover, sour orange and its hybrids are not susceptible to the pathogen (Paul *et al.,* 2005). In this regard, attempts have been made to develop tolerant hybrids using resistant citrus fruits as a source of resistance (Anon, 1974). Despite this, it is unlikely as conventional breeding did not produce commercially useful, tolerant cultivars (Anon, 1974). CBS fruit symptoms are wide-ranging and have many different names.

2.4 Symptoms of citrus black spot

The yield and commercial value of citrus fruit can be reduced by CBS infection, which results in premature fruit drop and unsightly blemishes that develop on the rind of the fruit (Baldassari *et al.,* 2009). In most instances, CBS occurs as a preharvest disease, although fruit that is asymptomatic at harvest may develop symptoms postharvest during transport or storage (Hlatshwayo, 2016). Different lesions characterize these CBS symptoms on the fruits at different growth stages. Symptoms can also occur on the leaves

(Figure 3 A) and stems (Figure not shown), even though they remain latent, and the symptoms are rarely visible on the leaves and stems. The asexual spores (conidia) on the leaves and stems can develop and serve as a secondary source of infection (Schreuder, 2017). These infections normally occur on the most susceptible citrus species, such as lemon (Fialho *et al.,* 2010). Under favourable conditions for their development, asexual spores (conidia) are closely covered over the entire leaf surface. They can occur on the dorsal or ventral surfaces of the leaf but are usually the thickest on the side exposed to the sun's radiation (Truter, 2010). The asexual spores (conidia) are produced on dead leaves beneath trees and occur in fruit lesions, on dead twigs, and sparsely within lesions on attached leaves or fruit stalks (Truter, 2010). Older leaf lesions have symptoms that include small, round, necrotic, sunken spots with a grey centre surrounded by a dark-brown ring (Kotzé, 1963; Kiely, 1948). Younger leaf lesions are generally small, reddish, and slightly raised, sometimes with a yellow halo (Schreuder, 2017). Symptoms on fruit are much easier to identify, and a variety of symptoms has been observed, such as (1) red spot; (2) hard spot; (3) false melanose/speckled blotch; (4) virulent spot; or (5) cracked spot. However, because of their variability, these symptoms can be very difficult to categorize in a specific class (Schreuder, 2017).

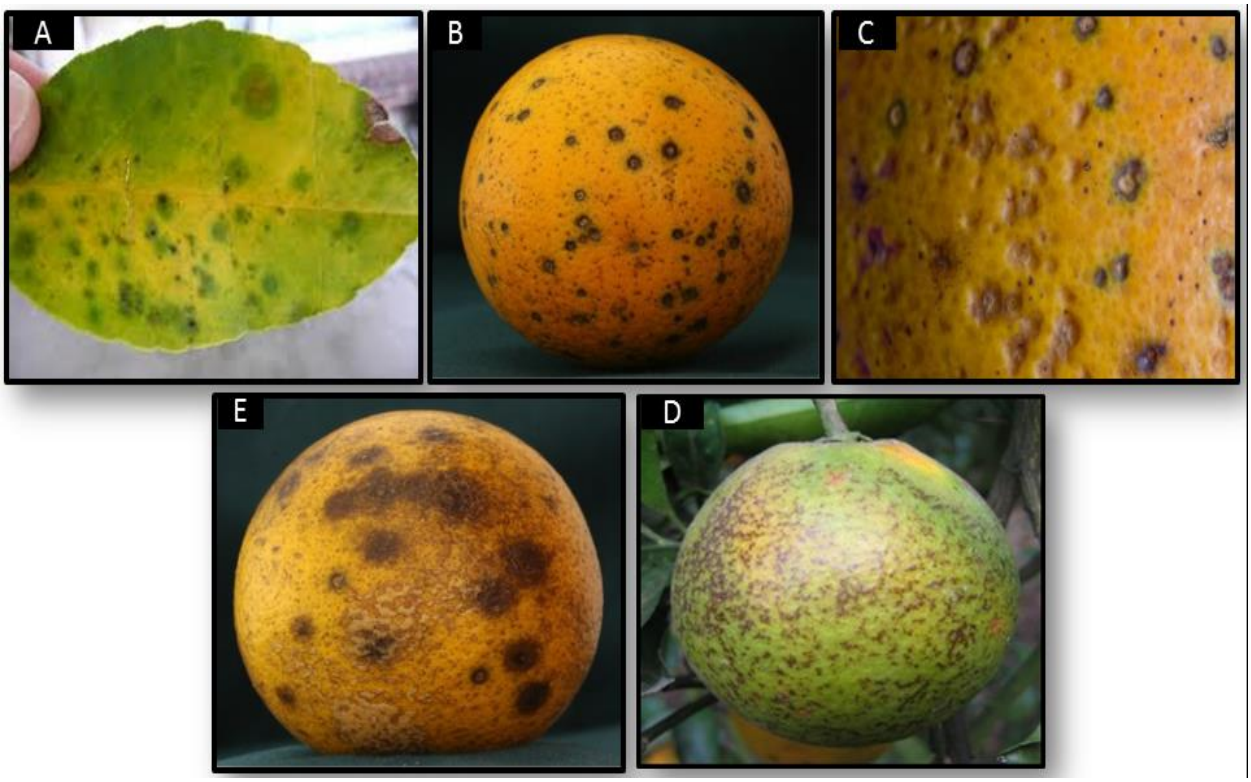

Figure 3. (A) Photograph depicts symptoms in mature leaves with tiny circular red to red-brown spots; (B) Photograph showing hard spot symptoms; (C) Photograph showing early virulent spot symptoms; (D) Photograph showing false melanose symptoms; (E) Photograph showing cracked spot symptoms, (cited from Magunga, 2016; Department of Plant Pathology, 2011).

2.4.1 Red spot

Bonants *et al.,* 2003; Korf, 1998; McOnie, 1967; Kotzé, 1963 are some of the authors that have reported the occurrence of red spot (Figure not shown), and, the red spot symptom category has increased, mainly because of the phytosanitary restrictions on the trade of symptomatic fruit and increased attentiveness to the presence of red spots on fruit at inspection sites (Truter, 2010). These symptom types are normally the first to develop postharvest on packed fruit, and symptoms require higher temperature and a longer incubation period for development. Lesions normally appear as round, sunken, reddish depressions on the fruit surface (Schreuder, 2017;Truter, 2010). The pathogen can be

isolated from a fruit demonstrating this symptom with little difficulty (Schreuder, 2017;Truter, 2010). These red spots later develop into hard spot (Truter, 2010).

2.4.2 Hard spot

Hard spot lesions (Figure 3 B) are the most distinctive and common CBS symptoms. These symptoms can be seen when fruit change colour from green to yellow or orange (Hlatshwayo, 2016). Hard spots are nearly circular, with grey necrotic tissue in the centre with a brick-red to a black margin that can be cracked around the edges. Fruiting structures (pycnidia) that produce the asexual spores (conidia) are often present in the centre of lesions and resemble slightly elevated black dots. Hard spot appears as the fruit begins to colour before harvest. They first occur on the side of the fruit with the greatest light exposure and may develop in the centre of cracked spot lesions and may also develop from speckled blotch (false melanose) lesions as fruit matures and changes colour (Dewdney *et al.,* 2010; Agostini *et al.,* 2006; Korf, 1998).

2.4.3 False melanose or speckled blotch

False melanose (Figure 3 D) symptoms appear on green fruit early in the season. They are slightly raised or depressed lesions 1-3 mm (0.04-0.1 inch) in diameter and can vary in colour from tan to chocolate brown as the fungus develops. The lesions can coalesce as the season progresses and do not contain fruiting structures (pycnidia) (Hlatshwayo, 2016). Under favourable infection conditions, false melanose can resemble the mud cake symptoms of authentic melanose but are very dark brown rather than rust red. False melanose symptoms can develop into hard spot as the season progresses (Dewdney *et al.,* 2010; Fialho *et al.,* 2010; Agostini *et al.,* 2006).

2.4.4 Cracked spot

Cracked spot (Figure 3 E) appears in fruit older than six months which are physiologically compromised and is characterized by the presence of superficial lesions which are variable in size and appear cracked (Truter, 2010). The symptoms are slightly salient, can occur individually or in groups and do not contain any fruiting structures (pycnidia) (Truter, 2010). These cracked spot lesions appear as large, slightly raised, dark brown patches that may occur on unripe and ripe fruit. (Truter, 2010). Cracked spot occurs during the interaction between rust mites and *P. citricarpa*. Furthermore, hard spots can form in the centre of these lesions (Dewdney *et al.,* 2010). According to Dewdney *et al.* (2010), cracked spot is a symptom only observed in the Americas.

2.4.5 Virulent spot

The most concerning CBS symptom is virulent spot (Figure 3 C), which usually causes heavy losses to postharvest fruits in transit (Kotzé, 1963). In most cases, the virulent spots arise from apparently healthy but latent infected fruits (Dewdney *et al.,* 2010; Kotzé, 1963). Early virulent spot (freckle spot) lesions start as irregularly shaped, sunken lesions with a reddish colour. Early virulent spot can either coalesce to cover a large proportion of the fruit surface or become hard spot. When spots coalesce, they turn brown to black, and the older lesion surface becomes leathery. Many pycnidia can be found in early and expanded lesions. Virulent spot occurs on mature, severely infected fruit at the end of the season (Dewdney *et al.,* 2010). Virulent spot symptoms can appear in postharvest on apparently symptomless fruit, sometimes in transit to markets. (Dewdney *et al.,* 2010; Fialho *et al.,* 2010; Agostini *et al.,* 2006). Symptoms can also occur on fruit that develops late in the season, and fruit that is symptomless at harvest may develop CBS in transport or storage. If symptoms develop before maturity, fruit often drop, resulting in yield loss. All these symptoms result from different stages of the life cycle of *Phyllosticta citricarpa*.

2.5 Life cycle of *Phyllosticta citricarpa*

During CBS epidemics, *Phyllosticta citricarpa* produces sexual and asexual spores (Figure 4). Each stage produces different spores responsible for spreading CBS using different dispersion modes (Magunga, 2016). These stages are discussed below.

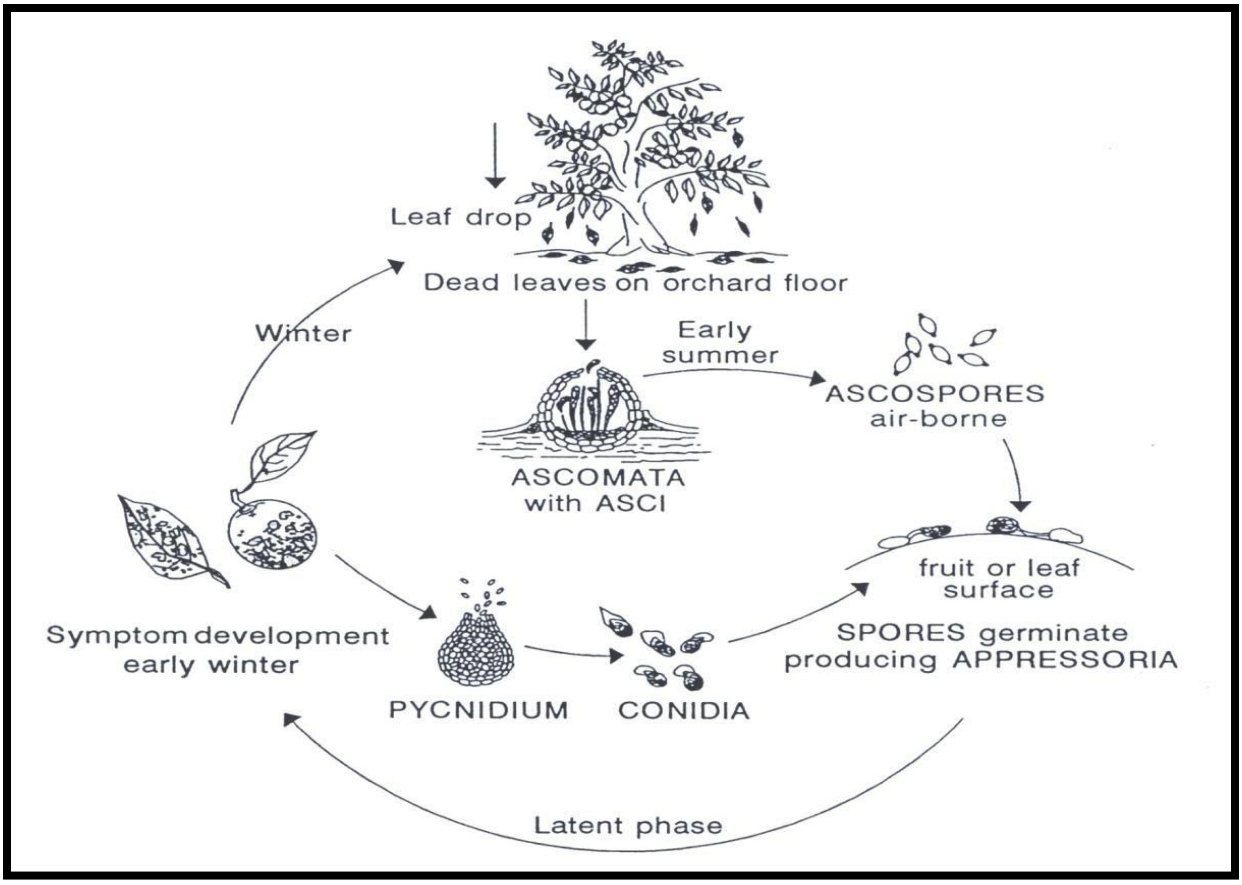

Figure 4: Life cycle and spreading of *Phyllosticta citricarpa* (Department of Plant Pathology, 2011).

2.5.1 Sexual stage

In the sexual phase, infected leaves drop and accumulate on the orchard floor from late winter to early spring. Pseudothecia with ascospores are then produced on the decomposing leaves on the orchard floor (Koltzé, 1981; McOnie, 1965). The production and maturation of ascospores are favoured by alternating dry and wet periods, a situation frequently observed during the rainy season (Kotzé, 2000). The active release of ascospores occurs when pseudothecia are moistened after maturity. Ascospores are ejected up to a height of about 1 cm and are dispersed by air currents (Kotzé, 1963; Kiely, 1948). On tree surfaces and in the presence of free water for periods longer than 15 hours at an optimal temperature of 27 °C, ascospores infect the host by direct penetration and form a mycelial mass in the sub-cuticular region. The fungus then remains quiescent until the leaves senesce, or the fruit begins to ripen (Kotzé, 2000; McOnie, 1967). Sexual stage or ascospore production is the primary inoculum for CBS, meaning the ascospores are injected and further dispersed by currents of air/ wind for long distances or water for short distances, where they are deposited on leaf and fruit surfaces and germinate to form quiescent infections (Magunga, 2016).

2.5.2 Asexual stage

In the asexual phase, pycnidia with conidia of *P. citricarpa* are produced in hard spot and freckle spot fruit lesions as well as on leaf litter prior to pseudothecia formation (Kotzé, 2000). When the pycnidia are mature, the conidia covered with a mucilaginous substance emerge from the ostiole. In contact with water, the mucilage is dissolved, and the conidia are dispersed by splashing or washed off by rain to nearby susceptible tissues, where new infections may occur (Koltzé, 2000; 1981). The infection and colonization processes of conidia are like those of ascospores (Spósito *et al.,* 2011), even though the conidia can only be distributed over short distances (<1 m) by water from the fruit on the top of the tree canopy to lower hanging fruit and leaves (Spósito *et al*., 2008; McOnie, 1965; Kiely, 1948). The spread of CBS can also be enhanced by human activity by moving infected citrus fruits from one area to another. As such, this fungal pathogen has led to strict restrictions being imposed across South Africa to prevent the spread of CBS and

phytosanitary restrictions to countries where the pathogen is known to be present (Department of Agriculture, Forestry and Fisheries, 2014).

2.6 Phytosanitary restrictions

Phytosanitary standards are measures taken to protect human, animal or plant life or health from risks associated with imported agricultural commodities (Gebrehiwet *et al.,* 2007). In order to prevent the use of phytosanitary standards as a trade obstacle, countries are encouraged to base their phytosanitary standards on international guidelines and recommendations (Gebrehiwet *et al.,* 2007). These standards allow the banning of imports as a precautionary step until an exporting country confirms its product and place are free from any potential risks that may affect the safety and health of consumers, animals, and plants (Gebrehiwet *et al.,* 2007). Failure to comply with these standards may result in the loss of export revenue. For example, it is estimated that African banana exporters lost US$ 410 million a year due to the failure to comply with the international standards for pesticide residue (Gebrehiwet *et al.,* 2007).

Losses due to non-compliance to the phytosanitary standards could be a significant setback for the promotion of the agricultural sector, which is the backbone of the economy of many African countries. This is a huge concern for domestic markets, as it reduces local revenue, and the loss is far greater on the international markets as most citrus growers export as much of their crop as possible because of the far greater returns obtained on the export market compared to the domestic market (Department of Agriculture, Forestry and Fisheries, 2014; Gebrehiwet *et al.,* 2007). Moreover, in 2013 the EU announced that it was adopting stricter restrictions on the number of allowable CBS interceptions, capping them at a maximum of five against 36 that were found in South African citrus exports in 2012 (Kapuya, 2015). Within this new regulatory framework, exceeding five CBS interceptions would trigger additional restrictions, the extreme measure being a ban on South Africa's citrus exports into the EU (Kapuya, 2015). Phytosanitary restrictions can sometimes be easily applied during shipment when visible

symptoms are spotted. Therefore, strict control measures to prevent the growth of *P. citricarpa* both post- and preharvest are required as these can limit losses in the field and avoid rejection of exported citrus fruits.

2.7 Movement control measures against spread of citrus black spot within South Africa

As a signatory member of the World Trade Organisation Agreement (WTO) on the application of sanitary and phytosanitary measures and the International Plant Protection Convention, South Africa has certain associated responsibilities (Carstens *et al.,* 2012). Some of these responsibilities are outlined in the National Control Measures in the Agricultural Pests Act, 1983, to prevent and control the spread of the CBS within the country to maintain pest-free areas from CBS (Department of Agriculture, Forestry and Fisheries, 2014). In terms of the Control Measures R.110 of 27 January 1984 (as amended) of the Agricultural Pests Act, 1983 (Act No. 36 of 1983), movement of citrus fruits and/or related plant propagation materials is prohibited from an infected area to a non-infected area within South Africa unless the movement is authorized by means of a permit, or the material is certified as pest-free. The movement of citrus fruits and/ or related plant propagation materials is also prohibited from CBS-infected areas to specified areas of low pest prevalence. For example, in South Africa, the disease is known to occur in the citrus-producing provinces of KwaZulu-Natal, Mpumalanga, Limpopo, and the Eastern Cape. Therefore, no movement of citrus fruits or trees is allowed to the citrus-producing provinces of the Western Cape and Northern Cape unless proper documentation is provided as CBS is known to be absent in these provinces (Carstens *et al.,* 2012).

Restricting the movement of contaminated fruit will assist in preventing the spread of CBS to non-infected areas. In terms of the R.110 of the Agricultural Pests Act, 1983 (Act No. 36 of 1983), no user of land (producers, farmers and landowners) shall remove any plant from the infected areas to pest-free areas. Any individual who refuses or neglects to

comply with the provisions of a control measure shall be guilty of an offence and liable to a fine or imprisonment or both in terms of section 13 of the Agricultural Pests Act, 1983 (Act No. 36 of 1983). Consequently, the protection of CBS-free citrus production areas in South Africa is paramount for the citrus industry because of its heavy reliance on export markets (Carstens *et al.,* 2012). As such, the South African citrus exporters will be able to retain their competitive edge in Europe and the USA and access new markets (Meyer *et al.,* 2006). Furthermore, South Africa sometimes relies on other countries to import other citrus fruits. Some of these citrus products can harbour CBS. As such, the South African Government has certain rules, regulations, and laws regarding the importation of citrus fruits to South Africa, as these can have a negative influence on the citrus industry (Meyer *et al.,* 2006).

2.8 Movement control measures against the spread of citrus black spot from other countries to South Africa

The rules, regulations and laws are in place to ensure that no alien pests and diseases enter the country which could damage the local citrus industry. Furthermore, these regulations aim to ensure the quality and safety of the citrus products imported to South Africa. This is outlined within the Agricultural Pests Act, 1983 (Act No. 36 of 1983) and its subordinate legislation to provide measures by which citrus diseases may be prevented and combated and for matters connected therewith. The Act also mandates the Directorate of Plant Health to regulate plants, plant products and other regulated articles when imported into South Africa, as these can harbour quarantine pests if they enter the country with imported commodities and establish, which may endanger the South African agricultural, horticultural or forestry sectors.

Before any person or company can import products to South Africa, an importer should find out which import conditions apply to that specific citrus commodity to be imported by consulting the Agricultural Pests Act, 1983 (Act No. 36 of 1983) or the National Plant Protection Organisation of South Africa (NPPOZA) within the Department of Agriculture,

Forestry and Fisheries (DAFF). Application for an import permit from the DAFF must be made if the commodity is not exempted from an import permit in terms of the Act referred to above. If the commodity is exempted from an import permit, compliance with phytosanitary measures for such exemption must be ensured. When the commodities arrive at the port of entry in South Africa, approved procedures should be followed: The South African Revenue Services (SARS) will detain the commodities for inspection. DAFF inspector/s from NPPOZA will inspect the consignment together with the accompanying documents. The following may happen following inspection of the imported commodities: (a) If the consignment meets the import requirements, it will be released by the DAFF inspector/s. (b) If the consignment does not meet the import requirements, risk management measures will be recommended; thereafter, a consignment may be treated and released, sent back to the country of origin, or destroyed. Once the consignment has been released by the DAFF inspector/s, the importer or his/her agent must take the import documents to SARS for final release. Similarly, some citrus products exported from South Africa can harbour dangerous pathogens like CBS that may endanger the countries to which they are exported; as a result, some trade partners have their own control measures for citrus fruits imported from South Africa.

2.9 Movement control measures against the spread of citrus black spot from South Africa to other countries

Studies conducted (Yonow *et al.,* 2013; Paul *et al.,* 2005) have shown that climatic conditions in citrus-growing regions within the EU are unsuitable for the establishment of *P. citricarpa* and the development of CBS disease. Only small, restricted Mediterranean coastal areas have climatic conditions with marginal potential suitability. Citrus plants were moved from Asia, where CBS is endemic and also regarded as the centre of origin of citrus, to Northern Africa and other countries around the Mediterranean Sea by traders as early as the 5th century BC (Ramón-Laca, 2003). It would, therefore, be expected that *P. citricarpa* may have been introduced to these citrus-growing countries along with the hosts, especially since infected plant material is regarded as the means of long-distance spread of this pathogen (Koltzé,1981; Kiely 1948). Likewise, there is always the possibility

of illegal movement of citrus plant propagating material (Koltzé, 1981; Kiely 1948). Generally, fruit infected with CBS is not acceptable for export to the EU, the USA and other countries because of phytosanitary regulations (Bonants *et al.,* 2003).

For example, the USA only permits the import of fresh citrus fruit from CBS-free areas, while Japan and India allow the import of consignments of fresh citrus fruit free from visible symptoms of CBS. Furthermore, the EU and Iran allow the import of fresh citrus fruit that has been produced in CBS-free areas or from production sites where no CBS-infected fruit has been detected in an official inspection., Even though these countries have climatic conditions that differ from South African conditions, which are unsuitable for the establishment of *P. citricarpa* and development, this fungal pathogen needs to be controlled (Carstens *et al.,* 2012).

2.10 Synthetic fungicides as control measures of citrus black spot.

Globally, losses in the field, during storage, and in transit result in major losses of harvest and income in the citrus fruit industry due to fungal pathogens, spoilage, and infections (Liu *et al.,* 2009; El-Ghaouth, 1997). Losses due to diseases in the field, during storage, transit and commercialization can amount to 25% of the total production in countries dependent on agricultural products. In developing countries, damages are often worse, exceeding 50%, because of the lack of adequate storage facilities (Spadaro and Gullino, 2004). Treatment programmes, including control of CBS, are extremely costly (Schreuder, 2017), but if left untreated, the crops could be lost due to CBS infections (Schreuder, 2017). In most citrus production areas where CBS is prevalent, production will be impossible without an effective CBS control programme (Schreuder, 2017).

When permitted, synthetic fungicides are the primary means of controlling the most devastating fungal pathogens, and estimations indicate that over 23 million kg of synthetic fungicides is used annually worldwide (Abbey *et al.,* 2019; Talibi *et al.,* 2014; Martınez-

Romero *et al.,* 2008; El-Ghaouth, 1997). It is generally accepted that the production and marketing of fruit would not be possible without the use of synthetic fungicides (Martınez-Romero *et al.,* 2008). These include imazalil (IZ), Thiabendazole (TBZ), sodium orthophenyl phenate (SOPP), fludioxonil (FLU), Bordeaux mixture, pyrimethanil or different mixtures of these compounds, amongst others. The fungicides are generally used on pack lines as the first line of defence against postharvest pathogens (Talibi *et al.,* 2014; Du Plooy *et al.,* 2009). Most of these are believed to reduce fungal risk by targeting structures responsible for the life cycle of the fungal pathogens, such as conidia and ascospores (Palou *et al.,* 2008). Treatments with synthetic fungicides result in curative action against pre-existing or established infections and persistent preventive action against potential new infections that can occur after their application in the packing house and may also inhibit reproduction structures such as ascospores and conidia, resulting in the breaking of infection life cycles of CBS (Du Plooy *et al.,* 2009; Palou *et al.,* 2008).

Literature has reported the beneficial effects of synthetic fungicides, for example, one or more of the following: imazalil (IZ), Thiabendazole (TBZ), sodium orthophenyl phenate (SOPP), fludioxonil (FLU) when sprayed onto orange and lemon shipments, can maintain freshness in transit and reduce rot during long shipping and storage periods (Ritenour *et al.,* 2004). Other trials proved that two applications comprising azoxystrobin and mancozeb during mid-November and mid-January could reduce CBS incidence by 95-100% (Schreuder, 2017). While in Brazil, control of the CBS pathogen has been achieved by using copper-based fungicides or dithiocarbamates and systemic fungicides, in combination or not (Possiede *et al.,* 2009). Control procedures are carried out in accordance with data generated by CBS control programs from other countries, particularly South Africa (Possiede *et al.,* 2009). In America, QoI fungicides are used for the control of CBS. Monthly applications of QoIs (azoxystrobin, pyraclostrobin, or trifloxystrobin) are recommended from early May to mid-September, but there is a label limit of four QoI applications in a season. QoI fungicides block electron transport at the quinol-oxidizing site of the cytochrome *b* complex (complex III) in the mitochondria,

disrupting adenosine triphosphate (ATP) production. Spore germination is the fungal growth stage particularly sensitive to QoIs (Hincapie *et al.,* 2014). However, as much as synthetic fungicides are effective in controlling major fungal infections, there is much criticism from the scientific community and the public against their use (Du Plooy *et al.,* 2009).

2.11 Problems associated with the use of synthetic fungicides

Even though synthetic fungicides are effective control measures for pre- and postharvest, they pose serious environmental problems (Du Plooy *et al.,* 2009). These conventional methods to control plant pathogens have affected the environment negatively as 90% of the applied synthetic fungicides are lost in the open field during application and as overland flow (Zhang *et al.,* 2020; Abd-Elsalam *et al.,* 2015). Moreover, these non-biodegradable synthetic fungicides accumulate in the soil, water sources (dams, rivers and lakes) and non-targeted crops that influence human and animal health (Abd-Elsalam *et al.,* 2015). This has been a matter of concern for scientists, and the National Academy of Sciences placed fungicides under special scrutiny when they studied the regulation of synthetic fungicides applied to food (National Research Council, 1987). Their report indicates that fungicides constitute 60% of the oncogenic risk among all the pesticides used on food, including insecticides (Gupta, 2018; Wilson and Wisniewski, 1992).

Additionally, fungal pathogens have shown a concerning trend of resistance against these fungicides (Deising *et al.,* 2008; Agostini *et al.,* 2006), thereby shortening the lifespan of protective products (Du Plooy *et al.,* 2009). These are linked to poor synthetic fungicide education, misuse and abuse of antifungal applications (Asogwa and Dongo, 2009). For instance, overuse for rapid killing of fungi is common among government-trained or agency-trained assisted small-scale farmers (Ugwu *et al.,* 2015; Asogwa and Dongo, 2009; Ivbijaro, 1998). It has also been noted that some farmers sometimes use pesticides for purposes other than what they are intended for (Asogwa and Dongo, 2009). For instance, Lindane, formerly used for controlling cocoa mirids in Nigeria, is poured into

rivers, lakes and streams to kill fish, which are then sold for human consumption (Asogwa and Dongo, 2009). Some farmers mix fungicides and insecticides during the fungicide application period to reduce the workload of spraying each differently and separately as advised. Sometimes expired pesticides or pesticide containers are used for domestic purposes (Asogwa and Dongo, 2009). All these problems result in farmers incurring huge financial losses due to wastage, as it is estimated that about 2.5 million tons of fungicides are used annually, and the universal financial harm caused by these fungicides reaches $100 billion annually (Ugwu *et al.,* 2015).

Furthermore, the application of synthetic fungicides depends on a wide range of factors, including plant species, formulation, and application methods, as well as climatic conditions such as rainfall, temperature, and sunlight (Martınez-Romero *et al.,* 2008). Concerns about human health risks associated with fungicide residues periodically led to regulatory reviews and potential restrictions or even cancellations of some synthetic fungicides (Palou *et al.,* 2008). These agents are known to remain on the plant or within its tissues following treatment resulting in potentially toxic and carcinogenic effects on human and food systems (Sellamuthu *et al.,* 2013). Public perception about the potential impact of traditional control practices on health and the environment led to an increased demand to reduce synthetic fungicides in food chains and increased research support to find alternative fungicides (Palou *et al.,* 2016; Gullino and Kuijpers, 1994). The development of biological control methods for the control of postharvest diseases has been generated due to emerging socioeconomic concerns over the use of synthetic chemicals (Palou *et al.,* 2016).

Traditional citrus export markets increasingly demand products with lower levels of pesticides to satisfy the safety demands of the public (Bravo *et al.,* 2017). In addition, new higher-value markets based on organically grown, sustainable, environmentally friendly, ecological, or green agricultural produce are becoming more popular (Bravo *et al.,* 2017; Palou *et al.,* 2008). Compounds that could equal or improve pathogen control yet

minimize disposal problems would be extremely valuable (Du Plooy *et al.,* 2009; Wilson and Wisniewski, 1992). Consequently, farmers and researchers started considering alternative methods to control fungal diseases (Punja and Utkhede, 2003). For example, salicylic acid (SA) treatments in fruit, either preharvest (Sayyari *et al.,* 2009; Yao and Tian, 2005) or postharvest reduced fungal decay in sweet cherry through induction of the defence resistance system (Sayyari *et al.,* 2009; Chan and Tian, 2006) and stimulation of antioxidant enzymes (Sayyari *et al.,* 2009; Xu and Tian, 2008). In addition, in chilling injury (CI-sensitive) fruit, pre-treatment with salicylic acid (SA) reduced CI in peaches (Wang *et al.,* 2006).

Kock *et al.,* 2007 reported that acetylsalicylic acid (ASA) possesses antifungal properties. A yeast bioassay was developed to expose the antifungal properties of ASA with *Eremothecium ashbyii* used as a test organism (Kock *et al.,* 2007). Similar findings were also observed by Ncango *et al.,* 2010 and Leeuw *et al.,* 2009 using *Mucor circinelloides* and *Aspergillus fumigatus,* respectively. Moreover, Magunga (2016) also showed the antifungal properties of ASA after exposing fungi *P. citricarpa* to this compound *in vitro*; the conidia structures were affected, as observed elsewhere (Magunga, 2016; Ncango *et al.,* 2010; Leeuw *et al.;* 2009; Kock *et al.,* 2007). Although alternative methods are being investigated to control postharvest decay during storage, natural plant products such as EOs and their hydrosol (a by-product of EO production) are well-documented. They are gaining global fame and the attention of researchers as they are biodegradable, eco-friendly, economical and safe. As a result, the last decades have witnessed a growing interest in natural fungicides, with EOs taking centre stage in the search for alternatives to synthetic fungicides (Hu *et al.,* 2019; Sivakumar and Bautista, 2014). The complex nature of EOs impedes the development of resistance by the pathogen. The application of EOs also leaves a smaller carbon footprint because they are biodegradable without being highly unstable. The application of EOs as fungicides is also far more acceptable to consumers and is suitable for the organic market (Abd-Elsalam *et al.,* 2015).

2.12 Essential oils (EOs) as possible antifungal agents

EOs are complex mixtures of secondary plant metabolites with relatively high vapour pressure and are poorly soluble in water (Feyaerts *et al.,* 2018). These aromatic oily liquids are obtained from plant materials such as flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits, and roots (Feyaerts *et al.,* 2018). They can be obtained by expression, fermentation, enfleurage or extraction, but steam distillation is commonly used for commercial production of EOs (Nazzaro *et al.,* 2013). Many of these volatile substances have diverse ecological functions, such as acting as internal messengers, as defensive substances against herbivores or as volatiles directing not only natural enemies to these herbivores but also attracting pollinating insects to their host (Chorianopoulos *et al.,* 2008). EOs are very interesting natural plant products with their use in food systems well documented in the literature (Chorianopoulos *et al.,* 2008; Koutsoumanis *et al*., 1998).

EOs differ from other oils (fixed oils such as olive oil) as they are volatile, evaporate when left open, and have certain therapeutic properties that can be used to promote the health and well-being of patients in healthcare settings (Sethlare, 2017). EOs are also employed in aromatherapy and treating several diseases, including cardiovascular disease, diabetes, Alzheimer's, and cancer (Sethlare, 2017; Swamy *et al.,* 2016). Furthermore, they are also known for their antimicrobial properties (Feyaerts *et al.,* 2018; Talibi *et al.,* 2014; Bakkali *et al.,* 2008). Antimicrobial properties of EOs include being antiviral, antibacterial, and antifungal (Burt, 2004). The EOs reported in various studies have shown to exhibit antifungal properties by targeting structures responsible for the life cycle of a fungal organism, such as ascospores and conidia, both *in vitro* and *in vivo* in different fresh produce (Imelouane *et al.,* 2009; Meepagala *et al.,* 2002; Caccioni and Guizzardi, 1994; Vaughn and Spencer, 1991).

The development of resistant strains of fungi against EOs may be less likely compared to many synthetic fungicides because several active components are often present in the final product, and synergistic interactions may exist between the different components of the oils (Talibi *et al.,* 2014; Bakkali *et al.,* 2008). For example, the chemical composition of thyme oil in some studies was shown to consist of mostly terpenes and/or terpenes derivatives (Plaza *et al.,* 2004). Terpenes are the major group of most plant natural products characterized by a wide variety of structural types. The most valuable compounds terpenes are monoterpenes (C10), sesquiterpenes (C15) and diterpenes (C20), but hemiterpenes (C5), triterpenes (C30) and tetraterpenes (C40) also can be found. Furthermore, terpenes containing oxygen are designated terpenoids (Faleiro, 2011). These terpenes and their derivatives are known to play a major role in the antimicrobial activities of EOs (Faleiro, 2011).

Plaza and others (2004) reported that thyme EOs significantly reduced the incidence of green and blue moulds on citrus. Moreover, thyme oil was reported to control most postharvest citrus rots, such as green mould, blue mould, and sour rot (Talibi *et al.,* 2014). A previous study *in vitro* by Magunga (2016) showed that thyme oil and thyme oil hydrosol could inhibit the fungal growth of *P. citricarpa* by targeting conidia structures responsible for the fungal growth and dispersal. The oil used in the Magunga (2016) study was analyzed using gas chromatography-mass spectrometry (GC-MS). The analysis revealed the presence of terpenes and/or terpenes derivatives which play a major role in the antimicrobial activities of EOs by targeting structures with increased mitochondrial activity in fungi.

2.13 Hydrosols as possible antifungal agents

Hydrosols are composed of water with dissolved EOs components with different names reported in the literature, including hydrolate, hydroflorate, plant aromatic waste, aromatic water, floral water, and essential aromatic water (D'Amato *et al.,* 2018). These are the by-

products of hydro- and steam distillation of plant material performed to obtain EOs (D'Amato *et al.,* 2018). These occur through the loss of EO constituents in the water during the distillation of aromatic plants, but the hydrosol remains as the condensate water left over after the process (D'Amato *et al.,* 2018). This aromatic water contains the very essence of everything that was contained within the plant when it was still alive and growing as well as microscopic droplets of EOs in suspension (Tornuk *et al.,* 2011). These also include traces of the EOs and several water-soluble compounds (Tornuk *et al.,* 2011).

Hydrosols have been used for many years in food formulations such as flavouring foods or beverages (D'Amato *et al.,* 2018). However, in the scientific community, for a long-time, hydrosols have been considered waste products and only recently have many researchers reconsidered them, trying to analyze their antimicrobial activity (D'Amato *et al.,* 2018). Hydrosols can also be used as antifungals against CBS, with the advantage over EOs of being water-soluble and less expensive (Nazzaro *et al.,* 2013). Their main advantages are that they are easy to produce and do not have any known health hazards for humans (Tornuk *et al.,* 2011). Hydrosols do not have many of the typical EOs side effects as they usually lack a strong scent capable of inducing headaches, do not cause contact irritation, and can usually be applied to the skin or ingested (Bajer *et al.,* 2017).

The ability of hydrosols to control microbial growth differs depending on the chemical composition and the microbial target. Thyme oil hydrosol is among the most efficient hydrosols against microorganisms (D'Amato *et al.,* 2018). Hydrosol of *Thymus capitatus L.* was tested against four phytopathogenic fungi (*Aspergillus niger, A. oryzae, Penicillium italicum and Fusarium solani*), causing the deterioration of *Citrus sinensis* fruits. The *in vitro* activity against the pathogens was revealed in all the pathogens (Tabti *et al.,* 2014). Afterwards, *Citrus sinensis* fruits were inoculated with *Penicillium italicum* and then treated *in vivo* with the hydrosol extract. This was sufficient for the total reduction of the orange infection, resulting in 100% inhibition of mycelial growth (Tabti *et al.,* 2014). The antifungal property of *Thymus capitatus L.* hydrosol was probably due to the high amount of carvacrol (95,1%) present (Tabti *et al.,* 2014). In 2008, Nebahat and co-workers

evaluated the effect of *Thymus vulgaris* hydrosol against *A. hydrophila,* which plays a role in the spoilage of freshwater fish. Hydrosols of thyme showed significant antimicrobial activity against *A. hydrophila* ($P<0.05$). The application of hydrosols in food products could soon become a reality, especially to control the growth of pathogenic and/or spoilage microorganisms, and this indicates that hydrosols have the potential to meet the demands of industry for natural antimicrobials. However, both hydrosols and EOs have modes of action to inhibit or kill fungal growth.

2.14 Essential oils' mode of action against fungi

In fungal cells, EOs can provoke depolarization of the mitochondrial membranes by decreasing the membrane potential, affect ionic Ca^{++} cycling (Bakkali *et al.,* 2008; Vercesi *et al.,* 1997; Novgorodov and Gudz, 1996) and other ionic channels and reduce the pH gradient, affecting the proton pump and the ATP pool. They change the fluidity of membranes, which become abnormally permeable, resulting in leakage of radicals, cytochrome *C*, calcium ions and proteins. Permeabilization of outer and inner mitochondrial membranes leads to cell death by apoptosis and necrosis (Bakkali *et al.,* 2008; Armstrong, 2006). It seems that chain reactions from the cell wall or the outer cell membrane invade the whole cell through the membranes of different organelles like mitochondria and peroxisomes. These effects suggest a phenolic-like prooxidant activity (Burt, 2004). Scanning and transmission electron microscopy observations reveal cell ultrastructural alterations in several compartments such as the plasma membrane, cytoplasm (swelling, shrivelling, vacuolations, leakage) and nucleus (Santoro *et al.,* 2007; Soylu *et al.,* 2006). The previous study demonstrated that EOs effectively control the growth of *P. citricarpa.* These results suggest that they may be considered a potential alternative to synthetic fungicides to be used as a control strategy for this devastating citrus fungal pathogen (Magunga, 2016). However, further studies *in vivo* still need to be done to determine the effectiveness of the EOs in the field and packing houses.

2.15 Rationale

2.15.1 Problem statement

The spread of CBS is a major concern in the citrus industry because the disease threatens fruit marketability and citrus tree health. In most countries, the disease is phytosanitarly important because of its role in international trade. If the fruits contain CBS lesions, this can result in the rejection of the whole imported batch; moreover, the disease can spread readily in the natural environment resulting in an increased incidence of CBS. Therefore, it is paramount to control and manage the disease; however, there is a great public concern about the safety and side effects of synthetic fungicides currently used to control CBS. Synthetic fungicides are known to have carcinogenic effects on humans and are also toxic to the environment. Furthermore, microorganisms tend to develop resistance to most synthetic fungicides. This problem has prompted research into identifying new ways with broad activity in treating microbial disease in plants. Although alternative methods are being researched to control citrus fruit diseases, natural plant products such as EOs and their hydrosol are gaining popularity and drawing the attention of researchers. In nature, EOs play an important role in the protection of plants. They contain a wide variety of secondary metabolites that can inhibit or slow the growth of bacteria, yeasts, fungi and even viruses. The oils and their components have activity against various microbial targets, particularly the membrane and cytoplasm, and in some cases, they completely change the morphology of the cells; EOs can be ideal candidates for use as alternative antifungal compounds.

2.15.2 Study aim

The overall aim of the study was to investigate the *in vivo* effect of thyme oil and thyme oil hydrosol against citrus fungal pathogen *Phyllosticta citricarpa*.

2.15.3 Objectives

To achieve the overall aim, the objectives of this study were:

- To characterize thyme EOs using GC-MS and GC × GCTOFMS.
- To evaluate the MIC of thyme oil.

- To evaluate the fungistatic and fungicidal effects of thyme EOs.
- To evaluate the inhibitory effect of both thyme oils and thyme oil hydrosol over *P. citricarpa in vivo* growth by:
    - Evaluating the inhibitory effect of thyme oils and thyme oil hydrosol on *P. citricarpa* pathogen grown on citrus leaves and citrus fruits.
- To map the mode of action of EOs using:
    - SEM and TEM to evaluate morphological changes that occur because of EOs on the organism.

These can create conditions conducive to prolonging the postharvest life of citrus fruits, thereby providing alternatives to synthetic fungicides.

2.16 References

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CHAPTER THREE

METHODOLOGY

METHODOLOGY

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Abstract

Introduction: As much as the use of synthetic fungicides as a treatment against citrus fungal pathogen has resulted in great health improvement of citrus fruits, their use has had many negative impacts such as increased cost and handling hazards resulting in economic loss to producers. Other harmful effects of synthetic fungicides are toxic and carcinogenic effects on human and food systems. Additionally, the resistance of fungal pathogens to fungicides has rendered some fungicides ineffective. Interest has increased in finding safer and biodegradable alternatives to replace synthetic fungicides, such as the use of essential oils (EOs) and their hydrosol as alternative antifungal strategies, as they have recently been proven to be a successful eco-friendly bio-control agent. EOs are aromatic and complex volatile liquids extracted from plant material using different methods such as steam distillation, in which an aqueous phase called hydrosol (HD) is obtained. EOs (such as thyme oil) have in the past effectively eliminated several bacterial, fungal and viral pathogens.

Purpose: This study investigates the antifungal effect of *Thymus vulgaris* and its hydrosol on *Phyllosticta citricarpa,* the causative agent of citrus black spot (CBS).

Methods: After thyme oil characterization using GC-MS and GC × GC TOFMS,their effectiveness, together with that of the hydrosol, was tested against *Phyllosticta citricarpa* (CBS causative agent) using methods such as broth microdilution assay (minimum inhibitory concentration (MIC) determination) and fungicidal or fungistatic activity. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were used to evaluate morphological changes that occurred due to EOs, while the inhibition of pathogen growth on leaves and fruits was also determined.

Results: The results, discussions and analysis are presented in chapter four.

Keywords: citrus black spot, *Phyllosticta citricarpa*, antifungal, essential oils, hydrosol

3.1 Introduction

Synthetic fungicide treatments are commonly used to contain the citrus black spot (CBS) spread in citrus orchards (Tonial *et al.,* 2017). In the past, their introduction has transformed citrus farming by revolutionizing the weaponry in the battle against fungal diseases, resulting in great health improvements for citrus fruits (Tzortzakis and Economakis, 2007). However, the complete eradication of the disease seems impossible. Moreover, the health benefits associated with synthetic antifungals have been questioned for a long time because of other harmful effects associated with their use (Tonial *et al.,* 2017). The widespread use of synthetic fungicides has significant drawbacks, including increased cost and handling hazards which can result in economic loss to producers (Tzortzakis and Economakis, 2007). Additionally, synthetic antifungal residues are known to remain on the plant or within its tissues following treatment, resulting in potentially toxic and carcinogenic effects on human and food systems (Tonial *et al.,* 2017). The National Academy of Sciences (NAS) reported that pesticide residues on food, including fungicides, pose more of a carcinogenic risk than insecticides and herbicides together (Wilson *et al.,* 1997). Fungal pathogens resistant to fungicides have rendered some fungicides ineffective, creating a need for new alternative modes of addressing fungal contamination (Wilson *et al.,* 1997).

Globally, large volumes of citrus are treated with fungicides annually, increasing the demand for organically produced fruit. Public awareness of the risks associated with synthetic fungicides has also increased interest in finding safer and biodegradable alternatives to replace synthetic fungicides (Du Plooy *et al.,* 2009). Many alternative antifungal strategies have been proposed, with fungicide activity, low or no mammalian toxicity, less environmental effects, and wide public acceptance (Du Plooy *et al.,* 2009). One such alternative is the use of natural plant protectants such as EOs and their hydrosol as alternative antifungal strategies, as studies have demonstrated their antimicrobial properties and highlighted their potential as successful eco-friendly bio-control agents.

Several authors have reported antimicrobial, antifungal, antioxidant, and radical-scavenging properties of EOs (Setlhare, 2017; Tonial *et al.,* 2017; Chutia *et al.,* 2009).

EOs are aromatic and complex volatile liquids extracted from plant material using different methods (Setlhare, 2017). One of these methods is steam distillation, in which an aqueous phase called hydrosol (HD) is obtained (Hay *et al.,* 2015). Hydrosols (a by-product of EOs after the purification procedure) are aqueous solutions and do not present the strong, usually undesirable smell of EOs (Chorianopoulos *et al.,* 2008). EOs have effectively eliminated several bacterial, fungal, and viral pathogens. The presence of different types of aldehydes, phenolics, terpenes, and other antimicrobial compounds indicates that the EOs are effective against a diverse range of pathogens (Sethlare, 2017). One such EOs is thyme (*Thymus vulgaris*) which has been found to be antibacterial and antifungal on various pathogenic microorganisms (Sethlare, 2017). Therefore, this study investigates the antifungal effects of *Thymus vulgaris* and its hydrosol on *Phyllosticta Citricarpa,* the causative agent of CBS.

3.2 Materials and methods

3.2.1 Strain to be used

Phyllosticta citricarpa that Kiely preserved at the national collection of fungi (ARC-PPRI) in Pretoria, South Africa, was used in the study. The fungus was cultivated on yeast malt (YM) agar at 25 °C in Petri dishes until spore-releasing structures were observed (Ncango *et al.,* 2010). Spore suspensions (10^6 spores/mL) were prepared after seven days of incubation by adding sterile ¼-strength Ringer's solution to colonized Petri dishes.

3.2.2 Preparation of suspensions of the test microorganism for minimum inhibitory concentration (MIC)

Fungal conidia cells were harvested after inoculation on YM for seven days. This was done using an inoculation needle with a loop (3 mm) and transferred to a test tube with a spore suspension (DMSO 0.5% agar in distilled water).

3.2.3 Essential oil characterization using gas chromatography-mass spectrometry (GC-MS).

GC-MS was used to characterize thyme oil (*Thymus vulgaris*). Briefly, the EO was dissolved in hexane (10% hexane) and injected in a Finnigan Focus Gas Chromatograph (GC), operated under the following conditions: the injector temperature was set at 230 °C. The GC was equipped with an AB-1MS (30 m x 0.25 mm id 0.25 µm) capillary column. Helium was used as carrier gas at a constant flow of 1 mL min^{-1} (at a split ratio of 50:1). The temperature programme was set at 40 °C for four minutes and then raised at 5 °C min^{-1} to 200 °C and then held at 200 °C for one minute and then raised at 5 °C to 220 °C, where it was held for 10 minutes. Mass analysis of the oils was done using a Finnigan Focus DSQ mass spectrometer. The ion source temperature was set at 250 °C with an ionization voltage of 70 eV and a mass scan range of 50-650 amu. Individual GC peaks and mass spectra were identified by searching commercial libraries, followed by expert matching of MS data (Leeuw *et al.,* 2007).

3.2.4 Essential oil characterization using GC × GC–TOFMS analysis

The thyme oil sample (10 µL) was diluted with n-hexane (1 mL). The GC × GC–TOFMS analysis was carried out on an Agilent 7890B gas chromatography system equipped with a Pegasus 4D TOFMS. The GC × GC system contained two chromatography columns. The first column was a nonpolar Rxi-5 Sil MS (5% phenyl-95% dimethyl arylene polysiloxane, 30 m ˆ 0.25 mm i.d. ˆ 0.25 µm film thickness), and the second column was a medium polarity Rxi-17 Sil MS with dimensions of 2 m ˆ 0.18 mm i.d. ˆ 0.18 µm film thickness, 50% phenyl-50% dimethyl arylene polysiloxane. Helium was used as a carrier gas at a constant flow rate of 1 mL/min. The initial temperature of the first column was set at 50 °C for 12 seconds and then ramped to 280 °C at a rate of 8 °C/min. The secondary oven was set at a 5 °C offset above the primary oven. The modulator offset and transfer line temperatures were 15 °C and 280 °C, respectively. The modulation period was four seconds, and the hot pulse was set at 0.8 seconds. The injection volume was 1 µL in split mode, at a ratio of 50:1. The temperature of the injector was kept at 240 °C. The mass spectrometer was set to scan in the range of m/z 33-550 at an acquisition rate of 100

spectra/s. The detector voltage was set at 1420 V, and the electron energy was set to 70 eV. The ion source temperature was kept at 250 °C. All the operations and analysis of data were controlled using LECO ChromaTOF software version 4.52.

3.2.5 Broth microdilution assay (minimum inhibitory concentration determination)

The antifungal activity of thyme (*Thymus vulgaris),* ethanol (negative control), and Thiabendazole (positive control) against the fungus *Phyllosticta citricarpa* were investigated using the microdilution method developed by Tonial *et al.,* (2017) with a slight modification. To determine the MIC, microdilution was performed in 96-well plates by adding 90 µL of yeast malt broth (YMB), 10 µL of the fraction being tested (thyme oil, Thiabendazole and ethanol, respectively), and 50 µL of *P. citricarpa* conidial suspension in saline solution (6×10^5 conidia mL^{-1}) to each well. The plates were incubated at 25 °C for seven days, where after 40 µL of 0.2 mg/mL, iodonitrotetrazolium salt solution (growth indicator) was added to each well and incubated for another day. After incubation, iodonitrotetrazolium salt solution changes colour from colourless (for no growth) to pink (for the presence of growth). The lowest concentration at which no growth was observed, indicated by colourless colour, was taken as the MIC value. The assay was performed in triplicate. Serial dilutions of the evaluated fraction and controls were performed to determine the MIC. The concentrations of thyme oil and Thiabendazole (stock solution of 2.0 mg. mL−1) were 100, 50, 25, 12.5, 6.25, and 3.13 µg. mL^{-1}.

3.2.6 Fungicidal or fungistatic activity

The fungicidal and fungistatic activity was assessed according to the method of Lombardo *et al.* (2016) with slight modification. When total fungal growth inhibition was observed in the MIC test, the viability of the pathogen was tested immediately after the end of day eight. All the wells with no growth observed, indicated by colourless colour, were sub-cultivated by transferring 2 µL and inoculated in yeast malt agar (YM) with no growth

inhibitors, then incubated for seven days at 25 °C. The commercial fungicide Thiabendazole was used as a positive control.

3.2.7 Scanning electron microscopy (SEM)

SEM was used to evaluate morphological changes that occurred due to the activity of thyme (*Thymus vulgaris)*, ethanol (negative control) and Thiabendazole (positive control) on the fungus *Phyllosticta citricarpa* in terms of shape and structure (Al-Bayaty *et al.,* 2011). Preparation of cells for analysis using SEM was carried out according to Ncango *et al*. (2010). Briefly, cells (from MIC results) of *P. citricarpa* treated with thyme oil (*Thymus vulgaris)*, ethanol (negative control) and Thiabendazole, respectively, were fixed using 3% v/v of a sodium phosphate-buffered glutaraldehyde solution at pH 7.0 and a similarly buffered solution (1% m/v) of osmium tetroxide for one hour. Subsequently, the material was dehydrated in a graded series of ethanol solutions (30%, 50%, 70%, 90%, and 100% for 30 minutes per solution). Next, the ethanol-dehydrated material was critical-point dried, mounted, and coated with gold to make it electrically conductive. This preparation was then examined using a SEM. Micrographs were taken to investigate the general pattern of the fungi for specific morphological features.

3.2.8 Transmission electron microscopy (TEM)

TEM was carried out according to the method of Van Wyk and Wingfield (1991). Cells (from MIC results) of *P. citricarpa* treated with thyme (*Thymus vulgaris)*, ethanol (negative control) and Thiabendazole (positive control), respectively, were used. Fungal cells were fixed using the same protocol described for SEM. After fixation, the material was dehydrated in a graded acetone (Merck, Darmstadt, Germany) series (50%, 70%, 95%, 2X 100%). Dehydration lasted 30 minutes for each step. Next, the material was embedded in an epoxy resin and allowed to polymerize in an oven at 70 °C for eight hours. Thin sections were made using an LKB III Ultramicrotome (Stockholm, Sweden) and stained with uranyl acetate for five minutes and lead citrate for one minute. Finally, these sections were viewed with a Phillips EM 100 transmission electron microscope (Eindhoven, the Netherlands).

3.2.9 Inhibition of pathogen growth on leaves

Fragments (2.0 cm x 1.5 cm) of healthy leaves of the lemon tree (*Citrus sinensis*) were washed and autoclaved at a temperature of 120 °C and 1 Pa pressure for 20 minutes. Each leaf fragment was placed on a water-agar plate and inoculated with a mycelial disc of *P. citricarpa* (1 mm diameter). Then, 10 µL of treatment (thyme oil and thyme oil hydrosol) or controls were applied over the inoculated leaf fragment, and the plates were incubated for 21 days at 25 °C. Ethanol and Thiabendazole were used as the negative and positive controls, respectively. The results were reported by determining the highest to the smallest growth of pathogen development on the leaf.

3.2.10 Inhibition of pathogen growth on citrus fruits

The experiment was done according to the method of Rodriguez *et al.* (2018) with slight modification. A total of 15 lemons were used in the study. Spore suspensions were prepared from 21-day-old cultures of *P. citricarpa* on yeast malt agar incubated in the dark at 25 °C. Spores were suspended in 2 mL of yeast malt broth and removed from sporulating colonies with a sterile pipette. Suspensions were adjusted to 10^6 conidia/mL. Lemons were wounded with a 1-mm-wide and 2-mm-deep sterile tip and were inoculated by injecting 5 µL of the spore suspension. Then, 10 µL of treatment (thyme oil and thyme oil hydrosol) was applied over the inoculated wounds. Ethanol and Thiabendazole were used as the negative and positive controls, respectively. Inoculated fruit was kept in sealed plastic trays in a security chamber at 25 °C and 80% relative humidity. Non-inoculated lemons wounded with a 1-mm-wide and 2-mm-deep sterile tip were used. Fruits were evaluated for the severity of symptoms by observing the lesion area without considering the point of inoculation (1 mm). The results were photographed weekly until the end of the experiment. All the experiments were done in triplicate.

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CHAPTER FOUR

RESULTS, DISCUSSIONS AND ANALYSIS

RESULTS AND DISCUSSIONS

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4.1 Results and discussions

This section focuses on the results and the discussion of the study findings. Moreover, the section provides logical explanations for the findings of the study and compares the results with previous studies done by other researchers, which further ensures a well-defined interpretation of the results and the suggestion thereof.

4.1.1 Essential oil characterization using gas chromatography-mass spectrometry

The chemical composition of thyme oil (*Thymus vulgaris)* was analyzed using gas chromatography mass-spectrometry (GC-MS,) and the results are shown in Table 4.1 in terms of the identity and the percentage content of the individual components that were identified. GC-MS is an analytical method that synergistically combines two powerful analytical techniques, the features of GC-MS, to identify different substances within a test sample (Kitson *et al.,* 1996). Gas chromatography works on the principle of separating components within a mixture in time, while mass spectrometry provides information that aids in the structural identification of each component (Kitson *et al.,* 1996). In the study, ten compounds were identified, representing 71% of the total oil composition of the test sample. Thyme oil (*Thymus vulgaris)* consisted mostly of terpenes as major compounds. Terpenes and their derivatives play a major role in the antimicrobial activities of EOs. These included thymol (32.1%) and ρ-cymene (20.4%) as main compounds, while other minor compounds were detected in ranges between 0.5 and 5.1%. The minor components identified included α-Pinene (5.1%), terpinene (3.0%) and other minor compounds (not reflected in the table). The results are aligned with those observed by other authors who indicated the chemical composition of thyme oil using GC-MS. A study by Kryvtsova *et al*. (2019) indicated that thyme oil presented high levels of the precursor monoterpenes – p-cymene and γ-terpinene (12.7%) and monoterpene phenols – thymol and carvacrol (more 70%). Another study by Keramat *et al*. (2017) indicated similar findings when analyzing thyme oil, oxygenated monoterpenes (thymol - 26.02%), and their corresponding monoterpene hydrocarbon precursors (p-cymene - 27.24%) and γ-terpinene (4.38%) that are considered as their major compounds, respectively. However, the study also had carvacrol (19.27%) as a major compound which differs from the current

study. The difference in composition was not surprising as the chemical composition of an EO can significantly vary according to several factors: the plant organ from which it is extracted, time of cultivation and harvest, climatic conditions of the place of cultivation, and extraction method, among others (Keramat *et al.,* 2017).

Table 4.1: Chemical composition of thyme oil, the essential oils used in the study obtained using GC-MS.

Essential oils plant composition	**(%)**
Borneol	0.5
Camphene	1.3
Caryophyllene	1.2
Caryophyllene oxide	1.4
Hexane, 3-methyl	4.5
Pinocarvone	1.5
Terpinene	3.0
Thymol	32.1
α-Pinene	5.1
ρ-Cymene	20.4
Total	**71**

4.1.2 Results of essential oil characterization using GC × GC-TOFMS

For many years, GC-MS has been used almost exclusively for the qualitative analysis of EOs and other compounds (Pasikanti *et al.,* 2008). The commercially available mass spectrometry (MS) libraries contain hundreds of mass spectra of different compounds. MS libraries allow tentative identification of EOs components. However, identifying individual components of EOs is not always possible using MS data alone. As mass spectra of these compounds are usually very similar, peak identification becomes very difficult and sometimes impossible (Baharum *et al.,* 2010). One-dimensional gas chromatography cannot provide sufficient separation for a complete qualitative, let alone

quantitative analysis, so it is ideal to use novel separation technologies to achieve improved analysis (Pasikanti *et al.,* 2008).

Comprehensive two-dimensional gas chromatography (GC × GC), which may be considered the most powerful separation tool in chromatography, is a technique highly suited for separating complex mixtures such as EOs samples (Könen *et al.,* 2019). With this technique, the peaks eluting from the first GC column enter a cold-jet modulator, which traps each subsequent small portion of eluate, focuses these portions and introduces them into a second column for further separation (Stevenson, 2019). GC × GC differs from conventional multidimensional separations in that the whole sample is subjected to both dimensions of the separation processes in a single run. For an EOs, different content ratios among the components will strongly influence the quality of the oil; meanwhile, even some minor components will be detected as they also contribute to the overall qualities of an EOs. Therefore, the analysis of an EOs should provide not only sufficient separation but also accurate quantitation of all individual components (Stevenson, 2019). In addition, a comparison was made between GC × GC and GC/MS to identify and quantify the components in thyme oil. Compounds found by GC × GC–TOFMS are shown in Table 4.2. The results showed that GC × GC–TOFMS had a great advantage over GC-MS because it is more sensitive and provides comprehensive data analysis. Based on GC × GC–TOFMS results of thyme oil, 26 compounds that accounted for 100% constituents were identified, compared to the ten compounds identified using GC-MS, representing 71% of the total oil composition of the same thyme oil. This means that more compounds were identified using a GC × GC–TOFMS than the results obtained using GC-MS for the same thyme oil. Out of 26 compounds observed through GC × GC–TOFMS analysis, only nine compounds were found when GC-MS was used for analysis. This may be due to the lower sensitivity of GC-MS compared to GC × GC–TOFMS. The compounds identified using GC × GC–TOFMS include major compounds such as thymol (13.56 %), camphene (11.83 %), o-cymene (11.09%), ç-terpinene (10.4%), carvacrol (10.01%), à-pinene (7.81%), and endo-Borneol (6.21%). However, other minor compounds were also detected, ranging from 0.59-3.77%. These minor compounds

identified included (+)-Borneol (3.13%), caryophyllene oxide (3.77%), bicyclo[3.1.0]hexane, 4-methylene-1-(1-methylethyl)- (2.23%), caryophyllene (2.28%), and other compounds listed in Table 4.2.

Table 4.2: Chemical composition of thyme oil, the essential oils used in the study obtained using GC-GC–TOFMS.

Essential oils plant composition	**(%)**
(+)-Borneol	3.13
1,2-Cyclohexanediol, 1-methyl-4-(1-methylethenyl)-	1.56
1,3-Cyclohexanediol, 2,5-dimethyl-2-nitro-, monoacetate (ester), [1s-(1à,2á,3à,5à)]-	0.84
1-Hexyl-2-nitrocyclohexane	1.38
Bicyclo[3.1.0]hexane, 4-methylene-1-(1-methylethyl)-	2.23
Bicyclo[3.1.0]hexane, 6-isopropylidene-1-methyl-	0.7
Camphene	11.83
Caryophyllene	2.28
Caryophyllene oxide	3.77
Isoborneol	1.52
Longipinene epoxide	0.85
Thymol	13.56
Tricyclo[3.1.0.0(2,4)]hexane, 3,6-diethyl-3,6-dimethyl-, trans-	0.68
endo-Borneol	6.21
geranyl-à-terpinene	0.68
m-Cymene, 5-tert-butyl-	0.76
Carvacrol	10.01
n-Hexanesulphonylacetonitrile	0.59
o-Cymene	11.09
p-Cymene	1.74
à-Guaiene	1.36
à-Pinene	7.81
à-Terpineol	1.52

á-Guaiene	0.76
á-Phellandrene	2.74
ç-Terpinene	10.4
TOTAL	**100**

The results show that GC × GC–TOFMS has increased accuracy in analyzing thyme oil over GC-MS. They also indicate that GC × GC–TOFMS has high-quality data sets, resulting in increased accuracy in thyme oil identification and, therefore, the overall diagnostic results obtained. Furthermore, these results are similar to a study by Baharum *et al.* (2010), where a comparison of GC-MS and GC × GC–TOFMS was made over *Polygonum minus* Huds EOs, commonly known as kesum, which is widely used in Malaysia for cooking and other traditional practices. Their results indicated that GC-MS only identified ten compounds in kesum essential oil, while 42 compounds were identified using GC × GC–TOFMS for the same essential oil. Another study done by Winnike *et al.* (2015), where they analyzed metabolite extracts from samples isolated in human serum, indicated that GC × GC–TOFMS detected three times more peaks than GC-MS and three times the number of metabolites were identified by GC × GC–TOFMS when compared to GC/MS. The reason that GC/MS is not as effective as GC × GC–TOFMS is mainly because of the limited resolution of chromatographic peaks by the GC-MS, which can result in peak overlap making subsequent spectrum deconvolution for metabolite identification and quantification difficult (Winnike *et al.,* 2015). The current study and previous studies demonstrate that GC × GC–TOFMS has more potential to be used in detecting and profiling EOs extracts and other compounds because of its powerful separation and identification tool that allows for the identification of a much larger number of complex, volatile oil components.

4.1.3 Broth microdilution assay (minimum inhibitory concentration determination)

There is an increasing demand for accurate knowledge of the MIC of EOs, and hydrosol to balance sensory acceptability and antifungal efficacy (Lambert *et al.,* 2001). MIC is

defined as the lowest concentration of an antimicrobial agent that will inhibit the visible growth of a microorganism after incubation for a period. This was done using the broth microdilution assay *in vitro*, in triplicate (100-3.13 µg/mL) using thyme oil and hydrosol, respectively, while ethanol was used as a negative control and Thiabendazole was used as the positive control (Results on Table 4.3 below).

Table 4.3. The minimum inhibitory concentration (MIC) of thyme oil against *Phyllosticta citricarpa* isolate.

Dilution of thyme oil, Thiabendazole, thyme oil hydrosol and ethanol (µg/mL)	≥ 100	≥ 50	≥ 25	≥ 12.5	≥ 6.25	≥ 3.13
Thyme oil	–	–	–	+	++	+++
Thiabendazole	–	+	+	++	+++	+++
Thyme oil hydrosol	+	+	++	+++	+++	+++
Ethanol (control)	+++	+++	+++	+++	+++	+++

Data are reported as '+++' indicates strong growth of fungi, '++' medium growth, '+' minor growth and '–' indicates inhibition of growth of fungi (sensitive to thyme oil, hydrosol, Thiabendazole and ethanol).

After the incubation period at 25 °C for seven days, 40 µL of 0.2 mg/mL iodonitrotetrazolium salt solution (growth indicator) was added to each well and further incubated for another day. Thereafter, the results of inhibition were analyzed based on the colour change. The following colour changes were observed, Light pink = (Minor

growth) denoted by (+), Pink = (Medium growth) denoted by (++), Dark pink = (Strong growth) denoted by (+++) and lastly, Colourless = (No growth/complete inhibition) denoted by (-) were observed. The analyses of growth inhibition of *Phyllosticta citricarpa* by thyme oil showed that at concentrations of 100–25 (µg/mL) (Table 4.3), there was complete inhibition of the fungi (-). Therefore, the MIC value observed was a concentration of 25 (µg/mL) presented in Table 4.3. This is the lowest concentration of the thyme oil capable of inhibiting the growth of *Phyllosticta citricarpa,* where growth was not observed. Meanwhile, at the 12.5 µg/mL dilution, minor growth (+) of the fungus *Phyllosticta citricarpa* was observed. Furthermore, 6.25 µg/mL dilution of *Phyllosticta citricarpa* indicated medium growth (++). At 3.13 µg/mL dilution, the *Phyllosticta citricarpa* indicated strong growth (+++), thereby showing no inhibition of the *Phyllosticta citricarpa* by *Thymus vulgaris* oil at 12.5 µg/mL until 3.13 µg/mL respectively. However, thyme oil hydrosol had no MIC value against the test organism. The hydrosol indicated strong growth (+++), medium growth (++), and minor growth (+), respectively (Table 4.3). The fungus demonstrated minor growth between 100 and 50 µg/mL, while medium growth was observed at 25 µg/mL. Lastly, at 12.5 µg/mL until 3.13 µg/mL dilution, the *Phyllosticta citricarpa* indicated strong growth (+++). The reason there was no MIC value when hydrosol was used might be because the hydrosol is a by-product of the EOs extraction process consisting of trace amounts of thyme oil that are not concentrated enough, which implied the bioactive compounds present were found in low concentrations. Ethanol used as the negative control did not show an MIC value against the test organism, indicated by a strong growth (+++) of the fungi; thereby, it is concluded that ethanol does not have any effect on the growth of *Phyllosticta citricarpa.* Thiabendazole used as a positive control only inhibited the growth of the fungus completely (-) at 100 µg/mL; therefore, 100 (µg/mL) given in Table 4.3 was the MIC value observed when using Thiabendazole against the test organism; however, between 50 and 25 µg/mL, there was minor growth (+) observed. At 12,5 µg/mL, there was medium growth (++) of *Phyllosticta citricarpa* fungi, but between 6,25 and 3.13 µg/mL, the fungus demonstrated strong growth (+++), thereby indicating no inhibition of the fungus. However, a study by Tonial *et al.* (2017) indicated different results from the current study, where the endophyte *Diaporthe terebinthifolii* was used against *P. citricarpa.* The MIC value of Tonial *et al.* (2017) was

17.3 µg.mL, which was different from the 12,5 µg/mL MIC value for thyme oil used in the current study against the same organism, but the MIC value of *Diaporthe terebinthifolii* has shown higher effectiveness than the Thiabendazole used as a positive as the MIC value was 100 (µg/mL).

As much as the MIC value of thyme oil (12.5 µg/mL) and *Diaporthe terebinthifolii* (17.3 µg.mL) were different, their effectiveness against *P. citricarpa* remains high. These results suggest that both thymus oil and *Diaporthe terebinthifolii* can be proposed as potential biological controllers of the *P. citricarpa*, which can be utilized to limit the spread of the CBS disease. Furthermore, the compounds identified in *Diaporthe terebinthifolii* are commonly found in EOs with biological activity. This explains the high effectiveness of these endophytes (Tonial *et al.,* 2017). In another antifungal study, the MIC of thyme oil (*T. vulgaris*) was compared to *Candida albicans* (one of the most prevalent human fungal pathogens). It was found that the MIC value of *T. vulgaris* EO was 25 µg/mL against this fungal pathogen (Jafri and Ahmad, 2020). The MIC value of thyme oil was similar to the current study; even though the test organisms were different, it is evident that thyme oil has antifungal activities against a wide range of fungal pathogens and can act as promising antifungal agents against many fungal pathogens, and these might assist in reducing the use of synthetic fungicides.

4.1.4 Results for fungicidal and fungistatic activity of thyme oil against *Phyllosticta citricarpa*

Generally, for new antifungal candidates, the MIC determination is one of the first steps in evaluating the antimicrobial potential of a compound. However, the MIC value alone does not indicate the mode of action (cidal or static) of the antimicrobial agent. Hence it was necessary to do a fungistatic and fungicidal activity test of thyme oil and Thiabendazole (positive control) against *Phyllosticta citricarpa*. These were observed after re-growing fungal cells that failed to grow (remain Colourless = (No growth/complete inhibition) denoted by (-)) on EOs-treated and Thiabendazole-treated cells respectively,

from the different concentrations used for the MIC tests. The results are shown in Table 4.4. In this study, thyme oil exhibited fungistatic (S) activity characterized by partial inhibition of mycelial growth and fungicidal (C) activity, causing complete inhibition of the development of *Phyllosticta citricarpa*. Furthermore, the mode of action (fungistatic or fungicidal) appeared to be strongly correlated with the thyme oil concentrations. The analyses of fungistatic or fungicidal of the thyme oil against *Phyllosticta citricarpa* showed that at 100-50 µg/mL, there was no growth of the fungi (viable fungal cells could not be re-isolated), indicating the fungicidal effect (C). However, at 25 µg/mL, there were viable fungal cells that were re-isolated, indicating fungistatic (S) activity. These results mean that when the thyme oil concentration is high, the oil demonstrates fungicidal activity, but as the concentration decreases, the oil demonstrates fungistatic activity. Lombardo ***et al***. (2016) indicated that EOs or their individual components might have fungistatic or fungicidal effects, depending on the concentration used. This could be the case for the current study, where thyme oil showed fungicidal and fungistatic activity. The current study is in line with the study done by Lombardo ***et al***. (2016), as the analysis of the fungistatic and fungicidal activity of the oils tested showed that the effectiveness of the oils depended on the concentration used.

Nevertheless, the high fungicidal activity of thyme oil could be explained by its balanced chemical composition among its major compounds, such as thymol (32.1%) and ρ-cymene (20.4%), as these compounds play a major role in the antimicrobial activities of EOs (Bakkali *et al.,* 2008). However, when Thiabendazole (positive control) was used, it was only fungistatic at 100 (µg/mL) and did not exhibit a fungicidal effect. These results further mean that thyme oil is more active against *Phyllosticta citricarpa* than Thiabendazole because thyme oil contains both fungicidal and fungistatic effects against *Phyllosticta citricarpa*, while Thiabendazole only has a fungistatic effect. However, when ethanol (negative control) was used, it did not have any fungicidal or fungistatic effect against the test organism (result not shown), indicating that ethanol did not eliminate the fungal growth. In brief, and from the presented results, thyme oil could be considered an option for controlling *P. citricarpa* on citrus fruit, but the need to determine the effect of

thyme oil on the conidia structures of *Phyllosticta citricarpa* is important. Detailed microscopic analysis of *P. citricarpa* treated with thyme oil (*Thymus vulgaris)* and ethanol were then conducted using SEM to determine the effects of the thyme oil (*Thymus vulgaris)* and ethanol on the conidia and thereby confirm the mode of action of the tested compounds.

Table 4.4 Fungistatic (S) and fungicidal activity (C) of thyme oil against *Phyllosticta citricarpa* isolate obtained after re-growing the MIC results.

Dilution of thyme oil and control (µg/mL)	**≥ 100**	**≥ 50**	**≥ 25**
Diluted thyme oil	**C**	**C**	**S**
Thiabendazole (Positive control)	**S**		

Data reported as 'S' indicates fungistatic effects, and 'C' indicates fungicidal activity of the tested antimicrobial agent.

4.1.5 Scanning electron microscopy

Scanning electron microscopy (SEM) was used to examine the morphological changes of *Phyllosticta citricarpa* conidia structures (size and shape) after being treated with thyme oil (*Thymus vulgaris),* Thiabendazole (positive control) and ethanol (negative control), respectively, using the MIC results that resulted in fungal growth at different concentrations. These conidia structures have increased mitochondrial activity and are responsible for the life cycle of *P. citricarpa,* including the involvement in the mechanism associated with CBS dispersion and symptom development. The thyme oil MIC results show the following patterns of growth: (Light pink = (Minor growth) denoted by (+) at 12,5

µg/mL, Pink = (Medium growth) denoted by (++) at 6,25 µg/mL, and Dark pink = (Strong growth) denoted by (+++) at 3,13 µg/mL concentration respectively, observed after the addition of iodonitrotetrazolium salt solution (growth indicator) in the MIC and further incubated for a day (Table 4.3). The results were further analyzed using SEM. The SEM indicated that conidia structures were damaged or lost after treatment with thyme oil (*Thymus vulgaris*) when analyzing the MIC results that resulted in the fungal growth (12,5, 6,25 and 3,13 µg/mL concentration, respectively) (Figure 5 A-C), where different concentrations indicated different growth patterns of the fungi. The damage to the conidia cells after the treatment with thyme oil at different concentrations varied between Damaged conidia cells (DCC) and Loss of conidia contents (LCC). When *Phyllosticta citricarpa* was treated with thyme oil at a concentration of 3.13 µg/mL ((Dark pink = Strong growth/major) denoted by (+++)), Damaged conidia cells (DCC) (Figure 5 A) were observed, although there was major growth of the conidia structures. At a concentration of 6.25 µg/mL (Pink = (Medium growth) denoted by (++)), the conidia cells were also assessed for possible damage and both Damaged conidia cells (DCC) as well as Loss of conidia contents (LCC) (Figure 5 B) were observed. Furthermore, at a concentration of 12.5 µg/mL ((Light pink = (Minor growth) denoted by (+)), the SEM micrograph of *Phyllosticta citricarpa* indicated Loss of conidia contents (LCC). These results are similar to those in the study done by Kohiyama *et al.* (2015), where the antifungal properties of *Thymus vulgaris* EOs were evaluated on *Aspergillus flavus in vitro* and then analyzed using SEM. Their study showed alterations in conidia structures after treatment with *Thymus vulgaris* at concentrations ranging from 50 to 500 µg/mL. The alterations included degenerative conidia, while some appeared degraded or with a complete absence of conidia (Kohiyama *et al.,* 2015). The Thiabendazole (positive control) MIC results have the following pattern of growth: Light pink = (Minor growth) denoted by (+), at 50 and 25 µg/mL, Pink = (Medium growth) denoted by (++), 12.5 µg/mL and Dark pink = (Strong growth) denoted by (+++), 6.25 and 3.13 µg/mL (Table 4.3), which were further analyzed using SEM as well (Figure 6 A-F). When the conidia structures were observed at different concentrations using SEM after treatment with Thiabendazole from the MIC results, the following damages were observed: At 50 µg/mL, (Light pink = (Minor growth) denoted by (+)), 25 µg/mL, (Light pink = (Minor growth) denoted by (+)) and 12.5 µg/mL, (Pink =

(Medium growth) denoted by (++)). Damaged conidia cells (DCC) were observed (Figure 6.1 A-C) at 6.25 and 3.13 µg/mL, (Dark pink = (Strong growth) denoted by (+++)), both Damaged conidia cells (DCC), as well as Loss of conidia contents (LCC), were observed (Figure 6 D-E).

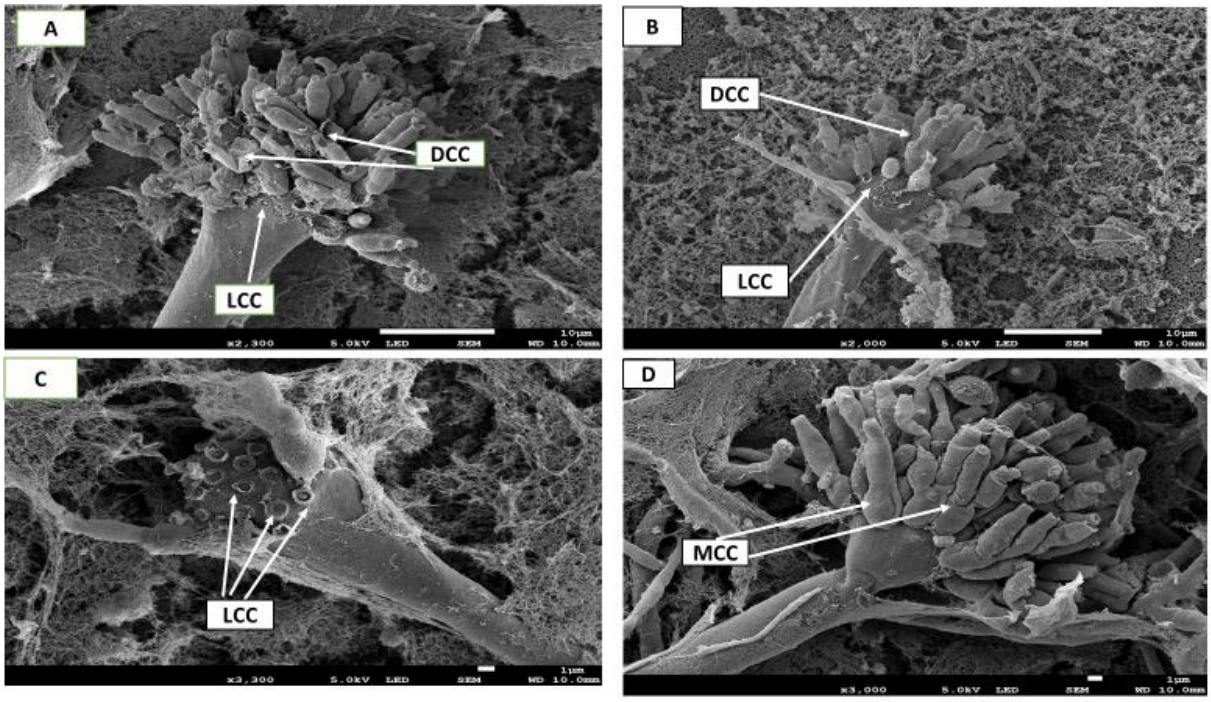

Figure 5: (A-C) SEM results of different types of injuries induced by thyme oil on the fungal conidia structures at different concentrations (D) control (ethanol) cells. DCC - Damaged conidia cells; LCC - Loss of conidia contents; MCC - Matured conidia cells.

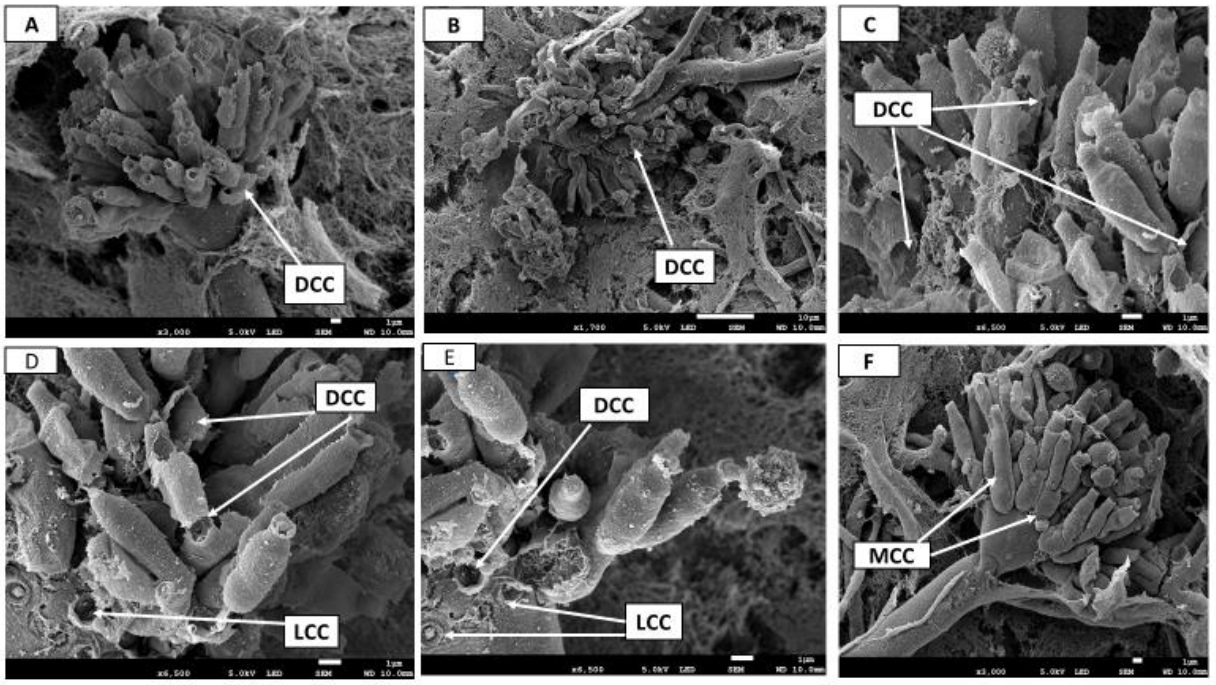

Figure 6: (A-E) SEM results of different types of injuries induced by Thiabendazole on the fungal conidia structures at different concentrations (F) control (ethanol) cells. DC - Damaged conidia cells; LCC - Loss of conidia contents; MCC - Matured conidia cells.

However, when *Phyllosticta citricarpa* was treated with ethanol (negative control), fungal growth was not inhibited and was represented by a high amount of intact and matured conidia structures (Figure 6 F). It can be concluded that a lower concentration of thyme oil can negatively affect conidia cells as damaged conidia cells were observed. An increased concentration resulted in even more damage where the conidia cells were disrupted, resulting in the loss of conidia content and the death of the organism. However, when *Phyllosticta citricarpa was* treated with ethanol (negative control) alone, the fungal growth was not disturbed, or there was a high number of intact and matured conidia structures (Figure 6 F). These findings suggest that thyme oil (*Thymus vulgaris)* can inhibit parts of the *P. citricarpa* life cycle by targeting conidia structures. Furthermore, it is believed that this occurs because thyme oil (*Thymus vulgaris)* consists of high amounts of terpenes, which play an important role in the plant defence mechanism against pathogenic fungi (Nazzaro *et al.,* 2013; Liu *et al.*, 2009). Furthermore, as much as

Thiabendazole (positive control) was effective against the test organism, it was not as effective as thyme oil. This might be because Thiabendazole has a single target side against the fungi, unlike thyme oil, which has many bioactive chemical compounds against fungal growth, preventing the growth of conidia structures. Furthermore, it is suggested that thyme oil can be utilized as an alternative control measure to replace the conventional synthetic fungicides currently in use, which may replace synthetic fungicides in future to reduce their persistence in the environment. However, ethanol (Figure 6 F) used as the negative control did not have any antifungal properties against the test organism, indicated by a high number of conidia, concluding that ethanol does not have any effect on conidia structures of *P. citricarpa.* The current study also showed that spore-releasing structures, such as conidia with increased mitochondrion activity, are more sensitive to mitochondrial inhibitors, including EOs, compared to vegetative cells and hyphae analyzed using SEM (Ncango *et al.*, 2010; Leeuw *et al.,* 2009; Kock *et al.,* 2007). This may be of value in combating fungi that depend mainly on these structures for dispersal. In the present work, the potential of thyme oil was assessed in the control of the phytopathogen *P. citricarpa*. This data is of great importance since the breakdown in the development of asexual spores is fundamental for interrupting the CBS disease cycle. These asexual conidia can further aggravate the disease within the plant and adjacent areas if not controlled (Perryman *et al.,* 2014). Furthermore, in countries such as the USA and Brazil, the conidia are the only ones responsible for the spread of the disease in the orchards since there is an overlap of fruits maturing and frequent summer rainfall present in this region, and sexual reproduction does not occur (Wang *et al.,* 2016).

4.1.6 Transmission electron microscopy

SEM offered clear, high-resolution images of the outer surface of *Phyllosticta citricarpa* conidia structures after being treated with thyme oil (*Thymus vulgaris),* Thiabendazole (positive control) and ethanol, respectively, at different concentrations, allowing observation and analysis of the outer cell and conidia damage. In addition to this analysis, a need to observe the internal structure of the same samples was the next logical step; hence a transmission electron microscope (TEM) was used. TEM transmits a beam of

electrons through a sample, creating an image of the internal structure of the sample, while SEM only analyzes the surface of a sample (Zhao *et al.,* 2018). The analysis of *Phyllosticta citricarpa* conidia morphology after treatment with thyme oil and Thiabendazole (positive control) at different concentrations using TEM revealed different morphological changes within the conidia structures (Figures 7 and 8). The TEM observations identified eight different morphotypes, which appeared at different concentrations of both antifungal treatments. These morphotypes were defined as CW - Cell wall; CM - Cell membrane; O - Organelles; DCW - Damaged cell wall; DCM - Damage cell membrane; LCMC - Loss of cell membrane content; LCWC - Loss of cell wall content; LO - Loss of organelles. When thyme oil was used against *P. citricarpa* at a concentration of 3,13 µg/mL (Figure 7 B), only DCW - Damaged cell walls of the conidia structures were observed, while at 6,25 µg/mL (Figure 7 C), DCW - Damaged cell wall; DCM - Damaged cell membrane; LCMC - Loss of cell membrane content and LCWC - Loss of cell wall content were observed. Lastly, at a concentration of 12,5 µg/mL (Figure 7 D), LO - Loss of organelles of the conidia was observed, resulting in cell death. However, the TEM of the ethanol-treated (negative control) *P. citricarpa* fungal conidia exhibited an image with well-defined structures such as CW - Cell wall; CM - Cell membrane; O - Organelles. These indicate that ethanol alone did not have any effect on the inhibition of the growth of this fungal pathogen (Figure 7 A) when Thiabendazole (positive control) was used. It also indicated similar patterns against *P. citricarpa,* at a concentration of 3,13 µg/mL (Figure 8 B). Conidia exhibited an image that has well-defined structures such as CW - Cell wall; CM - Cell membrane; O - Organelles, similar to the ethanol-treated cells (negative control) (Figure 8 A). At concentrations of 6.25 and 12,5 µg/mL (Figure 8 C, D), only DCW - Damaged cell wall of the conidia structures were observed, while at 25 µg/mL (Figure 8 E), only LCMC - Loss of cell membrane content. Lastly, at 50 µg/mL, LO-Loss of organelles was observed, resulting in cell death. These results emphasize the antifungal activity of thyme oil and Thiabendazole (positive control) against *P. citricarpa*. However, the SEM and TEM results proved that thyme oil was more effective. Furthermore, considering the eco-friendly properties of thyme oil and that *P. citricarpa* is less likely to be resistant to thyme oil, it indicated that it could be the future alternative strategy to address these fungal pathogen challenges.

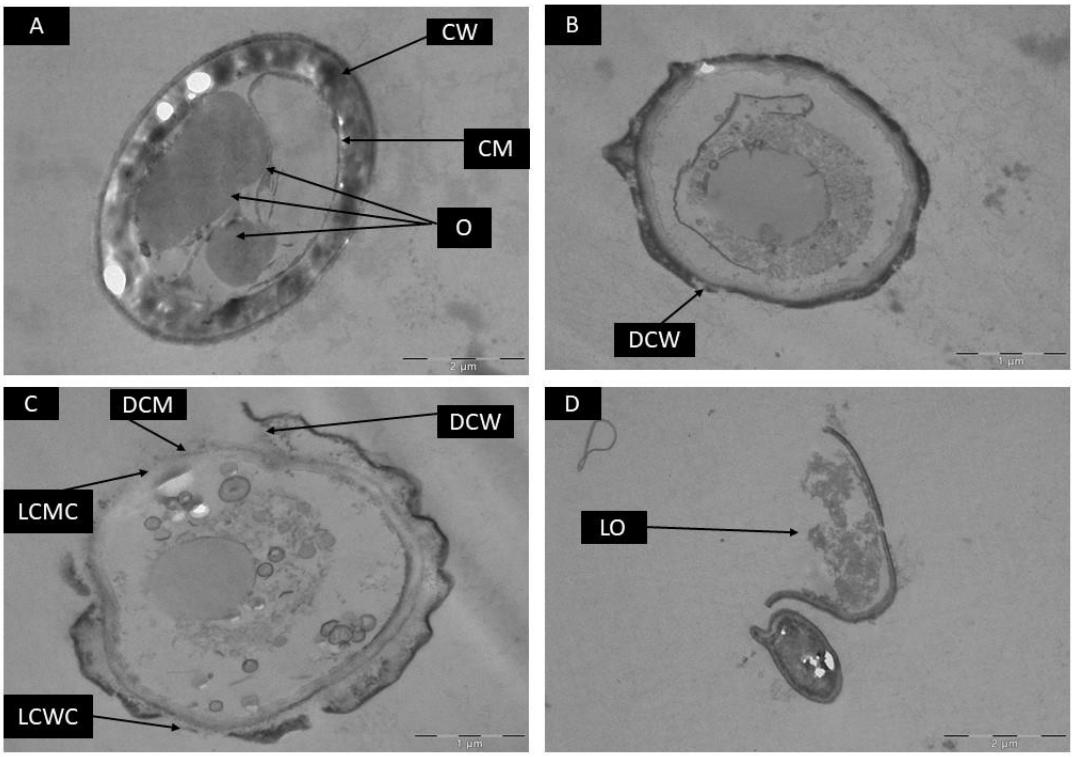

Figure 7: (A-D) TEM results of different types of injuries induced by thyme oil on the fungal conidia structures at different concentrations (A) control (ethanol) cells. CW - Cell wall; CM - Cell membrane; O - Organelles; DCW - Damaged cell wall; DCM - Damaged cell membrane; LCMC - Loss of cell membrane content; LCWC - Loss of cell wall content; LO - Loss of organelles.

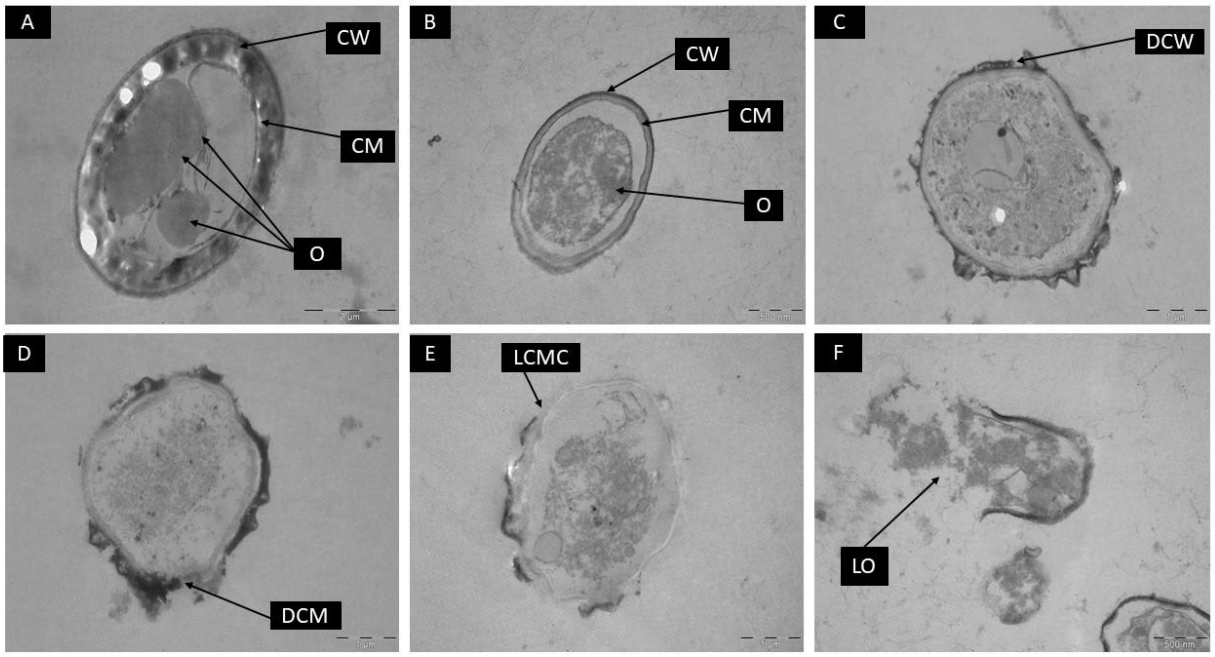

Figure 8: (A-F) Different types of injuries induced by Thiabendazole on the fungal conidia structures at different concentrations. (D) control (ethanol) cells. CW - Cell wall; CM - Cell membrane; O - Organelles; DCW - Damaged cell wall; DCM - Damaged cell membrane; LCMC - Loss of cell membrane content; LO - Loss of organelles cells.

4.1.7 Inhibition of pathogen growth on leaves

In this study, the antifungal activity of thyme oil was assessed *in vivo*, and the activity of Thiabendazole (positive control), hydrosol and ethanol (negative control) was also assessed on lemon leaves infected with *Phyllosticta citricarpa,* as shown in Figure 9. The activity of the abovementioned compounds was evaluated to determine their potential ability to inhibit or limit the development of *P. citricarpa* on the surface of sterile (20 min; 120 °C; 1 atm) lemon leaves. After a 21-day incubation period at 28 °C, the growth of *P. citricarpa* on the leaves of all the tested compounds was studied. The results of antifungal activity of the Thiabendazole (positive control), thyme oil, and hydrosol against *Phyllosticta citricarpa in vivo* on citrus leaves indicated a reduction of the severity incidence of *Phyllosticta citricarpa* compared with the untreated control.

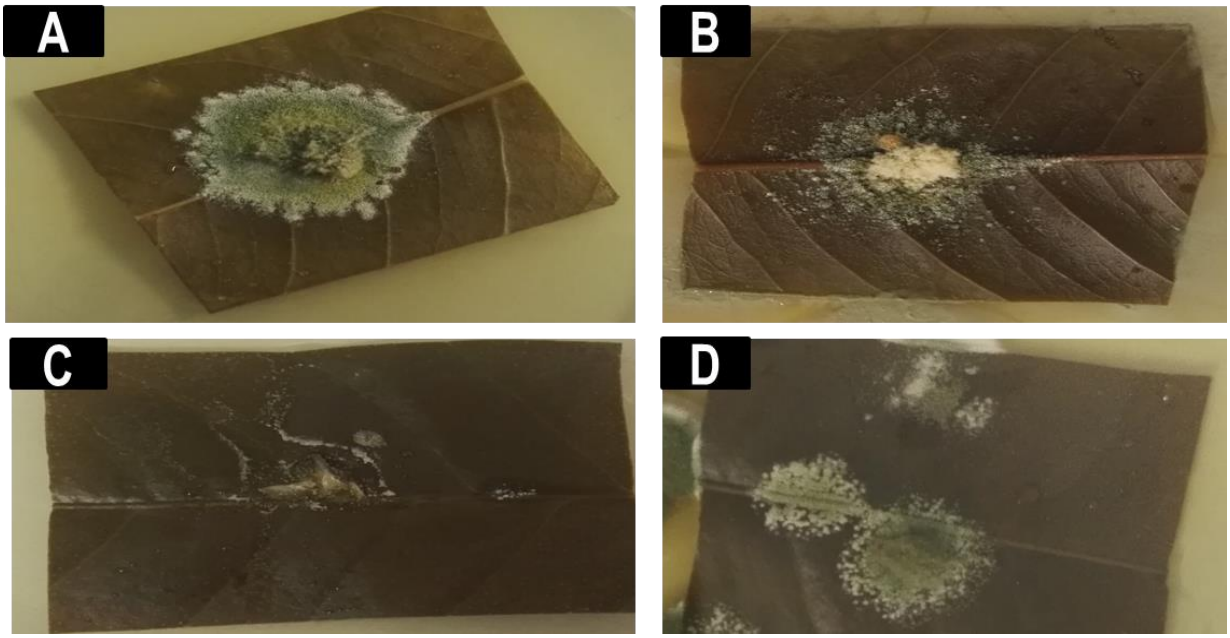

Figure 9. Growth inhibition of *Phyllosticta citricarpa in vivo* on leaves. (A) Ethanol (negative control), (B) Thyme oil hydrosol, (C) Thyme oil (*Thymus vulgaris)* and (D) Thiabendazole.

Among all compounds tested, thyme oil (*Thymus vulgaris)* demonstrated the highest severity of reduction because it almost completely inhibited fungal growth on the leaf surface (Figure 9 C). Savi *et al.* (2019) conducted a similar study where secondary metabolites 7-O-β-D-glucosyl-genistein and 7-O-β-D-glucosyldaidzein produced by the strain *Microbacterium* spp. were used to inhibit the formation of *P. citricarpa* in citrus leaves. Their results indicated that both compounds could considerably inhibit the development of CBS of *P. citricarpa* on leaves. The current study also showed that Thiabendazole and hydrosol reduced the growth of the fungi, but the reduction observed was not as effective as thyme oil. There was no reduction difference between Thiabendazole and hydrosol (Figure 9 B and D) in terms of size. The ethanol used as negative control indicated no reduction in the fungal growth on the leaves; therefore, it was concluded that ethanol alone had no effect against *P. citricarpa,* the causative agent of CBS *in vivo,* Figure 9 A. This data from the current study is of great importance since both thyme oil and thyme oil hydrosol inhibited the development of *P. citricarpa*. These

inhibitions are fundamental for the interruption of the CBS disease cycle. Furthermore, the results suggest that certain secondary metabolites produced by plants, such as EOs and their hydrosol, may represent an alternative to decreasing the use of synthetic fungicides in treating CBS and their negative impacts. These compounds (thyme oil and their hydrosol) repressed the development of CBS necrosis, suggesting that they can be an ideal eco-friendly alternative to reduce fungicide use in citrus orchards to control CBS disease.

4.1.8 Inhibition of pathogen growth on the citrus fruits

Phyllosticta citricarpa survives and continues sporulation during harvest, storage, and transportation. Therefore, antifungal applications are paramount to preventing pathogen infection that occurs postharvest. As a result, the inhibition of *P. citricarpa* development postharvest would have a great advantage for suppression of the infection due to this fungal pathogen. Mycelial growth of *Phyllosticta citricarpa* on citrus fruits (lemon) was monitored for 28 days (four weeks) when treated with ethanol (negative control), thyme oil hydrosol, thyme oil (*Thymus vulgaris*) and Thiabendazole (positive control), as well as a non-inoculated fruit, respectively. The results were monitored and recorded at a weekly (seven days) interval for four weeks (28 days). The results of *in vivo P. citricarpa* treatment with essential oil, hydrosol extract and Thiabendazole (positive control) on citrus fruits (lemon), respectively, are presented in Figures 10.1.-10.4. Throughout the four weeks, the most promising results were obtained from thyme oil (*Thymus vulgaris*) (Figure 10.4).

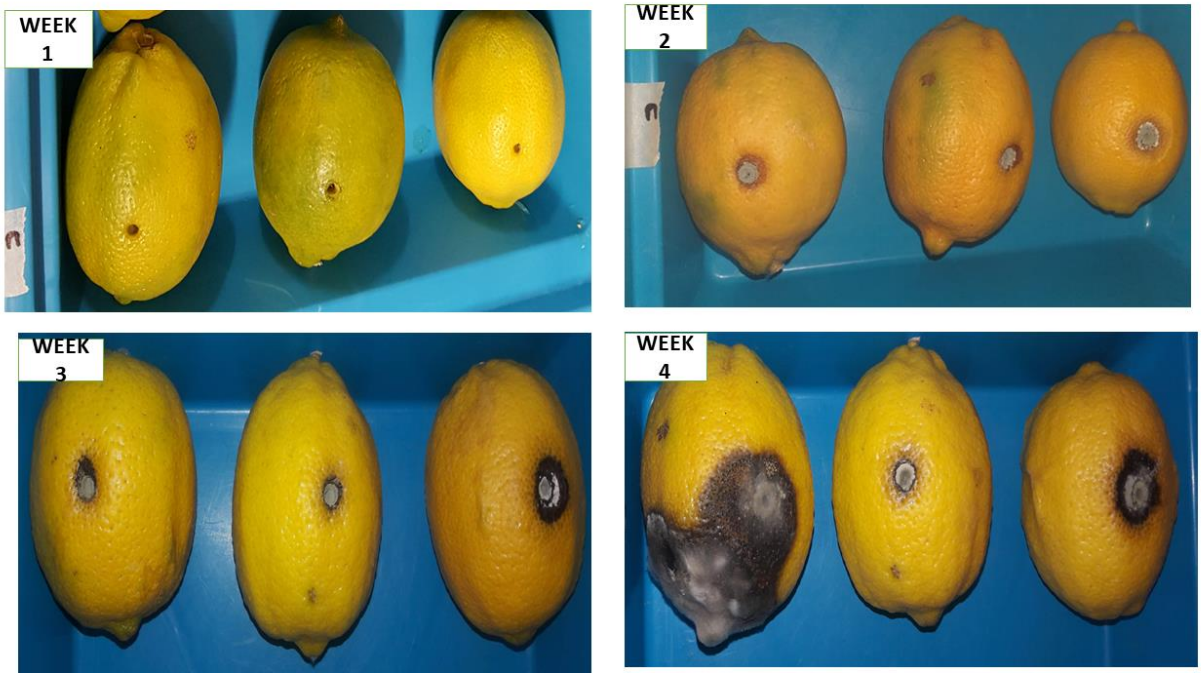

Figure 10.1 Growth inhibition of *Phyllosticta citricarpa in vivo* on citrus fruits treated with ethanol (negative control) monitored over four weeks.

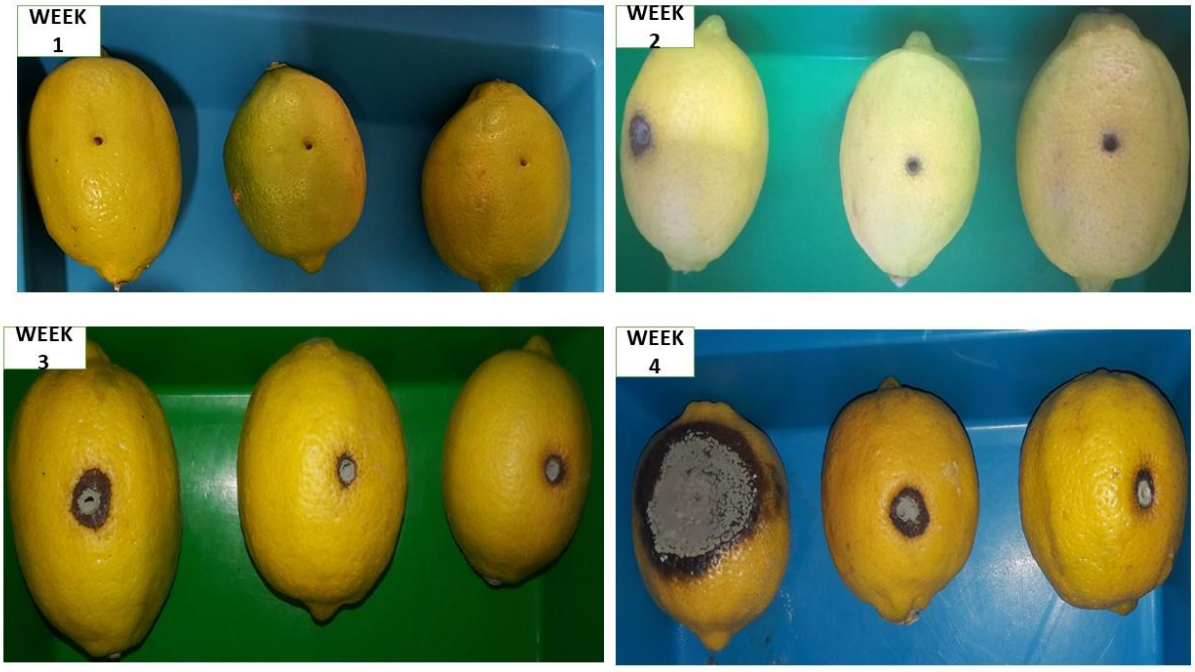

Figure 10.2. Growth inhibition of *Phyllosticta citricarpa in vivo* on citrus fruits treated with hydrosol monitored over four weeks.

Figure 10.3. Growth inhibition of *Phyllosticta citricarpa in vivo* on citrus fruits treated with Thiabendazole and monitored over four weeks.

Figure 10.4. Growth inhibition of *Phyllosticta citricarpa in vivo* on citrus fruits treated with thyme oil and monitored over four weeks.

According to the results, thyme oil had substantially prevented the growth of the fungi from week one until week four, as there was no growth of the fungal pathogen throughout. This indicates that thyme oil has high antimicrobial activity against *P. citricarpa,* the causative agent of CBS, even on the citrus fruits themselves. The results align with a study by Tabti *et al.* (2014) on *Citrus sinensis* (orange) fruits infected with *Penicillium italicum* and treated *in vivo* with thyme oil; their results indicated no infection on the oranges as there was no mycelial growth on the fruits.

However, the synthetic fungicide Thiabendazole (positive control) prevented the growth during week one (this was not surprising, as the organism normally takes days to start growing), but as the weeks progressed (weeks two, three and four), the growth was observed and increased as the weeks progressed (Figure 10.3). These results mean that the compound used as a positive control managed to control fungal growth. However, it does not completely limit the development of fungal growth postharvest. These results support the findings by Agostini (2006), where Thiabendazole indicated inhibitory effects against conidial germination of *P. citricarpa* in a postharvest fungicide application study after the citrus fruits (lemon) were infected with *P. citricarpa.* Furthermore, another *in vitro* study conducted by Korf *et al*. (1998) showed that CBS cultured on Thiabendazole-amended plates reduced conidial germination incident of *P. citricarpa*, leading to the conclusion that Thiabendazole is effective in controlling CBS *in vitro*. On the other hand, the hydrosol prevented the fungal growth during weeks one and two, while in week three, some growth was observed (Figure 10.2). However, the growth started to develop extensively during week four. These hydrosol results were not surprising as the hydrosol contains traces of thyme oil, which is why it was not as effective as thyme oil. However, these results differ from the study by Tabti *et al.* (2014), where the thyme hydrosol extracts were tested *in vivo* on orange citrus fruits infected with *Penicillium italicum,* the causative agent of citrus rot. In their study, the hydrosol showed a complete absence of infection on the oranges and no disease incidence of citrus rot throughout the experiment. These results might indicate that thyme oil hydrosol is more effective against *Penicillium italicum* than on *P. citricarpa*; however, these results will have to be investigated further in future

studies. The ethanol used as the negative control did not prevent the fungal growth; the growth was visible from week two and continued to grow extensively until week four (Figure 10.1). The results indicate that ethanol alone does not affect this fungal pathogen. Furthermore, uninoculated fruits did not develop CBS symptoms during the experiment (data not shown). This experiment also noted that all the compounds tested did not have any infection during week one, including the ethanol used as the negative control. These results suggest that at this point, the *P. citricarpa* conidia were not fully matured to cause severe infection, but from week two onwards, the fungal pathogen started to show infection growth in some of the tested compounds.

4. 2. References

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CHAPTER FIVE

Concluding remarks

5.1 Concluding remarks

Worldwide, postharvest fungal disease control relies on the use of synthetic fungicides such as imazalil (IZ), Thiabendazole (TBZ), sodium orthophenyl phenate (SOPP), fludioxonil (FLU), Bordeaux mixture, and pyrimethanil (Ritenour *et al.,* 2004). These synthetic fungicides play a huge role in preventing and controlling many fungal diseases in the agricultural industry. If these fungicides are not used, the damages and the loss could increase drastically (Talibi *et al.,* 2014; Du Plooy *et al.,* 2009). These fungal reduction methods minimize the loss in the agricultural industry, ease pressure on the production resources, and generate much-needed revenue. However, recently there has been public concern about human health and the environmental over-use of synthetic fungicides, coupled with the development of resistance by the fungus (Abd-Elsalam *et al.,* 2015). In this respect, natural bio-control agents have shown great potential as an alternative to synthetic fungicides and offer an environmentally friendly alternative to synthetic fungicides (Setlhare, 2017; Tonial *et al.,* 2017; Chutia *et al.,* 2009). An attempt to reduce crop losses caused by pathogenic fungi and, simultaneously, the increasingly evident defects of synthetic fungicides leads to a constant search for natural substances limiting the development of fungi. The risk of spreading dangerous pathogens (including *Phyllosticta citricarpa*) is increased because they can gradually become resistant to synthetic fungicides, and most are very ecotoxic. In addition, fungi often have a cross-resistance phenomenon with synthetic fungicides (Hu *et al.,* 2019; Sivakumar and Bautista, 2014). If a given phytopathogen species becomes resistant to one preparation, at the same time, it becomes resistant to the whole group of chemical substances to which this preparation belongs (Sivakumar and Bautista, 2014).

The current study has demonstrated that *Thymus vulgaris* EOs has fungal activity against *Phyllosticta citricarpa*. This citrus fungal pathogen indicated an appreciable sensitivity towards the EOs used in the experiment. Thyme EOs was found to damage the conidia structures of this fungal pathogen, one of the key reproductive structures of *P. citricarpa*. These conidia structures have increased mitochondrial activity and are responsible for the life cycle of *P. citricarpa,* including the involvement in the mechanism

associated with CBS dispersion and symptoms development. After treatment with the oil, damage to the conidia cells at different concentrations varied between Damaged conidia cells (DCC) and Loss of conidia contents (LCC) shown by SEM. Furthermore, when conidia structures were viewed using TEM, the structures indicated DCW - Damaged cell walls; DCM - Damaged cell membrane; LCMC - Loss of cell membrane content; LCWC - Loss of cell wall content; LO - Loss of organelles, which eventually leads to cell death. Furthermore, thyme oil hydrosol used in the study has indicated a moderate antifungal activity against the fungal pathogen used in the study in both the citrus fruits and the leaves, where the life cycle of *P. citricarpa* normally occurs in the field. The results imply that hydrosols can also be used as natural anti-microbials in agriculture. In fact, as aqueous solutions, the hydrosols could be easily rinsed off surfaces and do not have a strong, persistent smell, unlike EOs. From the results, thyme oil and its hydrosol have shown considerable damage to the citrus leaves and fruits, respectively. It is also important to note that both the citrus leaves and the citrus fruits are needed for the completion of the life cycle of this fungal pathogen. In addition, both the thyme oil and its hydrosol could be used to combat and prevent antifungal resistance. In fact, EOs and their hydrosols are unlikely to induce antimicrobial resistance, having multiple different targets in the microbial cells, unlike synthetic fungicides, which often only have a single target.

5.2 Future research

Due to the less effectiveness of hydrosol, future studies should explore the possibility of hydrosols being used synergistically with other molecules to enhance their antifungal properties in the citrus industry against CBS. Furthermore, more research needs to be done on thyme oil and its hydrosol to identify their sensory characteristics, and their persistence in the environment also needs to be investigated.

5. 3. References

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