Evaluating the Effect of Phenolic Compounds on the Growth of *Phyllosticta citricarpa*, the Casual Organism of Citrus Black Spot

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DECLARATION

I, Zinhle Valitha Hlatshwayo, declare that the research reported in this thesis; unless where otherwise indicated; is original work. This thesis has not been submitted for any degree or examination at any other institution.

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DEDICATION

This dissertation is dedicated to Zama Irmah Hlatshwayo

"I thank You Lord for natural, infinite and yes." – E.E. Cummings

ABSTRACT

Citrus black spot (CBS), caused by the ascomycete fungus *Phyllosticta citricarpa* [(McAlpine) Van der Aa] (Teleomorph: *Guignardia citricarpa*), is a phytosanitary disease that causes unsightly lesions on the rind of citrus fruit. South African farmers spend between R500 million and R1 billion annually, trying to comply with the phytosanitary regulations imposed by importing countries to control CBS. Control strategies include integrated pest management, quarantine methods and applying hazardous fungicides. Studies exploring alternative methods of control need to be undertaken as current control methods have proved expensive and inadequate. This study was conducted to evaluate if phenolic compounds can be used to control growth of *P. citricarpa*. Total phenolic compounds were extracted from tissues of navel oranges using the method described by Boehm *et al.* (2006). Oatmeal agar (OA) was amended using these phenolics extracted from the albedo (9.10 mg L^{-1}) and the flavedo (3.36 mg L^{-1}). *Phyllosticta citricarpa* was grown on this media at 30 mL total phenolics/ 250 mL growing media at 25^{o}C in the dark. Weekly observations on fungal growth and development were made and radial growth measurements taken. Flavedo and albedo tissues were removed from citrus fruit and placed on OA; the fungus was allowed to grow for four weeks after which a scanning electron microscope was used to determine whether the fungus was able to colonize these tissues. Growing *P. citricarpa* on OA amended with total phenolic compounds from the albedo or the flavedo extracted from navel oranges showed inconsistent results and no clear conclusions could be drawn regarding the ability of *P. citricarpa* to develop in the presence of phenolics extracted from either the albedo or flavedo tissues. The ability of the fungus to colonize different fruit tissues was also determined; results showed that despite the differences in the concentration of total phenolic compounds between the albedo and flavedo of navel oranges, the fungus was able to colonize both tissue types. These results contradict with the general opinion that *P. citricarpa* only colonizes and therefore affects the flavedo of the fruit and does not develop beyond the albedo into the flavedo of citrus fruit. From the experiments conducted in this study, it can be concluded that phenolic compounds, at physiological concentrations common in the citrus rind, cannot inhibit the development of *P. citricarpa*, the causal agent of CBS.

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GENERAL INTRODUCTION

Fruit in the genus *Citrus* belong to the family Rutaceae, a genus comprising a number of fruit crops, including *Citrus tangerina* Tanak. (tangerine), *Citrus aurantifolia* (Christm.) Swingle (lime), *Citrus paradisi* Macfad. (grapefruit), *Citrus sinensis* Osb. (orange), *Citrus limon* (L.) Burm f. (lemon) and *Citrus reticulata* Blanco (mandarin) (Mukhopadhyay, 2004). Citrus fruit is believed to have originated in South East Asia. All citrus species are trees characterized by fragrant flowers and edible juicy fruit (Davies and Albrigo, 1994). Citrus fruit are grown in over 100 countries, mainly to be eaten fresh, although many species are used for processing into fruit juices or added to dishes and beverages (Davies and Albrigo, 1994; Janick, 2005). Brazil, Spain, Greece, Egypt, Mexico and South Africa, amongst other countries, grow and export citrus fruit to other countries around the world (FAO, 2012). Although South Africa is only the 12th largest producer of citrus fruit, it is the third largest exporter of fresh citrus fruit in the world (Ginindza, 2015), with Limpopo, Eastern Cape, Mpumalanga, Western Cape and KwaZulu-Natal as the major citrus producing provinces in the country (DAFF, 2011; Ntombela and Moobi, 2013).

South Africa exports 70% of its fresh citrus produce to the European Union, India, Iran, Japan and the USA, with 55% of the total export going to the European Union (Mannya and Jaftha, 2013). Citrus exports generate about R8 billion a year in Gross Domestic Product for South Africa (Ginindza, 2015). The European Union (EU), along with other major South African citrus importing partners, has placed strict phytosanitary regulations for importing citrus from areas affected by pests and diseases to prevent the introduction of alien species into the EU (Carstens *et al.*, 2012). According to Pet Risk Assessments performed on the epidemiology and aetiology of *P. citricarpa*, the risk of introducing CBS into countries of the EU, is low, the European Commission, however, has stated that there is not enough scientific evidence to amend phytosanitary regulations that are currently in place (European Union, 2001). Disease symptoms or pest infestations found on citrus fruit can result in the rejection of an entire consignment, leading to financial losses for both, the exporting and importing countries (European Union, 2014). The South African citrus industry is currently severely threatened by restrictive phytosanitary regulations by importing countries on the presence of citrus black spot (CBS). This fungal disease, caused by *Phyllosticta citricarpa* [(McAlpine) Aa] (Teleomorph: *Guignardia citricarpa* Kiely), is found in six of the nine provinces of South

Africa (Carstens *et al.*, 2012). South African farmers incur financial losses between R500 million and R1 billion annually to comply with the phytosanitary regulations of the European Union (Mannya and Jaftha, 2013; Magwaza, 2014).

Citrus Black Spot affects and develops in the rind of citrus fruit (Davies and Albrigo, 1994; Kiely, 1948). The fungus causes unsightly lesions of the rind, reducing the aesthetic appeal of the fruit, thereby directly decreasing its economic value on local and international markets (Carstens *et al.*, 2012). Once *P. citricarpa* spores infect a fruit, a resting body within the rind tissue is formed. This resting body remains dormant, marking the beginning of latent period (Kiely, 1948). Latency occurs due to an increase in fruit resistance to develop disease symptoms, rather than a decrease in the amount of inoculum causing the disease (Whiteside, 1965). During the latent period, the disease remains dormant and fruit remain asymptomatic until environmental conditions and conditions in the rind tissue are favourable for disease progression. Disease symptoms usually only become visible at fruit maturity (Kotzé, 1996; McOnie, 1967). Fruit that was symptomless pre-harvest can, therefore, develop symptoms post-harvest during shipping and export leading to rejection of entire consignments of fresh fruit upon arrival at the destination despite having been cleared for shipping at the exporting terminal (Kotzé, 1996).

Pre-harvest control of CBS relies mainly on protecting young fruit in the orchard from infection over the period where the fruit is most susceptible by using fungicides, whilst post-harvest CBS management strategies have thus far been limited to the use of chemicals, sterilization and hot water treatment (Carstens *et al.*, 2012; Kotzé, 1996). During fruit growth and development, copper fungicides are effective and can reduce the inoculum of *Guignardia* in an orchard by up to 70% (Roberts and Dewdney, 2014); however, since pre-harvest lesions that are not associated with symptoms at the time of harvest can develop on fruit postharvest, effective postharvest methods of disease control are necessary (Kotzé, 1963; 1981).

Several compounds that naturally occur in plant tissues have been shown to play a role in protecting plants from infection by diseases; these compounds are usually secondary plant products (SPPs) that fulfill antioxidant functions in various fruit tissues (Bocco *et al.*, 1998). Such SPPS are also used by the plant as pigments, as well as compounds affecting growth and development as well as reproduction. Amongst the SPPs, the phenolic compounds form the largest group. Generally, phenolic compounds found in fruit tissue have been shown to protect fruit from physiological disorders caused postharvest stresses, such as rind pitting and

chilling injury, as well as from pathogens, often via elicitors, such as plant hormones (Cajuste and Lafuente, 2007; Siboza *et al*., 2014). Phenolic compounds include a diverse group of naturally occurring plant molecules found in various citrus plant tissues (Haminiuk *et al.*, 2012). Their main role is to allow the plant to adapt to changing environmental conditions and to act as signaling molecules within the plant. When plants are exposed to biotic or abiotic stresses, the concentration of phenolic compounds commonly increases and, through various signaling pathways, these compounds protect plants from stress and pathogens (Ali *et al.*, 2007; Bocco *et al.*, 1998; González-Aguilar *et al.*, 2004). Elicitors, such as plant hormones and biological control agents, have been applied to plants to artificially increase the level of phenolic compounds, thereby increasing the plant's tolerance to stress or to reduce the effect of stress on the plant (Ruiz-García and Gómez-Plaza, 2013). Artificially increasing phenolic compounds using elicitor has been shown to aid in protecting plants from several fungal diseases, including those caused by *Fusarium oxysporum*, *Penicillium brevicompactum*, *Streptomyces scabies*, *Verticillium alboatrum* and *Phytophthora infestans* (Lattanzio *et al.*, 2006; Zabka and Pavela, 2013).

The aim of this study was to evaluate the role of citrus rind phenolics on the growth of *P. citricarpa*. Many of these phenolics are known to exhibit biological and antimicrobial activity in citrus and are able to activate induced systemic resistance (ISR) in plants. Such a resistance is elicited by a local infection by a pathogen to which plants respond with a salicylic-acid-dependent signaling pathway that results in the expression long-lasting disease resistance against pathogens throughout the plant. This ISR may also result in a hypersensitive response by the plant and delay disease development leading to pathogen control (Heil and Bostock, 2002). It was hypothesized that increasing the phenolic concentration in the citrus rind may lead to the conditions in the rind being unfavourable for the resting body to develop and cause disease.

The objectives used to achieve this aim were to:

1. Determine optimum growth media and conditions for culturing *P. citricarpa in vitro* on different growing media to determine the best media sustaining optimal growth *in vitro* of this pathogen.

2. Determine the initial phenolic concentration present in the albedo and flavedo of navel oranges.

3. To evaluate the effect of total phenolics extracted from citrus rinds on the *in vitro* mycelial growth of *P. citricarpa* grown on growing media to evaluate addition of phenolics as a possible control method of *P. citricarpa*.

4. Evaluate the ability of *P. citricarpa* to infect the albedo and flavedo *in vitro*.

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CHAPTER 1

BLACK SPOT DISEASE OF CITRUS AND PHENOLIC COMPOUNDS IN CITRUS FRUIT: LITERATURE REVIEW

1.1 INTRODUCTION

Citrus exports are an important source of foreign exchange for South Africa and these exports are threatened by restrictive quarantine regulations linked to certain fruit pests and diseases. Fruit displaying symptoms of diseases cannot be sold, both on the local and the international market. Citrus Black Spot (CBS), caused by the fungus *Phyllosticta citricarpa* [(McAlpine) Van der Aa] (Teleomorph: *Guignardia citricarpa* Kiely) causes devastating losses to the citrus industry due to unsightly lesions that form on the fruit, rendering them unmarketable (ISPM, 2014). Symptomatic fruit may contain spores that could introduce CBS to areas where the disease does not yet occur and, as a result, strict phytosanitary restrictions have been placed on shipments of citrus coming from areas affected by CBS. Such regulations are in place to prevent the introduction of alien species into countries where the organism does not yet occur. According to the European Food Safety Authority (EFSA) Panel on Plant Health, the risk of *P. citricarpa* entering a region where it is absent through citrus plants and citrus fruit is highly likely. The entry of the pathogen into a country through shipments containing only citrus leaves is unlikely, and very unlikely if the shipment consists of Tahiti lime (*Citrus latifolia*) only, as this species has been shown to be resistant to the fungus (Kotzé, 1981; 1996).

Countries that import South African citrus fruit place strict rules on accepting such fruit from areas where CBS is known to occur, although, in some countries, fruit imports from countries where this phytosanitary disease is known to occur are not entirely forbidden (Carstens *et al.*, 2012). South Africa cannot export citrus fruit to the USA, if these fruit come from trees grown in areas that have not been certified as 'free from disease' during official inspections. Japan and India only allow imports of citrus shipments that are free from any visible signs of CBS. Iran and countries that form part of the European Union (EU) only allow South Africa to export fruit from the Western Cape, the Free State and the Northern Cape, production areas in South Africa where so far no phytosanitary citrus diseases have been detected during official inspections (Carstens *et al.*, 2012). Disease symptoms found by EU inspectors on imported citrus fruit may result in the rejection of an entire shipment, leading to large

financial losses of exporting countries (Magarey and Borchert, 2003; Carstens *et al.*, 2012). In April 2016, exports of organic lemon to the EU were suspended after CBS symptoms were detected on lemon fruit, costing the South African growers R50 million (Magwaza, 2016).

In South Africa, CBS was first discovered on bud wood imported from Australia and the disease has since developed to epidemic proportions (Carstens *et al*., 2012). Yield and commercial value of citrus fruit may be reduced by CBS, due to premature fruit drop as well as unsightly blemishes that develop on the rind of the fruit (Spósito, 2008; Baldassari *et al.*, 2008). Most commonly, CBS occurs as a pre-harvest disease, although fruit, asymptomatic at harvest, may develop symptoms postharvest, during transport or storage (Kotzé, 1996). Several symptoms occur on the fruit, twigs and leaves of citrus trees. Symptoms of CBS may vary with climatic condition, the type of citrus, as well as with fruit development in relation to disease development. As this disease is not considered harmful to humans, symptomatic fruit may be used for the production of juice and other secondary products (Kotzé, 1981; 2000).

Effective CBS control relies mainly on protecting young fruit from infection, as during the early fruit growth period fruit most susceptible to the fungus (McOnie and Smith, 1964). In South Africa, the critical infection period is, in summer rainfall areas, from October to January during the rainy season. Infection begins when ascospores are released by the fungus. Pre-harvest and postharvest control of CBS has been mainly through the use of copper and strobilurin fungicides. Hot water and wax treatments are also used to prevent the development of CBS postharvest ..; these are, however, not specific to *P. citricarpa* but have been shown to decrease the viability of pathogens in general and, therefore, may reduce the viability of *P. citricarpa* (Wild, 1981; Korf *et al.*, 2001). Postharvest disease management strategies are generally less effective than pre-harvest control of CBS (Korf *et al.*, 2001; Carstens *et al.*, 2012). Using certified disease-free nursery stock, effective orchard sanitation and removing leaf litter from the orchard floor can also help reduce CBS inoculum, thereby reducing the risk of pathogen infection (Kotzé, 1996; Agostini *et al.*, 2006).

1.2 REVIEW OF CITRUS BLACK SPOT

Citrus Black Spot is thought to have originated in South East Asia but was first reported in New South Wales, Australia, in 1895 (Smith *et al.*, 1997). Shortly after Benson discovered CBS; McAlpine (1899) described the asexual stage of the fungus found on symptomatic fruit and named it Phoma *citricarpa* McAlpine. The name was later changed to

Phyllosticta citricarpa (McAlpine). Sutton and Watterson (1966) reclassified the asexual stage, and named it *Phyllosticta citricarpa* (McAlpine) Petrak. The sexual stage of the fungus, *Guignardia citricarpa,* was described in 1948 by Kiely as present on citrus leaf litter. The accepted scientific names today are *Guignardia citricarpa* for the sexual stage and *Phyllosticta citricarpa* for the asexual stage of the causal fungus (Van der Aa, 1973; Baayen *et al.*, 2002; Van der Aa and Vanev, 2002; ISPM, 2014).

In any citrus orchard where CBS can be found, another *Guignardia* species, *G. mangiferae* (Anamorph: *Phyllosticta citrisiana*) can also be isolated from branches, leaves and fruit of asymptomatic plants (McOnie, 1964). This fungus, *G. mangiferae*, can also be associated with citrus fruit and is morphologically similar to *G. citricarpa*. For this reason, *G. mangiferae* has often been misclassified as an avirulent form of CBS (Baayen *et al.*, 2002; Glienke *et al*., 2011). There are, however, several ways in which these two fungi can be distinguished; firstly, through the use of amplified fragment length polymorphisms (AFLP) that investigates genetic diversity and differences in fungal populations using polymerase chain reaction (PCR) to amplify DNA. This ALFP technique produces polymorphic fragments and has been extensively used to differentiate between isolates of *G. mangiferae* and *G. citricarpa* that have previously been misclassified as *G. citricarpa*. Spores of *G. mangiferae* produce thick mucoid sheaths in culture, while those of *G. citricarpa* produce mucoid sheaths that are thin or even absent (Baayen *et al.*, 2002). On oatmeal agar, *G. mangiferae* grows relatively faster (40 mm in 7 days) than *G. citricarpa* (25 mm in 7 days) (Meyer *et al.*, 2001). *G. mangiferae* has been reported to be present in many citrus (Balakumaran *et al*., 2015) and non-citrus species (Baayen *et al*., 2002). The fungus has a more widespread geographic distribution than *G. citricarpa*, occurring as an endophyte by entering plant cells and establishing symbiotic relationships with plants (Sutton and Waterston, 1966; Glienke *et al*., 2011).

Both, the sexual and asexual stage are able to cause CBS on citrus fruit. Ascospores produced by *G. citricarpa* are dispersed by wind and are the primary source of infection. Pycnidiospores produced during the asexual, *P. citricarpa*, stage of the fungus, are disseminated from plant to plant via rain splash. Despite the potential for spore movement over large areas, long-distance dissemination occurs mainly through the trading of infected seed and vegetative propagules and not via rain or wind. Pycnidiospores may be found inside the lesions in the rind of symptomatic fruit. These spores do not only initiate infection but

may contribute to secondary infection during the growing season; a secondary infection may occur when pycnidia, which develop as a result of the primary latent infection in fruit, give rise to macroconidia, these may mature and develop black spot lesions, which may initiate a secondary infection (Kiely, 1948; Kiely, 1949; Kotzé, 1963; McOnie, 1964).

1.2.1 DISTRIBUTION OF CBS

Citrus black spot has been reported to occur in many citrus-growing countries of the world, including Australia, Brazil, China, Ghana, Indonesia, Kenya, Mozambique, Nigeria, Philippines, South Africa, Swaziland, Taiwan, USA, Uruguay, West Indies, Zambia and Zimbabwe (Fig. 1.1). The disease has not been found in Mediterranean and European countries, nor in Chile and Japan (Baayen *et al.*, 2002; Paul *et al.*, 2005; Schubert *et al.*, 2012). Citrus black spot was previously thought to be present in New Zealand, Egypt, Uruguay, India and Singapore, but it was later discovered that *G. mangiferae*, and not *G. citricarpa*, was present (Everett and Rees-George, 2006; EPPO, 2014). The extension of the distribution range of CBS is restricted by climatic factors, but the disease is generally present in summer rainfall areas suitable for citrus production (Paul *et al.*, 2005; Martínez-Minaya *et al.*, 2015).

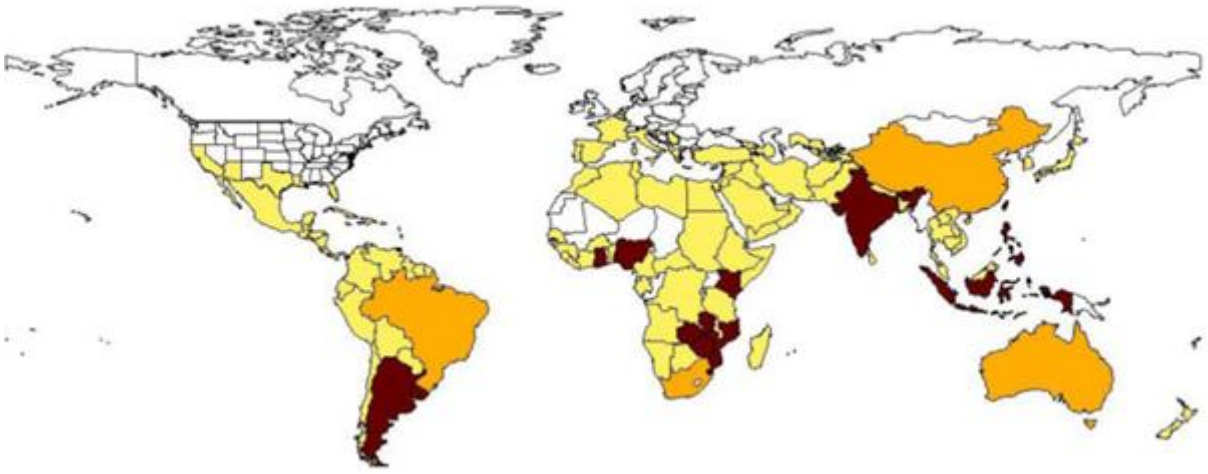

Fig. 1.1: World distribution of CBS (Paul, 2006).

KEY:

Countries/States where CBS is present
Countries/States where CBS is partially present
Areas where citrus is cultivated and CBS is absent or has never been recorded
Areas where citrus is not cultivated

In South Africa, CBS is found in summer rainfall areas characterized by warm and wet climates (Carstens *et al.*, 2012; Martínez-Minaya *et al.*, 2015*)*. The disease has been found in KwaZulu-Natal, Mpumalanga, Limpopo, North-West and Eastern Cape (Fig. 1.2).

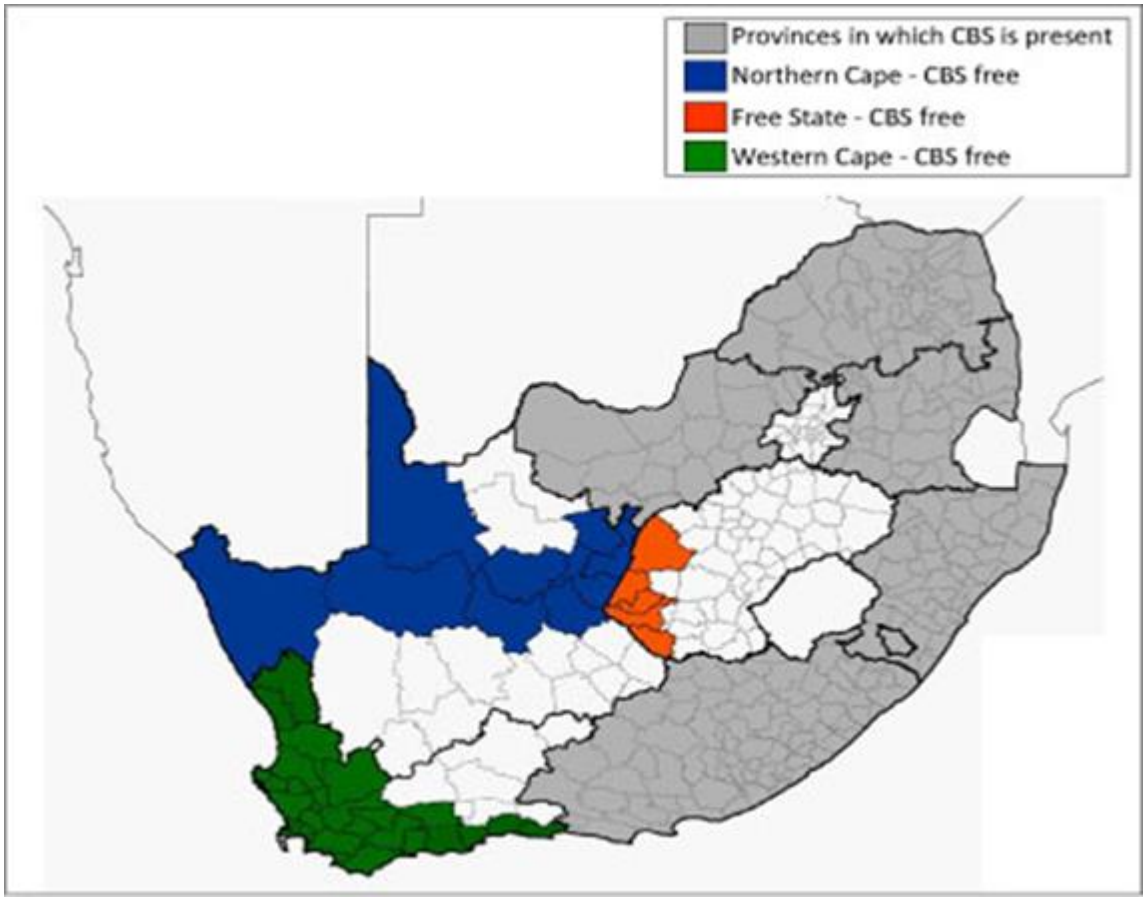

Fig. 1.2: Distribution of CBS in South Africa (Carstens *et al.*, 2012).

1.2.2 HOST RANGE

All commercially sold citrus species and varieties are susceptible to *P. citricarpa* infection, with the exception of Tahiti lime (*C. latifolia* Tan.), sour orange (*C. aurantium* L.) and sour orange hybrids. The pathogen readily infects lemon (*C. limon* (L.) Burm. f.), different varieties of sweet orange (*C. sinensis* Osb.), grapefruit (*C. paradisi*) and lime (*C. aurantifolia*) and can cause significant losses, particularly under favourable environmental conditions. The disease has also been reported on citron (*C. medica* L.), pummelo (*C. grandis* L. Osb.) and mandarin (*C. reticulata* Bla.) (Kiely, 1948; Brodrick, 1969; Kotzé, 1981; Baldassari *et al.*, 2008). *Guignardia* species have been isolated from leaves and fruit of Seville sour orange (*C. aurantium* L.) and Tahiti acid lime (*C. latifolia* Tan.) trees but the fruit remain asymptomatic, even in orchards characterized by high inoculum pressure and favourable

fungal growth conditions. Tahiti limes and Seville sour oranges are regarded as alternate hosts of CBS. Rough lemon (*C. jambhiri* Lish.) has been said to be tolerant to CBS. This tolerance is still unexplained (Kiely, 1948; Wager, 1952; McOnie, 1964; Kiely, 1970).

1.2.3 EPIDEMIOLOGY

Ascospores of *G. citricarpa* and pycnidiospores of *P. citricarpa* are responsible for infection and development of CBS symptoms on citrus fruit (Kotzé, 1981). Ascospores are thought to be a more important source of infection than pycnidiospores and generally form when temperatures range between 21 and 28 °C and relative humidity is above 60% (Sutton and Watterson, 1966; Lee and Huang, 1973). Ascospores are produced in pseudothecia fruiting bodies which are dark brown in *G. citricarpa* and develop on the orchard floor, 40-180 days after leaf drop of infected leaves and twigs (Fig. 1.3). These fruiting bodies are produced on both, the abaxial and adaxial surfaces of decomposing leaves and have yet to be reported on infected fruit, in fruit lesions or even on leaves that are still attached to the tree (Kiely, 1948; Van der Aa, 1973). Pseudothecia maturation is mainly determined by weather conditions, as these structures require alternative periods of wetting and sun-drying of leaves coupled with fluctuations in temperature to develop fully (Kiely, 1948; McOnie, 1967; Kotzé, 1981). Ascospores produced are hyaline to grey in colour. They are commonly aseptate but may have one septum near the end of the spore. Aseptate fungi have no pores between cells and form long cells that contain many nuclei and other cellular organelles that share the same cytoplasm. Septate fungi, however, have pores between cells that allow cytoplasm and nutrients to flow through the mycelium; cellular organelles, including the nucleus, can also fit through these pores and flow between cells of different mycelia (Kiely, 1948; Huang and Chang, 1972).

Pycnidiospores are produced and borne in pycnidia, which can be found on infected citrus fruit, tree twigs and leaves. They have never been reported on symptomless fruit but may be present, together with ascospores, in leaf litter on the orchard floor (McOnie, 1964). Pycnidia are brown to black on fruit and sub-hyaline to brownish on leaves (Kiely, 1948; Kotzé, 1996; Kotzé, 2000). Pycnidiospores develop on both sides of the leaf surface but are mainly concentrated on the side of the leaf exposed to the sun. These spores are thought to be a source of secondary infection (Darnell-Smith, 1918; Kiely, 1948).

Dissemination of fungal inoculum occurs when *Guignardia* ascospores are ejected from pseudothecia as leaf litter decomposes in the presence of water (Fig. 1.3). For this reason, the onset of CBS in an orchard is closely related to irrigation and rain incidences. Ascospores can be carried by wind over distances. Pycnidiospores ooze from pycnidia as gelatinous masses and are dispersed by rain splashes into the tree canopy or when spores from infected fruit are washed to young fruit and leaves by rain or dew. They are transported over long distances by orchard machinery, man and infected nursery stock (Kiely, 1948; Kotzé, 1963; Kotzé, 1996; Dewdney *et al.*, 2013).

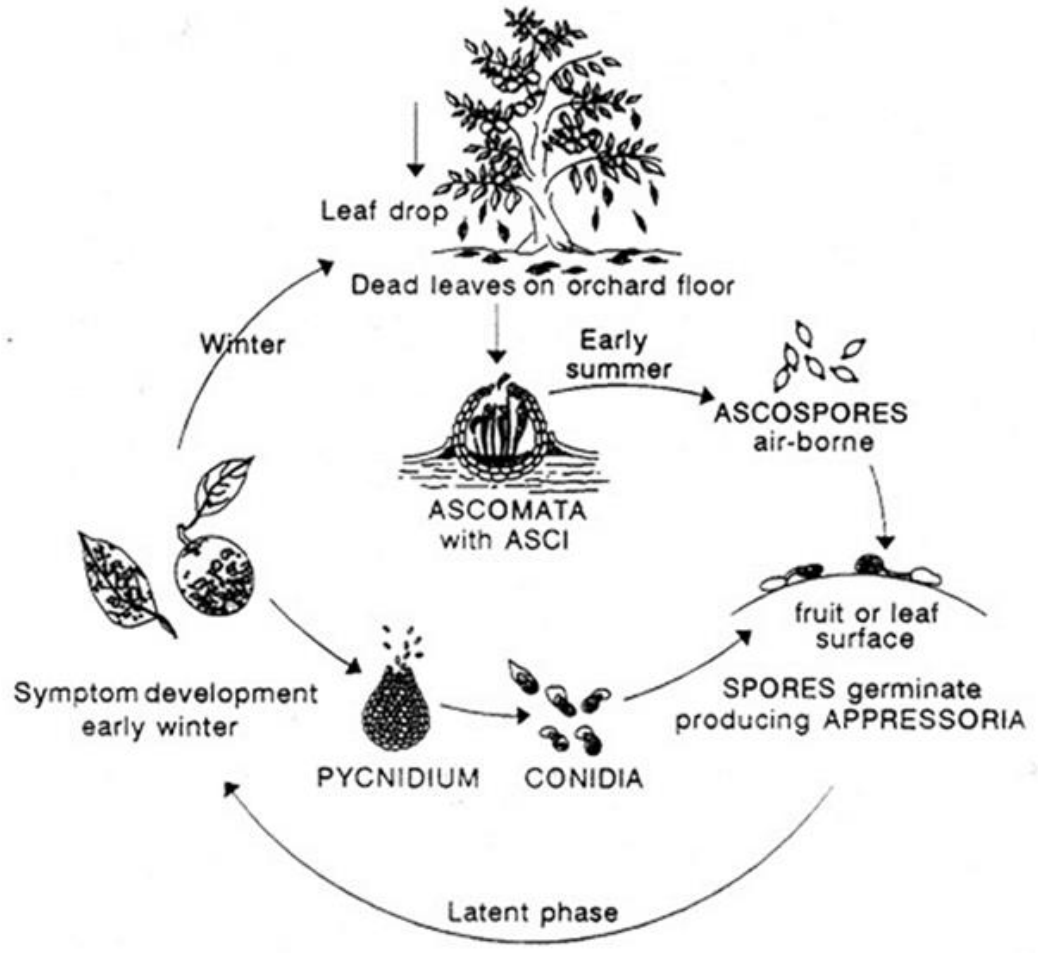

Fig. 1.3: Disease cycle of CBS.
(http://www.crec.ifas.ufl.edu/crec/websites/fungal/BlackSpotlifeCylces.html; Date accessed: 13/4/2015)

When ascospores or pycnidiospores land on susceptible plant tissues, they germinate and produce appressoria. These appressoria consist of hyphae and are used by the fungus to penetrate the host tissue (Timmer, 1999; Kotzé, 2000). The appressorium attaches to the

surface of the plant and an infection peg forms between appressorium and plant tissue. The peg allows the fungus to anchor itself into the plant tissues (Fig. 1.3). Then, under favourable temperature and relative humidity a peg penetrates the cuticle of the fruit and the epidermal wall of the albedo using mechanical pressure and species-specific enzymes (McOnie, 1967; Truter, 2010). After this, the fungus forms a resting body within the flavedo or below the leaf cuticles. Once this occurs, the latent period begins and only ends when the fruit is mature and conditions are favourable for fungal growth and symptom development. The latent period appears to be an essential part of CBS development and the reasons for its occurrence are not yet fully understood (Kiely, 1969; Baldassari *et al.*, 2008; Truter, 2010).

Optimal conditions for fungal growth and symptom development in the field include temperatures ranging from 18 to 30 °C for 15 hours with adequate relative humidity throughout the period of infection (Kotzé, 1981). The frequency of infection is determined by rainfall and inoculum availability; disease severity is affected mainly by climate (Whiteside, 1967). The onset of CBS is closely related to rain and moisture, temperatures between 18 and 30 °C and inoculum pressure (Kotzé, 1963; McOnie, 1964).

Only young fruit and young leaves are susceptible to CBS infection. Ascospores and pycnidiospores do not infect mature fruit, and any symptom development on mature fruit and older leaves is thought to be from previously dormant resting bodies. Fruit are susceptible to CBS up to five months after anthesis, while leaves are susceptible to up to nine months after the start of a new flush. Leaf infections may remain latent until leaf drop, although lesions may appear on mature, attached leaves (Whiteside, 1965; Kotzé, 1996).

1.2.4 SYMPTOMS

Citrus black spot symptoms may develop on fruit, leaves and stems of citrus trees. Fruit symptoms are more distinctive and common than leaf symptoms (Kiely, 1949). Leaf symptoms seldom occur and most commonly appear on lemon trees than on trees of any other citrus species or cultivar. Infected leaves develop necrotic spots, usually with a grey centre that is surrounded by a dark brown ring and yellow halo (Fig. 1.4). Symptoms occur on leaves three to ten months after infection (Wager, 1952; Kotzé, 1981). Leaf lesions may contain pycnidia. Leaf colonization occurs mainly when the leaf has dropped and the fungus produces fruiting bodies that harbour disease inoculum.

Fruit symptoms vary in appearance and develop at different phenological stages of tree and fruit maturity (Wager, 1952; Whiteside, 1965). Symptoms that appear on fruit can be classified into several categories, namely: speckled blotch lesions, hard spot lesions and cracked spot lesions - all developing pre-harvest - and freckle spot lesions and virulent spot lesions, which develop postharvest (Katzi, 1996; Kotzé, 2000). Due to the variable types of symptoms caused by CBS, symptoms can be easily mistaken for symptoms caused by other citrus-infecting pathogens, such as those caused by *Diaporthe citri* [(Faw.) Wolf], the causal agent of false melanoses on citrus (Bonants *et al.*, 2003). Hard spot, freckled spot and virulent spot lesions are widely recognized symptoms and may be seen on citrus fruit grown all over the world, while false melanose and cracked spot predominantly occur in South Africa and Brazil (McOnie, 1965; de Goes *et al.*, 2000).

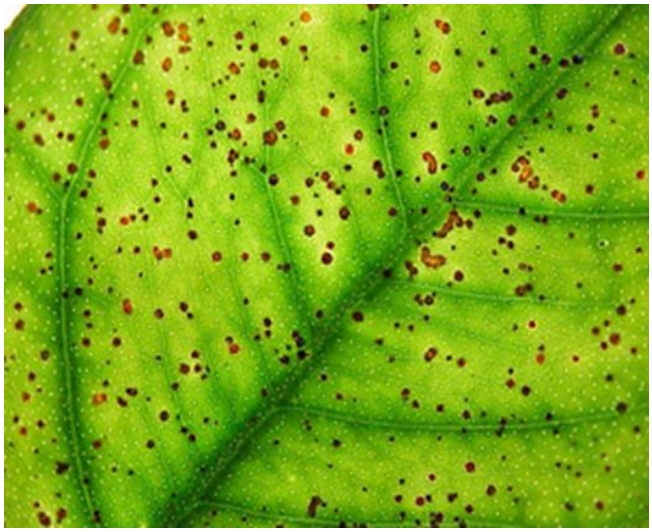

Fig. 1.4: Leaf lesions of *P. citricarpa* on citrus leaves (Gomez, 2013).

Speckled blotch lesions are characterized by small, separate lesions that do not contain pycnidia. The lesions may be depressed or slightly raised (Fig. 1.5). Speckled blotch lesions are initially red and turn dark brown as the fungus develops. Lesions are found mainly on unripe fruit (Kiely, 1960; Kotzé, 1981; 2000).

Fig. 1.5: Speckled blotch lesions on a Valencia orange (Gomez, 2013).

Hard spot lesions are the most distinctive and most common CBS symptom. These symptoms can be seen when fruit change colour from green to yellow or orange. Hard spots are round with dark-red to brown margins on yellow fruit and a yellow halo on green fruit (Fig. 1.6). The lesions are sunken and usually do not increase in size as disease progresses (Kiely, 1948). Hard spot lesions contain pycnidia and may develop in the centre of cracked spot lesions. Hard spot lesions may also develop from speckled blotch lesions as fruit mature and change colour (Korf, 1998; de Goes *et al.*, 2000).

Fig. 1.6: Hard spot lesions of *P. citricarpa* on Valencia fruit (Gomez, 2013).

Cracked spot lesions appear as large, slightly raised, dark brown patches which may occur on both, unripe and ripe fruit (Fig. 1.7). The patches are characterized by cracked surfaces and irregular margins (de Goes *et al.*, 2000).

Fig. 1.7: Cracked spot lesions of *P. citricarpa* on a Valencia orange (Gomez, 2013).

Freckle spot lesions may be seen pre-harvest on mature fruit and during postharvest storage. They are numerous lesions that are small, sunken and orange to red-brown in colour (Fig. 1.8). The size and severity of freckle spot lesions is dependent on temperature as lesions have been shown to enlarge and coalesce when temperatures increase (Kiely, 1948; Kotzé, 1981).

Fig. 1.8: Freckle spot lesions of *P. citricarpa* on a Valencia orange (Gomez, 2013).

Virulent spot symptoms may appear when freckle spot lesions coalesce or they become visible independent of any other CBS symptoms. Virulent spot lesions develop late in fruit development, principally when the fruit is fully mature and temperatures in the orchard begin to increase. Pycnidia may develop within these small, irregularly shaped lesions (Fig. 1.9). Lesions are surrounded by necrotic tissue and with severe infections; they may develop deep into the rind of the fruit and may cover the entire rind. Virulent spot symptoms contribute greatly to postharvest losses (Kiely, 1948; Calavan, 1960; Kotzé, 1981).

Fig. 1.9: Virulent spot of *P. citricarpa* on immature (left) and mature (right) Valencia fruit (Gomez, 2013).

Several factors contribute to symptom development and disease severity. Symptom expression is affected by increased temperatures and increased day-lengths that coincide with fruit maturity (Kotzé, 1981). Lack of water and other abiotic stresses occurring during fruit development may increase symptom expression. Mature citrus fruit express symptoms more rapidly than younger fruit; older trees tend to develop symptoms more intensely than younger trees (Kotzé, 1963; Whiteside, 1967; Kiely, 1969; Kotzé, 1981).

1.2.5 DISEASE MANAGEMENT

Control of CBS is achieved mainly through the use of agro-chemicals. Using fungicides together with orchard sanitation has proved to be the most effective method of reducing CBS

incidence in citrus orchards. Practices such as quarantine through exclusion and using CBS-free planting material have proved effective methods of preventing CBS establishment in new areas. Cultivars resistant to CBS have not yet been discovered and efforts to breed resistant cultivars have been unsuccessful (Calavan, 1960, cited by Truter, 2010). Biological control agents have also been tested as CBS antagonists. The bacterium *Bacillus subtilis* and fungal species of *Trichoderma* were found to partially prevent the development of new lesions on citrus fruit when tested on CBS in the field (Kupper, 2011).

1.2.5.1 QUARANTINE AND PHYTOSANITARY REGULATIONS

Phytosanitary regulations restrict the import of citrus fruit from countries where CBS is known to occur through preventative quarantine measures. Countries are not permitted to export citrus from regions where CBS has been reported. In South Africa, provinces where CBS is known to occur are not permitted to transport citrus fruit to CBS-free areas within the country. This method is used mainly as a preventative strategy and is effective in precluding CBS from entering and establishing in areas where it is not present (Carstens *et al.*, 2012; Mannya and Jaftha, 2013; Martínez-Minaya *et al*., 2015). Fruit may only be imported into South Africa, if sufficient evidence can be provided that effective management strategies were practiced during fruit production. Evidence given depends on the importing countries as regulations differ from country to country. If no disease symptoms are observed in official inspections of consignments at entry ports, the fruit is accepted (Bonants *et al.*, 2003; Baker *et al.*, 2008).

Since the occurrence of CBS depends largely on climatic conditions, introduction of diseased material into some areas will not necessarily result in a CBS disease epidemic. This observation was made when repeated introductions of suitably infectious material were made in inland citrus growing areas of Australia and the Western Cape Province of South Africa (Smith, 1962; Whiteside, 1967; Barkley, 2003; Mabiletsa, 2003).

1.2.5.2 SANITATION

Trees from certified CBS-free nurseries are used when establishing new orchards to exclude the fungus from occurring where it is not currently present (Whiteside, 1965) but in areas where CBS is present sanitation practices in the orchard are the most important cultural management practices that may be used to reduce CBS inoculum. These include the removal of leaf litter from the orchard floor as fallen leaves may harbour spores. Late-hanging fruit

can transfer pycnidia to new fruit flushes, especially in citrus varieties where new and old fruit flushes can overlap; removal of late-hanging fruit in such cultivars may prevent CBS infection of new fruit flushes (Calavan, 1960; Kiely, 1969; Kotzé, 1996; Dewdney *et al.*, 2013).

Reducing tree stress and maintaining tree vigour during fruit growth, using timely fertilization and irrigation regimes, may reduce CBS incidence as stressed trees and trees in poorly maintained orchards are more susceptible to CBS infection. Skirting and pruning the tree canopy increases airflow which helps reduce leaf wetness periods, disease incidence and pycnidiospore dissemination (Calavan, 1960; Kotzé, 1981; Dewdney *et al.*, 2013).

Before postharvest storage, water-wax emulsions may be applied to fruit as these have been shown to reduce the development of CBS during storage (Seberry *et al.*, 1967). Waxing of citrus fruit and hot water treatments not only specific to *P. citricarpa* may also be used to reduce symptom development (Korf *et al.*, 2001). Postharvest symptom development on citrus fruit is dependent on light and temperature as citrus fruit stored in the dark at low temperatures tend to have fewer symptoms than those that kept in warmer temperatures (Kiely, 1970; Korf, 1998; Korf *et al.*, 2001).

1.2.5.3 CHEMICAL CONTROL

Effective chemical control relies heavily on well-timed applications of protectant and systemic fungicides integrated with cultural practices. Properly timed fungicide applications protect trees from infection, may eradicate infections and prevent symptom development on fruit (Kellerman and Kotzé, 1977). Bordeaux mixture applications prevent CBS incidence but may cause copper toxicity (Kiely, 1948, Kotzé, 1964). Dithiocarbamates may also be used to prevent CBS incidence and are more effective than copper-based fungicides. Oils added to fungicides increase fungicide penetration into plant tissues. This method is often used to enhance fungicide efficiency (Kellerman and Kotzé, 1977). Benomyl fungicides were found to be both preventative and curative for CBS before the disease developed resistance in the 1980's. This occurred due to frequent use of these fungicides (Herbert and Grech, 1985; De Wet, 1987). Strobilurin fungicides replaced benomyl fungicides a few years later. Strobilurin fungicides are not only protective; they are also curative but may eradicate CBS (Gold and Leinhos, 1995; Schutte *et al.*, 2003). Rotations of strobilurin fungicides with copper-based fungicides or mancozeb are recommended for effective CBS control (Schutte *et al.*, 2003;

Miles *et al.*, 2004). Postharvest fungicide applications of Imazalil and thiabendazole have been shown to reduce spore viability but do not inhibit pathogen development (Seberry *et al.*, 1967; Korf *et al.*, 2001; Agostini *et al.*, 2006).

1.3 PHENOLIC COMPOUNDS AS A POSSIBLE POSTHARVEST CONTROL STRATEGY

1.3.1 BIOCHEMISTRY OF PHENOLIC COMPOUNDS

Phenolic compounds occur naturally in fruit and are known to be able to protect fruit from biotic and abiotic stresses. They are produced as the plant develops as well as in response to pathogen infection, wounding and plant exposure to ultra-violet radiation and extreme temperatures (Naczk and Shahidi, 2006; Ruiz-García and Gómez-Plaza, 2013). Quantitative differences in total phenolic content in plants depend largely on storage conditions, the type of cultivar grown, the ripening processes of the plant, stage of maturity and environmental conditions during cultivation (Abad-Garcia *et al.*, 2012).

Phenolic compounds can be simple, consisting of a single aromatic ring or large, complex polyphenolic compounds. They are characterized by a benzene ring with one or more hydroxyl groups (Fig. 1.10). They are aromatic secondary plant metabolites which are widely spread throughout the plant kingdom and have various structures and functions.

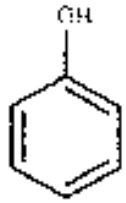

Fig. 1.10: Structure of a simple aromatic ring phenolic compound (Giada, 2013).

Phenolic compounds are synthesized from phenylalanine and tyrosine produced via the shikimic acid pathway (Robbins, 2003; Haminiuk *et al.*, 2012; Ruiz-García and Gómez-Plaza, 2013). Phenolic compounds can be classified into two groups, flavonoids and non-flavonoids that are conjugated to sugars through phenolic hydroxyl groups and to organic acids as conjugated esters (Benavente-Garcia *et al.*, 1997; Bocco *et al.*, 1998; Li *et al.*, 2006; Naczk and Shahidi, 2006; Hamaniuk *et al.*, 2012). In citrus, the phenolic compounds are made up of

phenolic acids and flavonoids. Flavonoids are represented by polymethyloxylated flavones and glycosylated flavanones (Bocco *et al.*, 1998). Flavonoids are characterized by a benzo-pyrone structure and act as antioxidants in plants. Four types of flavonoids occur in citrus, namely: flavanones, flavones, flavonols and anthocyanidins - which only occur in blood oranges (Ogah *et al.*, 2014). Polymethyloxylated flavones are phenolic compounds which are associated with oil glands that are found in the citrus flavedo (Tatum *et al.*, 1978). Nobiletin, sinensetin and tangeretin are flavones that do not occur in large quantities but exhibit the highest biological and antimicrobial activity of any phenolic compound in citrus (Benavente-Garcia *et al.*, 1997).

1.3.2 PHYSIOLOGICAL IMPORTANCE OF PHENOLICS IN DISEASE INFECTION

Phenolic compounds protect plants from biotic and abiotic stresses (Ruiz-García and Gómez-Plaza, 2013). When the plant recognizes an attack, defence mechanisms within the plant are induced; both, at the site of initial infection and at distant uninfected tissues (Matern and Kneusel, 1988). Phytoalexins, for instance, are phenolic compounds that are produced in response to pathogen attack and phytoanticipins are phenolic compounds that have been shown to inhibit pathogen infection (Chérif *et al.*, 2007; Ruiz-García and Gómez-Plaza, 2013). Plant pathogens also induce metabolic changes in the plants they infect. Plants respond in various ways to pathogen attack. Some plants synthesize large quantities of phenolic compounds at the site of infection resulting in a hypersensitive response (Matern and Kneusel, 1988). A hypersensitive response isolates the pathogen to the site of infection and prevents the pathogen from spreading to the rest of the fruit. Following the activation of hypersensitive responses, distant plant tissues may develop resistance to further infection through systemic acquired resistance (SAR). Some plants may produce pathogenesis-related proteins that eradicate the pathogen before it is able to spread deeper into the fruit while other plants reinforce their cell walls restricting the pathogen to only a few cells (Matern and Kneusel, 1988).

The concentration of phenolic compounds in fruit can be enhanced using elicitors. Elicitors may be natural or synthetic compounds that induce defence responses in plants. Plants respond in a similar manner to elicitors as they do to pathogens. Elicitors do not kill diseases; they merely trigger the plants defence mechanisms; some of which trigger the synthesis of phenolic compounds (Ruiz-García and Gómez-Plaza, 2013). Signalling pathways in the plant are triggered to synthesize specific compounds in response to pathogen attack. Increased

levels of phenolic compounds have been shown to protect fruit from chilling injury and some pathogens and may be useful in protecting citrus fruit from infection by *P. citricarpa* (Chérif *et al.*, 2007; Matern and Kneusel, 1988).

The rind of citrus fruit, made up of the waxy cuticle, the flavedo and the albedo, is an abundant source of flavonoids, phenolic acids and other phenolic compounds. The rind contains about 15% more phenolic compounds than the edible portions whilst the albedo contains three times more phenolic compounds than the flavedo. By enhancing phenolic compounds that are known to be biologically active in the citrus rind, effective protection against CBS incidence and symptom development of CBS could be achieved.

1.4 CONCLUSION

Citrus Black Spot has been categorised as a disease of economic importance in South Africa. The fungus largely affects citrus production and citrus trade on international markets. Some countries have opted to only import fruit from certified CBS-free areas of South Africa, while others will only import shipments of asymptomatic fruit from South Africa. The use of fungicides pre-harvest in CBS management is effective but costly and not environmentally friendly. Postharvest control measures do not eradicate or hinder disease development and because of the latent period of the disease, symptoms may only appear on fruit postharvest, therefore, more effective methods of postharvest control need to be investigated. Disease resistance in plants is dependent on pre-existing barriers and inducible defence mechanisms in plants.

Induction of resistance to pathogens using elicitors is a promising approach for controlling postharvest diseases, especially since postharvest control of many pathogens, including CBS, is limited to fungicidal dips and hot water treatments that do not completely hinder pathogen growth. Citrus Black Spot causes blemishes on fruit that render the fruit unmarketable and, in severely affected orchards, may reduce yields. The use of elicitors to enhance phenolic compounds and induce the plants natural defence mechanisms could lead to a more effective method of CBS control both, pre- and postharvest.

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CHAPTER 2

COMPARISON OF RADIAL GROWTH RATES OF *Phyllosticta citricarpa* ON DIFFERENT GROWING MEDIA

2.1 Abstract

Citrus black spot (CBS) is a phytosanitary disease caused by the ascomycete fungus *Phyllosticta citricarpa* [(McAlpine) Aa] (Teleomorph: *Guignardia citricarpa*]. The disease causes unsightly lesions on the rind of citrus fruit. The specific infection and epidemiological mechanisms used by this fungus are well-documented, but little information has been published regarding the *in vitro* growth of *P. citricarpa* isolates on different culture media. This experiment evaluated and compared radial growth of *P. citricarpa* at 25 °C on three solid growing media, namely: malt extract agar (MEA), potato dextrose agar (PDA) and oatmeal agar (OA). Media were prepared and 5 mm^2 fragments of *P. citricarpa*, previously isolated on OA, were transferred onto the media in Petri dishes and incubated at 25 °C (±1ºC) in the dark. Weekly radial growth measurements were taken from the edge of the initial inoculum fragment to the most extreme area of fungal development. After one week, the mean growth of *P. citricarpa* on all three media was significantly different; fungal growth was fastest on OA, followed by PDA and slowest on MEA. After the second and third week, fungal growth on PDA and MEA was not significantly different. Fungal growth on OA was significantly faster than on PDA and MEA. After four weeks, growth of *P. citricarpa* on PDA and on OA was not significantly different from each other, but fungal growth on MEA was significantly less. Spores on OA and PDA were counted after four weeks. Significant differences were found between the three media each week with OA consistently more growth promoting, while MEA proved each week to be the least suitable growing medium. Overall, OA was the most suitable medium for *P. citricarpa*; because of the visual transparency of OA, radial measurements were easier and more precise. Oatmeal agar allowed efficient differentiation between isolates on a Petri dish even when mixed with other fungal isolates because of the characteristic yellow halo surrounding colonies of *P. citricarpa*.

2.2 Introduction

The epidemiology of CBS is influenced by the availability of inoculum, the occurrence of environmental conditions favourable for infection (*i.e.*, warm, wet and humid conditions), the

growth cycle of the citrus tree, and the age of the fruit and leaves in relation to their susceptibility to infection (Kotzé, 1981). CBS is known only to affect the flavedo of the rind and is not known to develop deep into the albedo. Several types of lesions are associated with CBS, namely: hard spot, freckle spot, cracked spot, virulent spot and speckled blotch. One or more of these symptoms may occur on fruit, depending on climatic conditions, the type of citrus, and fruit development in relation to disease development (Katzi, 1996; Kotzé, 2000). Disease develops from ascospores produced by *Guignardia citricarpa*, the sexual stage and conidia produced by *P. citricarpa*, the asexual stage, of the fungus. Ascospores are produced in pseudothecia in decomposing leaves on the orchard floor (McOnie 1965; Kotzé, 1981). Conidia are produced and borne in pycnidia which are found on fruit, twigs and lesions on the fruit (Kotzé, 1963). Conidia may also be found in hard spot and freckle spot lesions as well as on dead branches and leaf litter (Huang and Chang, 1972).

Several growing media are used for isolating and culturing *P. citricarpa*, nonetheless, cultures of *P. citricarpa* grow slowly on culturing media. This poses a problem when isolating fungus from infected tissues as *P. citricarpa* is easily overgrown by other fast-growing fungi that may be found on citrus. A selective growing medium specific to *P. citricarpa* has yet to be established (McOnie, 1964; Baldassari *et al*., 2008; Truter, 2010).

Effective isolation and growth of *P. citricarpa* coupled with understanding the effects of phenolic compounds on growth and development of *P. citricarpa* are both important and relevant, as this understanding will contribute to the development of techniques to manage and control CBS. The aim of this study was to evaluate the ability of the fungus to colonize three different growing media (MEA, PDA and OA) and to determine radial growth development of *P. citricarpa* at 25 °C on these media. Results from this study should provide insights as to which medium will be most efficient when carrying out *in vitro* experiments on *P. citricarpa*.

2.3 Materials and Methods

2.3.1 Isolation of *P. citricarpa* on OA, PDA and MEA

Oatmeal agar (OA) was prepared according Gams *et al*. (1998); briefly, 30 g oatmeal flakes were placed in a volumetric flask and 250 mL distilled water was added. The mixture was brought to a boil, then left to simmer for approximately 2 h after which the mixture was filtered and squeezed through cheesecloth. The flakes were discarded and to the watery

extract, 7 g agar was added. Thereafter, the medium was sterilized for 15 minutes at 121°C. Commercially available PDA and MEA (Biolab, Johannesburg, South Africa), was prepared according to manufacturer's instructions. All three media were amended with 50 μg mL^{-1} penicillin and 50 μg mL^{-1} streptomycin, to prevent bacterial contamination. The media were allowed to cool to 50 °C before pouring into 90 mm Petri dishes. These Petri dishes were kept in a laminar flow overnight.

Navel oranges showing characteristic hard spot lesions of CBS were obtained from Ukulinga Research Farm in Pietermaritzburg, KwaZulu-Natal. The fruit were surface-sterilized by immersion in 70% ethanol for 1 minute, followed by immersion in a sodium hypochlorite: sterile water (1:3 v/v) solution for 3 minutes and finally fruit were rinsed in double-sterilized water for 30 s before blot drying with sterile filter paper (Baldassari *et al*., 2008). Lesion-containing fragments from the flavedo of each fruit were removed using a scalpel and transferred onto Petri dishes containing OA, PDA or MEA. In total, 15 plates were used with five plates per medium. Plates were incubated at 25 °C for two weeks and observed regularly for the appearance of characteristic colonies of *P. citricarpa*.

2.3.2 Sub-culturing of *P. citricarpa* on OA, PDA and MEA

Phyllosticta citricarpa cultures, isolated by Elma Carstens, were obtained from the Citrus Research Institute (CRI) in Stellenbosch. When received, cultures were seven days old and had been grown on OA. A wet mount of the cultures was carried out by placing a drop of water and a sample from the *P. citricarpa* culture on a microscope slide, and examining under a light microscope for mycelia and spores.

Plates were incubated at 25 °C (±1 ºC) in the dark. Two perpendicular straight lines were drawn underneath each Petri dish. The crossing point of the lines coincided with the centre of the 5 mm^2 initial fungus containing disc. Weekly, radial growth measurements were taken from the edge of the initial inoculum fragment to the most extreme area of fungal development. Radial growth development was recorded weekly by measuring the distance from the edge of the initial inoculum to the furthest area of mycelial development, with a ruler, following the four segments formed by the two perpendicular lines. The final measurements were taken when fungal growth reached the Petri dish wall in any dish. The daily fungal growth rate was calculated and expressed as mm $week^{-1}$.

2.3.3 Data analysis

Radial growth rates were subjected to analysis of variance (ANOVA) using Statistical Analysis System (SAS) software, Version 9.3 (SAS Institute Inc, 2011). Treatment means were separated using Duncan multiple range test at 5% probability level.

2.4 Results

After 7 days of incubation, only one out of the five OA plates from which *P. citricarpa* was isolated, contained a colony surrounded by a yellow halo. None of the plates containing MEA or PDA showed any of the known identifying features of *P. citricarpa*. On 14 out of 15 plates, mixed infections were visible, but no plate had the characteristic yellow halo of a *P. citricarpa* colony (Fig. 2.1). As a result, *P. citricarpa* was sub-cultured from existing cultures as described under section 2.3.2 Sub-culturing of *P. citricarpa* on OA, PDA and MEA.

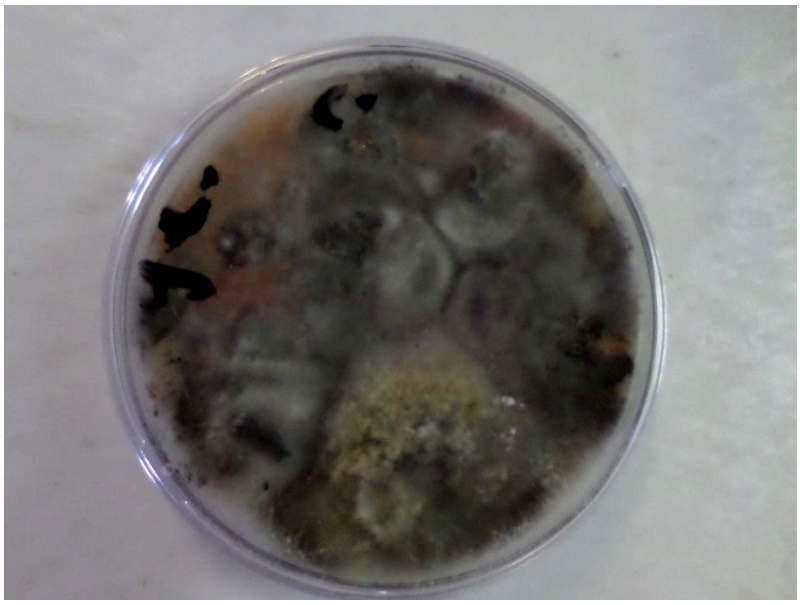

Fig. 2.1: Petri dish containing a number of pathogens from navel orange flavedo tissue symptomatic of *P. citricarpa*

After one week, the mean growth of *P. citricarpa* differed significantly ($P \leq 0.05$) between the different media after one week. The fungus had, initially, the highest growth rate on OA, followed by PDA and lastly, MEA. After the second and third week, growth of *P. citricarpa* on PDA and MEA was not significantly different (Table 2.1). During the first three weeks, fungal growth had the highest rate on OA, significantly different from that of growth on PDA

and MEA. At week 4, the fungus had grown similarly on PDA and on OA, while fungal growth on MEA was still significantly slower. Colonies on OA were dark mycelial masses, surrounded by a yellow halo; on PDA and MEA dark, raised masses of mycelia were observed with the yellow halo only slightly visible.

Table 2.1. Mean radial growth over the four weeks observation period of *P. citricarpa* on OA, MEA and PDA with levels of significance, P-values, F-values and % Coefficients of Variation

	Time in Weeks			
	Week 1	Week 2	Week 3	Week 4
Media	Colony diameter (mm/week)			
Potato dextrose agar (PDA)	20.1b	37.1b	41.9b	73.4a
Malt extract agar (MEA)	14.9c	32.4b	44.7b	57.8b
Oatmeal agar (OA)	24.9a	48.9a	68.3a	79.4a
F-value	16.47	37.85	45.42	35.65
P-value	0.0004	0.0001	0.0001	0.0001
CV%	13.8	9.4	9.3	6.5

[1]Within each column, values followed by the same letter indicate no significant difference at P = 0.05, according to Duncan Multiple range test (DMRT)

2.5 Discussion

Oatmeal Agar seemed to best supply *P. citricarpa* with the nutrients and phytochemicals necessary for growth. From week 3 to week 4, *P. citricarpa* growth on PDA increased significantly (Table 1), opening the possibility that *P. citricarpa* grows exponentially, once a certain stage of development is reached; by week 4 the fungus had covered a similar area of the plate, whether grown on PDA or OA. This suggests that for efficient culturing of *P. citricarpa*, either PDA or OA can be used. Information on how long the fungus can grow *in vitro* before nutrients are used up and growth slows down from exponential to stationary (Werner-Washburne *et al.,* 1993) could be useful in determining the efficiency of each of the media over a long period of time. According to the results presented above, the weekly growth rate of *P. citricarpa* is highest on OA and lowest on MEA. According to the standard diagnostic protocols set by the International Standards for Phytosanitary Measures (ISPM), PDA is the recommended medium when isolating *P. citricarpa* from symptomatic fruit (ISPM, 2014); OA can be used as an alternative to PDA as the fungus has the fastest radial growth rate on OA. The characteristic yellow halo was observed on OA and allowed easy identification of *P. citricarpa*, and was not clearly visible on PDA. Cherry decoction agar has

been suggested as another possible medium to culture *P. citricarpa*, although it is more expensive as it is made using fresh cherries and is not commercially available (ISPM, 2014).

One problematic area when isolating *P. citricarpa* from symptomatic fruit lies in the accumulation of contaminants in the Petri dish. Fruit material is surface sterilized before being transferred onto the plate, but this does not eliminate all pathogens from the fruit; consequently; a plate consisting of several non-pathogenic and citrus-infecting fungi is produced (Fig. 2.1). Faster growing fungi, such as green mould (*Penicillium digitatum*) and blue mould (*Penicillium italicum*), grow and colonize the plate leaving only little space and possibly only low concentrations of nutrients and carbohydrates for *P. citricarpa* to develop (Kotzé, 1981). This problem is closely related with the slow growth rate (24.9 mm week^{-1}) of *P. citricarpa in vitro*, resulting in less than 10% of plates used for isolation containing clearly visible *P. citricarpa* (Baldassari *et al*., 2008).

Oatmeal Agar was the most efficient medium for growing *P. citricarpa* and because of its simple composition and visual transparency; radial measurements were easier and more precise using this medium. The yellow halo, characteristic for *P. citricarpa*, is clearly visible on OA, making cultures easily identifiable (Baayen *et al*., 2002). When isolating *P. citricarpa* from citrus, a number of other bacterial and fungal species, including other *Phyllosticta* species, may also be isolated, these include *P. citriasiana* (Wulandari *et al*., 2009), *P. capitalensis* (Baayen *et al*., 2002) and *P. citribrazilliensis* (Glienke and Crous, 2011). These species are closely related to *P. citricarpa* and may be difficult to distinguish on a Petri dish containing different fungi and isolates; however, *P. citricarpa* can be clearly told apart from these fungi by the production of the yellow halo that is clearly visible on OA (Baldassari *et al*., 2008).

2.6 Conclusion

Radial growth measurements have been shown to be a good means to determine fungal growth, even though only horizontal growth is measured and vertical growth or the number of spores produced by the fungus on the Petri dish is not taken into account (Loeck *et al*., 2004). Significant differences were found between the three media each week; OA proved consistently more efficient in providing compounds or conditions necessary for fungal development, while MEA proved each week to be the least efficient of the evaluated three media. The measurements used in this study are estimative and are based on visual

observation; therefore, future repetitions of measurements should be carried out by several researchers. Future research building on this experiment should include measurements of the number of spores produced each week by the fungus on each medium, combined with a determination of the number of germinated spores. Furthermore, methods to determine the vertical growth of fungi on growing media also need to be evaluated before the potential inhibitory effect of specific phenolics on growth of *P. citricarpa* can be determined.

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CHAPTER 3

COLONIZATION OF CITRUS TISSUES BY *Phyllosticta citricarpa*, THE CAUSAL AGENT OF CITRUS BLACK SPOT

3.1 Abstract

Citrus black spot (CBS) is an economically important disease caused by the ascomycete fungus *Phyllosticta citricarpa* [(McAlpine) Aa]. Fruit infected with *P. citricarpa* develop one or more of five typical lesions, namely: virulent spot, freckle spot, speckled blotch, hard spot and cracked spot on the rind of citrus; however, rind biochemical conditions under which CBS develops or what hinders development are not well understood. This study was conducted to determine whether the inability of *P. citricarpa* to grow and develop CBS symptoms could be attributed to differences in the concentration of total phenolic compounds in the flavedo and/or albedo tissue of citrus fruit. Fragments of flavedo and albedo tissues from navel oranges were sterilized and exposed to *P. citricarpa* on Petri dishes containing oatmeal agar. Total phenolic compounds were extracted from the same fruit and the concentration of these compounds was quantified. The results showed that the albedo of navel oranges can, *in vitro*, be colonized with the mycelia and spores of *P. citricarpa* under optimal growing conditions. The results also suggest that, under optimal growing conditions, the concentration of total phenolic compounds in navel flavedo and/or albedo tissues does not have an effect on the development of *P. citricarpa*.

3.2 Introduction

South Africa exports about 70% of its fresh citrus produce to several countries across the globe. Citrus exports generate approximately R8 billion a year in GDP (Gross Domestic Product) (Ginindza, 2015). Shipments of citrus fruit containing diseases, amongst them, citrus black spot (CBS) have been intercepted by countries importing South Africa citrus fruit, on a number of occasions (ISPM, 2014). Those shipments have been rejected, leading to financial losses to the South African citrus industry (Magarey and Borchert, 2003). South Africa has been banned from exporting citrus to countries in the EU on a number of occasions over the past few years because of interceptions of fruit containing CBS symptoms. The country is only allowed five interceptions of CBS symptoms on fruit per year after which a ban is placed, and South Africa may not export anymore citrus until the ban is lifted (Sherry, 2013).

As a results, many citrus importing countries do not accept symptomatic fruit or fruit from areas in South Africa where the disease has been known to occur (Carstens *et al.*, 2012).

Fruit infected with the ascomycete fungus *Phyllosticta citricarpa* [(McAlpine) Aa] (Teleomorph: *Guignardia citricarpa* Kiely) develop one or more of five types of lesions, namely: virulent spot, freckle spot, speckled blotch, hard spot and/or cracked spot on the rind; these symptoms render the fruit unmarketable, both locally and abroad (Katzi, 1996; Kotzé, 2000; Carstens *et al.*, 2012). Lesions that develop on symptomatic fruit are unsightly and reduce the aesthetic appeal of the fruit (Kotze, 1981).

Successful infection of citrus by *P. citricarpa* is determined by the availability of inoculum, favourable environmental conditions, health of citrus trees, as well as the age of the fruit (Kotzé, 1981, 2000). The fungus *P. citricarpa* is known to only affect the rind of fruit; which consists of the waxy cuticle, the flavedo and the albedo (Kotzé, 1981, Truter, 2010). The fungus causes lesions only on the flavedo and is not known to affect the albedo or the endocarp of citrus fruit pre-harvest (Katzi, 1996; Kotzé, 1981, 2000). Virulent spot lesions may penetrate the surface of the albedo but do not develop deep into the albedo or grow into the endocarp of the fruit. This process has not yet been described but has been shown to occur only during postharvest, under conditions of heavy infection and high inoculum pressure. Such a scenario is, however, very rare and has therefore been considered the only time the fungus can grow into the albedo (Kiely, 1948; McOnie, 1964; Kotzé, 1981). The reason for this growth into the albedo or why the fungus cannot develop in the albedo pre-harvest is not yet known.

Citrus fruit contain phenolic compounds that may restrict fungal growth on the rind (Cherif *et al*., 2007; Arcas *et al*., 2000, Yao *et al.*, 2012). These compounds occur in varying concentrations in the different rind tissues and may be synthesized by the plant in response to pathogen attack. Phenolic compounds consist of a group of simple and complex chemical compounds produced by plants in response to abiotic or biotic stresses (Ruiz-García and Gómez-Plaza, 2013). A significant increase in the level of phenolic compounds in plants has been shown to hinder fungal growth and protect against environmental stress (Lattanzio *et al.*, 2006; Oliveira *et al.*, 2015). The exocarp of citrus fruit contains about 15% more total phenolic compounds than the inner edible portions made up of the juice sacs (Naczk and Shahidi, 2006). The phenolic compounds in citrus rinds can be divided into two groups, namely, phenolic acids and flavonoids. The latter can be further divided into two classes,

namely, the polymethoxylated flavones (PMFs) and the glycosylated flavanones (Bocco *et al.*, 1998; Lattanzio *et al.*, 2006). These PMFs are predominantly found in the flavedo, while glycosylated flavanones are dominant in the albedo. Despite occurring in much lower concentration than glycosylated flavanones, PMFs are more biologically active in protecting plants from biotic and abiotic stresses. Phenolic compounds occur in different concentrations depending on cultivar and the phenological stages of citrus fruit (Benavente-Garcia *et al.*, 1997; Bocco *et al.*, 1998). Understanding the conditions under which *P. citricarpa* is unable to grow into the albedo of citrus fruit may enhance application of strategies to control the development of the pathogen on the citrus rind pre-harvest. Since phenolic compounds have been shown to affect disease development in many plants (Cherif *et al.*, 2007; Yao *et al.*, 2012), they may also help in developing more effective methods of controlling CBS.

This study was conducted to determine whether the inability of *P. citricarpa* to penetrate into the albedo, while being able to grow on the flavedo, is attributed to differences in the concentration of total phenolic compounds in the flavedo and albedo tissues of citrus fruit. The colonization of the flavedo and albedo tissues of citrus fruit using an artificial method of inoculation under optimal growth conditions was also evaluated. It is hypothesized that differences in the amount of total phenolic compounds in the different fruit tissues result in the inability of the fungus to grow in the albedo.

3.3 Materials and Methods

3.3.1 Extraction of total phenolic compounds from navel oranges

Navel oranges were harvested from Bounty Farm, Winterskloof (29°28'S; 30°161'E) in KwaZulu-Natal, South Africa. Free and bound phenolic compounds were extracted from the albedo and flavedo tissues of six navel oranges; with three replications per orange; using a method described by Boehm *et al.* (2006). This method simultaneously results in the extraction of both free and bound phenolic compounds. Briefly, 500 mg flavedo and albedo tissues were weighed out and placed into centrifuge tubes. The samples were mixed with 5 mL hydrochloric acid (1.0 mol L^{-1}) and incubate at 37 °C for 30 minutes in a water bath. Five mL NaOH (2.0 mol L^{-1} in 75 % methanol) was added for alkaline hydrolysis and the samples were incubated a second time at 37 °C for 30 minutes. Thereafter, 5 mL meta-phosphoric acid (0.56 mol L^{-1}) and 5 mL acetone/water mixture (1:1) were added to complete the extraction.

Finally, the mixture was centrifuged at 3000 G for 5 minutes. Supernatants were stored at 4 °C until further use.

3.3.2 Quantification of total phenolic compounds in navel oranges

A mixture of 10 mL ultrapure water, 2 mL sample and 2 mL Folin-Ciocalteu reagent (Sigma®) was made up in a 50 mL volumetric flask and left to stand at room temperature for 5-8 minutes;. 20 mL 7 % sodium carbonate solution was added, followed by an addition of ultrapure water to volume (50 mL). The solution was gently mixed and left to stand at room temperature for 2 h, after which it was filtered through Whatman® 0.45 µm filter paper. The absorbance of the total phenolic compounds in each sample was determined using a spectrophotometer at wavelength 750 nm (Boehm *et al.*, 2006) and expressed as mg L^{-1} GAE using a standard curve (Fig. 3.1).

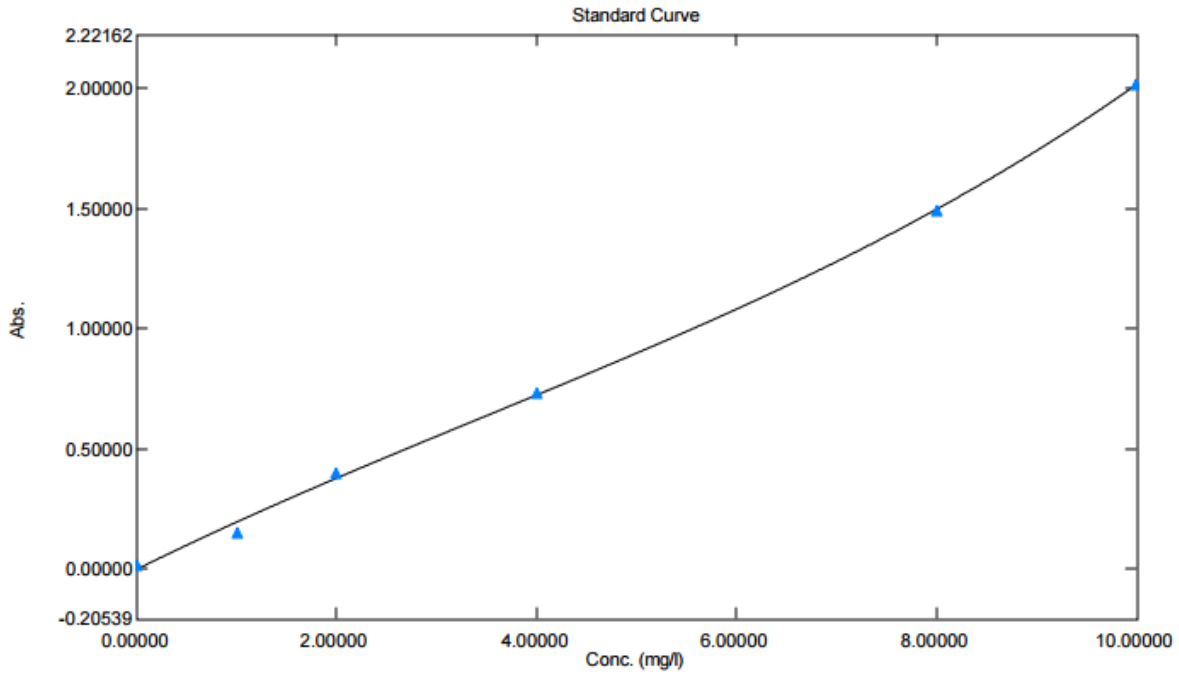

Fig. 3.1: Standard calibration curve for the quantification of total phenolic compounds using Gallic acid

3.3.3 Artificial inoculation of oranges with *P. citricarpa*

Flavedo and albedo tissues of navel oranges were removed using a scalpel. The excised tissues were cut into 5 mm^2 fragments. These fragments were sterilized first in 70 % ethanol followed by a sodium hypochlorite: sterile water (2:1) solution and then rinsed three times in

double-sterilized water. Four sterilized tissue fragments were placed on oatmeal agar (OA), in a Petri dish, equidistant to a 5 mm^2 piece of *P. citricarpa*, previously grown on OA (Fig. 3.2). Cultures of *P. citricarpa* were obtained from the Citrus Research Institute (CRI) in Stellenbosch, South Africa. Three replications were made for each of the types of oranges tissues.

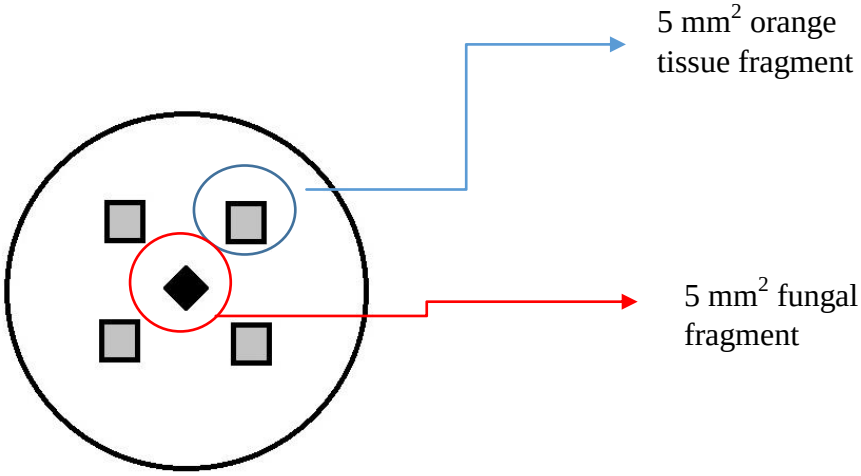

Fig. 3.2: Typical experimental setup in a 90 mm Petri dish containing four sterile navel tissue fragments and *P. citricarpa*.

The plates were incubated at 25 °C for four weeks. After four weeks of growth, the fragments from the different tissues of the fruit were analysed using a ZEISS EVO LS 15, high vacuum and X-ray microanalysis conventional scanning electron microscope (SEM). The samples were fixed in 3% Glutaraldehyde for 3 hours followed by sodium cacodylate buffer wash. They were then dehydrated using 10%, 30%, 50%, 70%, 90% 100% Ethanol. Following this, samples were dried using a Quorum K850 critical point dryer machine. The samples, now dry, were mounted onto stubs using carbon double-sided tape and sputter coated with Gold using an EIKO IB3 Ion coater machine before being viewed though a ZEISS EVO LS15 SEM.

3.3.4 Data analysis

Differences in total phenolic concentration between the albedo and flavedo were evaluated using SAS statistical software version 9.4. Means were separated using Duncan's multiple range at 5% level of significance.

3.4 Results

The albedo phenolic concentration was significantly higher - more than 2.5 times higher - than the flavedo phenolic concentration (Fig. 3.3).

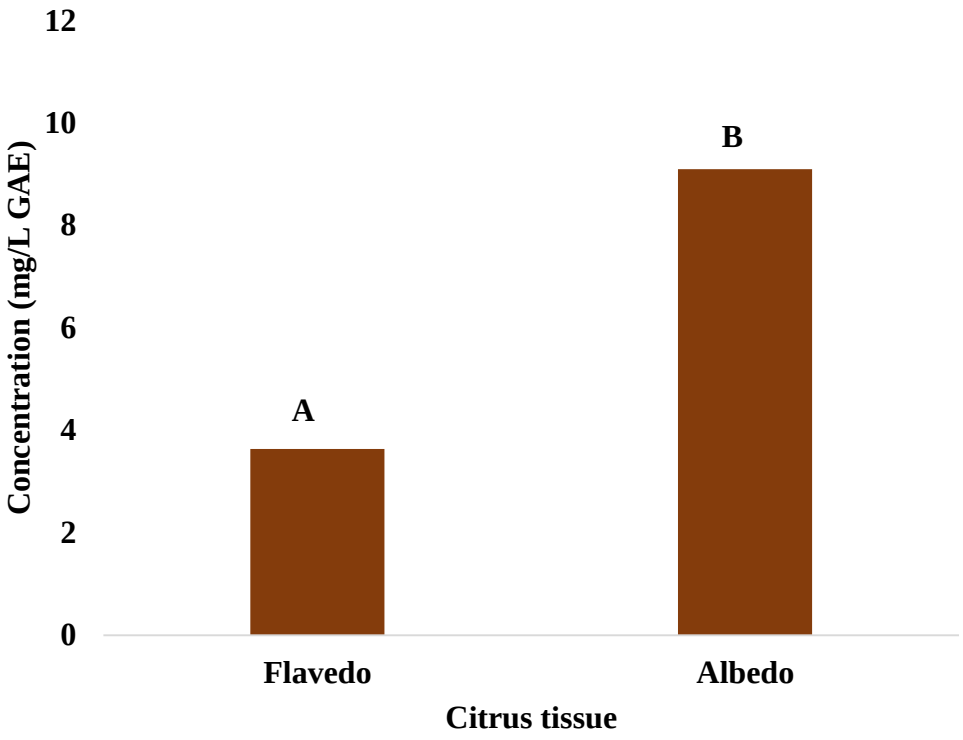

Fig. 3.3: Total phenolic concentration (mg L^{-1} GAE) in albedo and flavedo tissues of navel oranges. Bars marked with the same letter in each column are not significantly different from one another at $P \leq 0.05$.

Neither un-inoculated flavedo nor albedo tissue showed presence of spores or mycelia; however, spores of *P. citricarpa* were observed both, on the surface and in the interior cell layers of flavedo and albedo tissue. The surface of the albedo and flavedo is represented by the side of the tissue that was exposed to the fungus on the Petri dish. Few to no mycelia were observed in the flavedo (Fig. 3.4).

Spores of *P. citricarpa* were observed in all the flavedo samples of navel oranges. The circle represents a part of the flavedo containing spores under a layer of the tissue (Fig 3.4). Spores were observed under the layers of the flavedo although they were not observed to be embedding into the flavedo tissues. Spores in the flavedo were not grouped together and were spread out over the surface and on the inside of the flavedo.

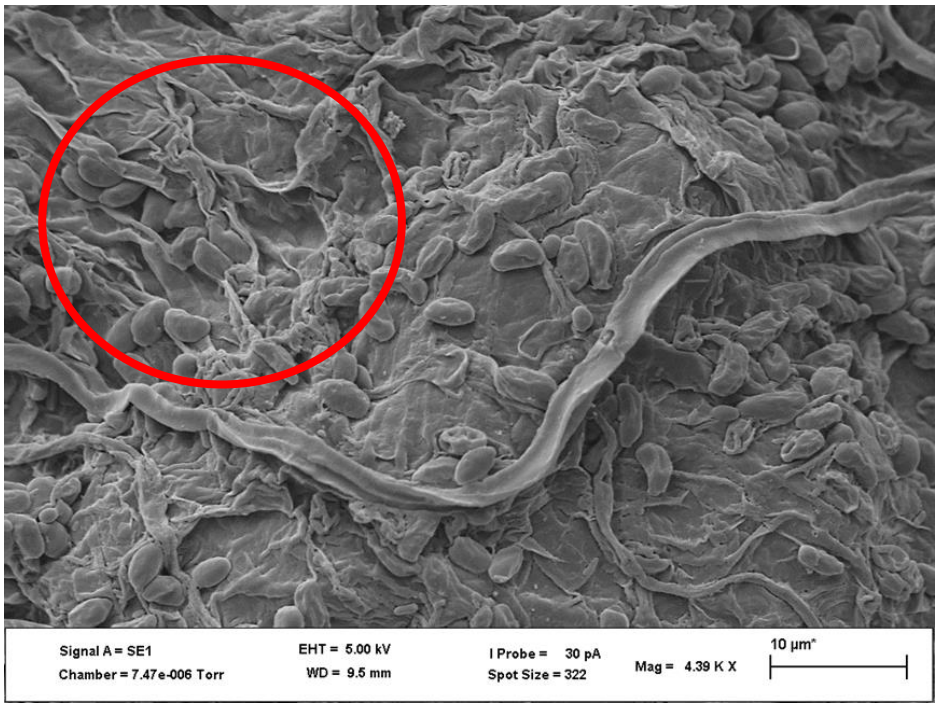

Fig. 3.4: Flavedo tissue of navel colonized by *P. citricarpa* (X4.39 K). Circle shows the area between the flavedo tissues containing spores.

The cross section of the albedo allowed spores and mycelia to be observed inside the albedo (Fig 3.5). A number of spores were visible inside the albedo (Fig. 3.6) and mycelia were found inside the albedo tissues (Fig. 3.6), therefore, both the albedo and flavedo tissues were successfully colonized by *P. citricarpa*. A larger number of mycelia seemed to be present on the surface of the albedo than on the inside of the albedo, whilst a larger number of spores seemed to exist inside the albedo than on the surface of the albedo; similarly, a larger number of spores was observed in the albedo samples than in the flavedo samples (Fig. 3.5; 3.6).

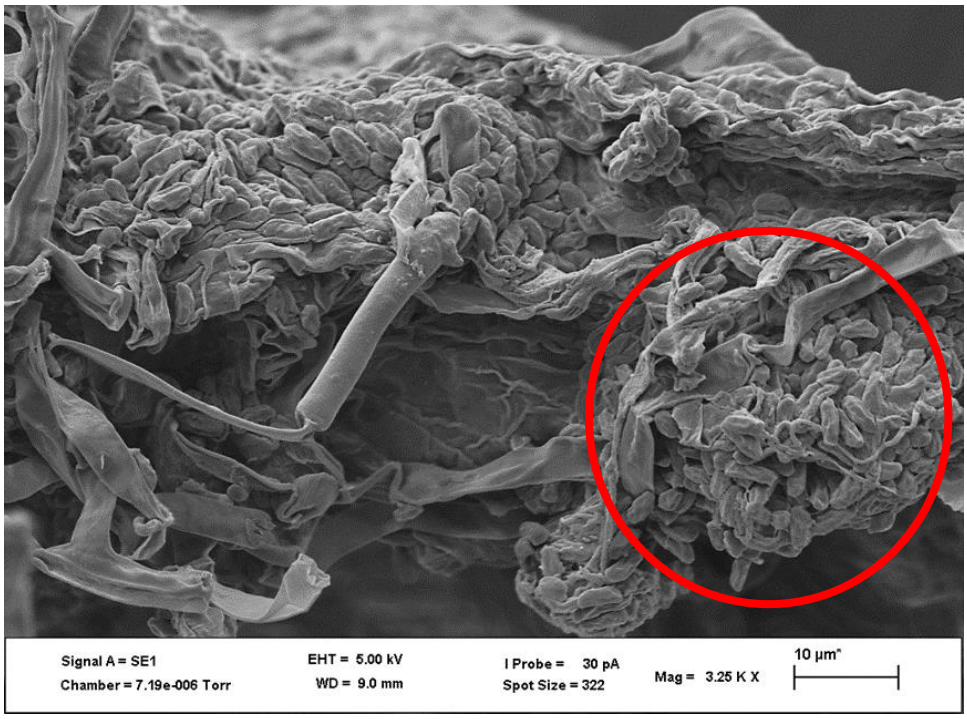

Fig. 3.5: Cross-section of albedo tissues showing clutters of *P. citricarpa* spores (X3.25 K)

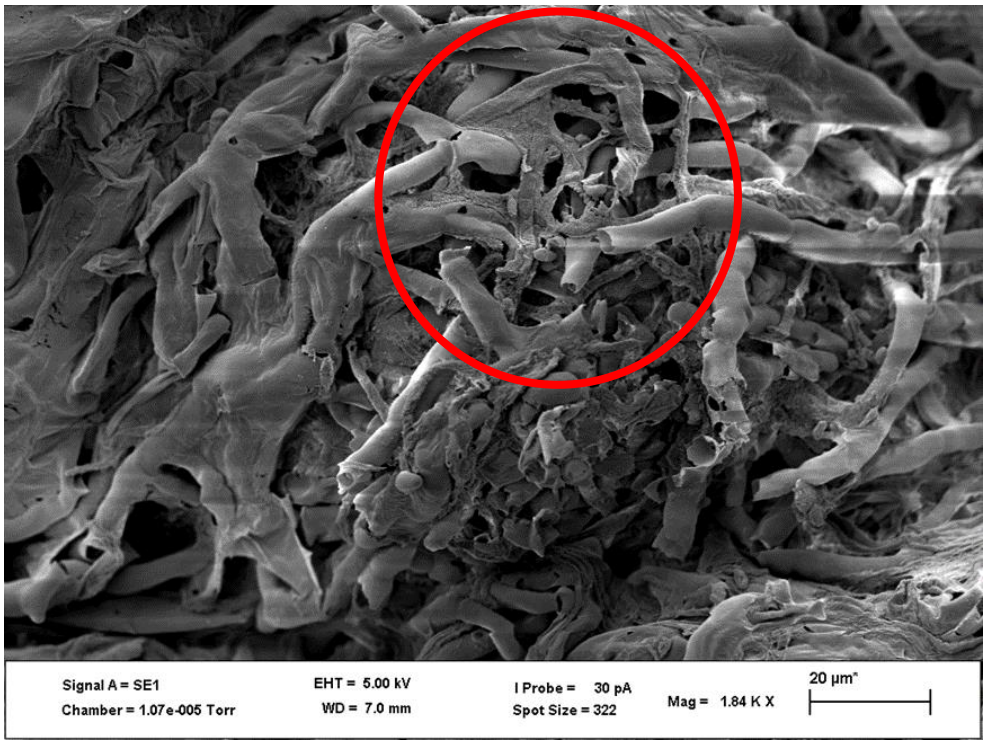

Fig. 3.6: Mycelia seen inside the albedo. Area in the circle shows mycelia of *P. citricarpa* tearing through the albedo tissue of a navel orange (X1.84 K)

3.5 Discussion

This study was aimed at determining whether the inability of *P. citricarpa* to colonize the albedo tissue of navel oranges could be attributed to differences in the concentration of total phenolic compounds in the flavedo and albedo tissue of the fruit. The results revealed that *P. citricarpa* could colonize both, flavedo and albedo of the orange rind under optimal growth conditions *in vitro*. Colonization of flavedo tissues by *P. citricarpa* was expected, as the fungus is known to infect and develop in the flavedo tissues of infected lemon and orange fruit (Kiely 1948; Kotze, 1981). Growth in the albedo, however, was not expected as the fungus is not known to develop in the albedo in nature, except under conditions of heavy infection and high inoculum pressure postharvest (Kiely, 1948).

Disease symptoms of *P. citricarpa* are considered superficial, limited to the flavedo in orchards where the disease is present. It is well-known that *P. citricarpa* does not develop deep into the albedo of citrus fruit in nature, but the processes involved have not yet been described or well-explained (Kotze, 1981; Cardoso-Filho, 2003). One of the few reports of *P. citricarpa* developing on or near the albedo tissues of citrus was a histological analysis of symptomatic fruit using light microscopy, showed that inter-cellular hyphae of *P. citricarpa*, may be observed between the flavedo and albedo tissues (Marques *et al.*, 2012).

Although the concentration in phenols between the two tissues was significantly different, and more or less 2.5 times higher in the albedo than in the flavedo, *P. citricarpa* successfully colonized the albedo. The fungus produced a higher number of spores in the albedo than in the flavedo tissue, indicating that the flavedo provides better growing conditions for the fungus. The large number of spores produced and the ability of *P. citricarpa* to imbed spores and use the mycelia it produces to tear through albedo tissues may be due to the degradation of the tissues, owing to the long incubation period of the navel tissues on the growing media during the experiment, exposure of the tissues to high temperatures for four weeks as well as the direct exposure of the tissues to the pathogen. The concentration of phenolic compounds increases during postharvest transport and handling in citrus (Daayf and Lattanzio, 2009; Tiwari *et al.*, 2013); it has, therefore, been hypothesized that this increase further prevents *P. citricarpa* from colonizing the albedo tissues; the colonization of the albedo by *P. citricarpa* (Fig 4, 5) seems to disprove this hypothesis.

Results suggest that under optimal growing conditions of *P. citricarpa*, the concentration of total phenolic compounds in various navel fruit tissues does not have an effect on the development of *P. citricarpa*. This may depend on the citrus host infected and may not apply for all hosts of *P. citricarpa*. Studies have shown that artificially increasing the concentration of total phenolic compounds in citrus and several other plants, increases plant resistance to disease infection (Naczk and Shahidi, 2006). It has also been shown that plants increase total phenolic content in response to pathogen attack (Ruiz-García and Gómez-Plaza, 2013). In a study conducted on Valencia oranges, Marques *et al.* (2012) showed that after infection with *P. citricarpa*, in the area surrounding stomatal guard cells, phenolic compounds were present, suggesting that the host was able to react to pathogen infection. The results of the present study show clear and significant differences in the concentration of phenolic compounds between the two tissues and the fungus not only colonized both the tissues, but more spores where observed in the albedo, which had a higher total phenolic concentration than the flavedo. This suggests that although several reports have suggested that phenolic compounds protect plants from infection in several ways, these are not effective against *P. citricarpa* infection and plant resistance to pathogen attack cannot always be attributed to the presence of phenolic compounds. This could also mean phenolic compounds may work synergistically with other chemicals through complex pathways to protect the plant from pathogen attack. The total phenolic compounds in the different citrus tissues contain varying concentrations of phenolic compounds and this may contribute to the efficiency of total phenolics to inhibit or prevent pathogen attack (Lattanzio *et al.*, 2006).

In citrus orchards, temperature and humidity tend to fluctuate over the growing season and concentrations of phenolic compounds of the fruit vary over the different phenological stages of the tree (Nebauer *et al.*, 2006). The amount of *P. citricarpa* inoculum available for successful infection may also vary from orchard to orchard and, depending on the citrus type, infection may not always be successful (Kotze, 1981). The experiment conducted in this study does not reflect natural conditions, but the results found suggest that the inability of the pathogen to develop deep into the albedo under natural conditions and may not depend on the concentration of total phenolic compounds. The presence of complex chemical processes that may include some phenolic compounds and other compounds produced by the tree working synergistically to prevent pathogen development should be further investigated. These processes and pathways may only allow the fungus to grow in the flavedo tissues when the fruit is still attached to the tree and may be inactivated when the fruit is harvested, hence the

ability of the pathogen to develop into the albedo during postharvest when virulent spot symptoms are visible. When citrus fruit is harvested, it undergoes several physiological changes, all of which may contribute to its degree of susceptibility to pathogen attack (Kotze, 1981; Iglesias *et al.*, 2006). Disease severity may be perpetuated by light intensity as is the case when hard spot symptoms develop; hard spot symptoms have been shown to be more severe on the side of the fruit facing the sun (Kiely, 1948, Kotze, 1981). The fungus may not develop into the albedo but rather in the flavedo where the most light can be intercepted and the most disease can be caused on fruit.

An increase in plant phenolic compounds may decrease disease severity and disease incidence, but several factors may also contribute to plant resistance to pathogen infection. In nature, *P. citricarpa* is only known to develop into the albedo tissues of citrus fruit during postharvest and only under conditions of high inoculum pressure, in the tissues of highly susceptible fruit; the results from this study showed that when conditions for growth are optimal, *P. citricarpa* will colonize the albedo tissues of navel oranges. In the case of *P. citricarpa*, research that focuses on the specific processes involved in preventing the fungus from developing in the albedo under natural conditions needs to be investigated. A description of processes that occur in the albedo that allow it to hinder the development of *P. citricarpa* in those tissues may lead to a clearer understanding of the pathogen and allow for more effective control methods of the pathogen in citrus orchards were the disease is known to occur.

3.6 Conclusion

Under optimal disease conditions, *P. citricarpa* colonizes and develops in the albedo and flavedo of navel oranges in vitro, even with significantly different concentrations of total phenolic compounds present in the tissues. Further studies should be conducted to determine the specific processes involved in *P. citricarpa* infection of navel oranges and the phenolic compounds that may contribute to inhibition of pathogen development by the fruit further into the edible portions of the fruit.

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Chapter 4

EVALUATION OF THE EFFECT OF PHENOLIC COMPOUNDS ON *Phyllosticta citricarpa IN VITRO*

4.1 Abstract

Phenolic compounds play an important role in protecting plants from pathogen attack and disease development. Induction of natural plant defences using phenolic compounds has been studied in several plant species against a number of pathogens, but is yet to be studied as a possible disease management strategy against *P. citricarpa. Phyllosticta citricarpa*, the causal agent of citrus black spot, a disease causing unsightly lesions on the citrus rind; was grown on growing media amended with phenolic compounds extracted from navel oranges (*Citrus sinensis* Osb.). The effect of phenolic compounds on *P. citricarpa in vitro* was evaluated. Oatmeal agar (OA) was amended with 30 mL of total phenolic compounds extracted from the flavedo and the albedo tissues of navel oranges. Fragments of *P. citricarpa* (5 mm^2) were transferred onto the media in Petri dishes which were incubated at 25 °C (±1ºC) in the dark for 4 weeks. No growth was observed in the media amended with total phenolic compounds extracted from the flavedo; whilst in the media containing total phenolic compounds from the albedo, growth was observed; this was the case on several attempts. The opposite proved true in some cases and growth was observed in the OA amended with flavedo phenolics, while no growth was observed on the flavedo phenolics containing plates. Results were found to be inconsistent and it was not possible to determine a distinct effect of total flavedo or albedo phenolic compounds on the growth of *P. citricarpa*.

4.2 Introduction

Disease resistance in plants is dependent on pre-existing barriers and inducible defence mechanisms. Inducible defences can be activated at the site of infection as well as in distant plant tissues. Depending on the type of attack, the plant may activate different signalling pathways to synthesize defensive compounds, such as phenolic compounds (Beckers and Spoel, 2006). Phenolic compounds, therefore, play an important role in disease resistance in many plants and for this reason, several methods to improve the content of phenolic compounds in plants have been developed with the aim of plant protection against pathogen attack and disease. These methods include cultural management practices, such as thinning,

pruning and controlled irrigation deficit (Perez-Lamela *et al.*, 2007; Basile *et al.*, 2011; Soufleros *et al.*, 2011). Classical breeding methods and genetic engineering have also been used to develop plants with a high phenolic content (Martens *et al.*, 2003; Tusa *et al.*, 2007). Natural and chemical elicitors that trigger defence mechanisms in the plant – amongst them triggers of phenolic compound synthesis - have also been used; this has led to the enhancing of plant responses that prevent disease development (Ruiz-García and Gómez-Plaza, 2013).

Phenolic compounds are secondary metabolites naturally occurring in many plants, synthesized via the shikimic acid pathway and vary in chemical structure (Naczk and Shahidi, 2006; Ruiz-García and Gómez-Plaza, 2013). Phenolic compounds are characterized by a benzene ring with one or more hydroxyl groups and can be classified either as flavonoids or as non-flavonoids (Waterhouse, 2002; González-Aguilar *et al.*, 2004). Phenolic compounds of both types are known to protect plants from biotic and abiotic stresses. Some phenolic compounds are constitutive and are therefore always present in the plant, while others are adaptive and are only synthesised in response to environmental stress, injury or pathogen attack (Ruiz-García and Gómez-Plaza, 2013).

In citrus plants, several phenolic compounds have been recorded; their occurrence and concentration varies from species to species as well as within the plant itself (Marini and Balestrieri, 1995; Ooghe and Detavernier, 1997). The rind of citrus fruit, made up of the waxy cuticle, the coloured flavedo and the white spongy albedo, is an abundant source of flavonoids, phenolic acids and other phenolic compounds (Bocco *et al.*, 1998). Flavonoids occur as aglycones, glycosides, and methylated derivatives. The basic three-ring flavone structure contains either an α-pyrone (flavonols and flavanones) or its dihydroderivative (flavonols and flavanones). Various positions of the ring structure can be hydroxylated. Methyl- as well as acetyl-esters occur in nature. Flavonoids can therefore be divided into polymethoxylated flavones (PMFs) and the glycosylated flavanones, depending on the esters formed. In citrus, PMFs are concentrated in the flavedo, while flavanones occur mainly in the albedo (Mizuno *et al.*, 1991; Kanes *et al.*, 1992). These PMFs are highly active against pathogens, although they occur in low concentrations compared to the flavanones. Phenolic compounds found in citrus, particularly in the rind, have been shown to act as fungitoxins, partially inhibiting disease development (Rio *et al*, 2004; Zabka and Pavela, 2013; Oliviera *et al.*, 2015). The location and relatively high concentration of these PMFs in the rind suggest that they play a role in protecting fruit from pathogenic attack (Horowitz and Gentili, 1977).

Phyllosticta citricarpa is known only to develop on the flavedo of the rind and does not develop into the albedo tissues in any of the citrus hosts that it infects (Kotzé, 1981). Disease management strategies of CBS rely mainly on the use of fungicides and cultural practices to reduce inoculum (Carstens *et al.*, 2012, Kotzé, 1996). An integrated pest management (IPM) approach is mainly used in citrus orchards to manage CBS as efforts to develop resistant varieties have proved unsuccessful (Calavan, 1960 cited by Truter, 2010). The induction of natural plant defences, such as induced systemic resistance (ISR) and systemic acquired resistance (SAR), have been explored as possible methods of control against many plant diseases but have not yet been investigated for *P. citricarpa*. These mechanisms (ISR and SAR) may inhibit or limit pathogen attack and compounds exerting such resistance may be artificially induced in plants using elicitors to emulate pathogen attack and elicit the plant's defences. When exposed to elicitors, plants react as they would to pathogen attack and activate a variety of resistance mechanisms, including the synthesis of large quantities of phenolic compounds at the site of infection, resulting in a hypersensitive response (Matern and Kneusel, 1988). Hypersensitive responses isolate the pathogen to the site of infection and prevent it from spreading to other areas of the organism. Following the activation of hypersensitive responses, distant plant tissues may develop resistance to further infection (Matern and Kneusel, 1988).

Phenolic compounds have proved partially effective in hindering disease development against several pathogens in plants and may be a promising addition in the integrated pest management control of CBS. The aim of this study was to evaluate the growth of *P. citricarpa* on growing media amended with phenolic compounds previously extracted from navel orange (*C. sinensis*) fruit. It was hypothesized that the fungus would grow in the presence of phenolic compounds extracted from the flavedo, but growth in the presence of the albedo extract would be hindered. These results could provide insight into the effect of total phenolic compounds on the growth of *P. citricarpa*.

4.3 Materials and Methods

4.3.1 Extraction of total phenolic compounds from navel oranges

Navel oranges were harvested from Bounty Farm, Winterskloof (29°28'S; 30°161'E) in KwaZulu-Natal, South Africa. Total (free and bound) phenolic compounds were extracted from the albedo as well as the flavedo tissue of six navel oranges, with three replications per

orange, using the method described by Boehm *et al*. (1998). Flavedo and albedo tissue (500 mg) was weighed out and placed into centrifuge tubes. Samples were mixed with 5 mL hydrochloric acid (1.0 mol L^{-1}) and incubated at 37 °C for 30 minutes in a water bath. Thereafter, 5 mL NaOH (2.0 mol L^{-1} in 75 % methanol) was added for alkaline hydrolysis and samples were incubated a second time at 37 °C for 30 minutes. Then, 5 mL meta-phosphoric acid (0.56 mol L^{-1}) and 5 mL acetone/water mixture (1:1) were added to complete the extraction. Finally, the mixture was centrifuged at 3000 G for 5 minutes; supernatants were stored at 4 °C until further use.

4.3.2 Sub-culturing of *P. citricarpa* on OA amended with phenolic extracts

Oatmeal Agar (OA) was prepared according Gams *et al.* (1998); briefly, 30 g oatmeal flakes were placed in a volumetric flask and 250 mL distilled water was added. The mixture was brought to the boil, then left to simmer for approximately 2 h; thereafter, the mixture was filtered and squeezed through cheesecloth. The flakes were discarded and to the watery extract, 7 g agar was added. Thereafter, the medium was sterilized for 15 minutes at 121°C. The OA was amended with 50 µg mL^{-1} penicillin and 50 µg mL^{-1} streptomycin, to prevent bacterial contamination. The media was then amended with phenolic compounds extracted as described above. Thirty mL supernatant, containing phenolic compounds extracted from albedo and flavedo tissue of navel oranges, was added into volumetric flasks containing 250mL growing media at 55 °C. The OA was allowed to cool to 50 °C, before pouring into 90 mm Petri dishes. These Petri dishes were kept in a laminar flow overnight.

Phyllosticta citricarpa cultures, isolated by Elma Carstens, were obtained from the Citrus Research Institute (CRI) in Stellenbosch. When received, cultures were seven days old and had been grown on OA. Fragments (5 mm^2) of *P. citricarpa* were transferred onto the centre of the Petri dishes containing OA prepared as described above. Five plates of phenolic compounds from the flavedo and albedo were prepared and incubated at 25 °C (±1ºC) in the dark for four weeks.

4.4 Results

After 4 weeks of incubation, inhibition of *P. citricarpa* growth in the presence of phenolic compounds could not be clearly evaluated. The experiment was repeated several times and at various temperatures and results was inconsistent between study repetitions. On several attempts, no growth was observed in the media amended with total phenolic compounds

extracted from the flavedo, whilst in the media containing total phenolic compounds from the albedo, growth was observed. In some cases the opposite was observed and growth was observed in the OA amended with phenolic compounds from the flavedo plates and none was observed in the plates.

No clear conclusions could be drawn from the results and it was not possible to evaluate to what degree phenolic compounds could inhibit the growth of *P. citricarpa in vitro.*

4.5 Discussion

This study attempted to test whether phenolic compounds endogenous to citrus flavedo or albedo were effective in inhibiting the growth of *P. citricarpa*. A crude extract of free and bound phenolic compounds from navel oranges was used and after several weeks, no clear conclusions could be drawn on whether *P. citricarpa* could grow in the presence of flavedo or albedo phenolic compounds *in vitro.* The fungus behaved in a similar way in all the repititions of the experiment, on in the presence of both the albedo and the flavedo extracts, therefore no specific reasons can be cited for inconsistent performance of the phenolic extracts and their effect on *P. citricarpa*. Phenolic compounds have been shown to inhibit pathogen growth at varying degrees of efficiency, with some fungi being completely inhibited by the presence of phenolic compounds and others being only partially inhibited (Zabka and Pavela, 2013; Oliviera *et al*., 2015).

The fungus, *P. citricarpa,* is known only to develop in the flavedo of the citrus rind and only under conditions of heavy infection, does it develop in the albedo (Kiely, 1948) and, as the albedo is known to contain higher concentrations of phenolics than the albedo (Bocco *et al*., 1998) fungal growth in the presence of phenolic compounds extracted from the flavedo was expected, but not in the presence of the concentration or type of phenolics present in the albedo.

The different tissues in citrus fruit differ in the phenolic compounds they contain (Mizuno *et al.,* 1991; Kanes *et al.,* 1992). The flavedo is abundant in PMFs, phenolics which are known to be biologically active and to prevent pathogen attack (Rio *et al*, 2004). These phenolic compounds were not tested against *P. citricarpa* in the present study as preliminary studies were not successful. The albedo has been reported to have a high concentration of flavones, however, these compounds do not play a major role in inhibiting disease development or preventing pathogen attack (Rio *et al*, 2004; Ruiz-García and Gómez-Plaza, 2013). Levels of

phenolic compounds change in response to *P. citricarpa* infection in Valencia orange or Eureka lemon leaves (Truter, 2010) but no resistance against *P. citricarpa*, developing due to phenolic compounds has been reported. *P. citricarpa* has been shown to produce metabolites that allow resistance to antagonists during studies in biological control, these metabolites may be used by the fungus to allow it to develop under unfavourable conditions, as the case may be in the presence of phenolic compounds found in the albedo (Kupper *at al*., 2011).

Disease resistance in plants may be due to several synergistic interactions that occur in plants and may not be attributed to a single compound or mechanism (Kuc, 1995).The albedo tissues contain three times more total phenolics than the flavedo tissues but the composition and concentration of the different compounds is dissimilar. The ability or inability of *P. citricarpa* to develop in the presence of phenolic compounds, may therefore, not only be dependent on the concentration of total phenolic compounds, but also the type of compounds and the concentration of the different compounds present in the respective tissues.

4.6 Conclusion

In this study, 30 mL of total phenolics extracted from both the albedo and flavedo was used and the fungus was able to develop in the plates containing extracts from both tissues. The concentration of phenolic compounds used remained unchanged for all the treatments and in all the repetitions of the experiments, the fungus behaved the same way. Inconsistent results were observed and no clear conclusions could be drawn as fungal growth was observed both in the absence and presence of flavedo or albedo phenolic compounds into the albedo may be due to the type of phenolic compounds present as well as the concentration.

Results from this study contribute important preliminary information, particularly pointing towards a role of that certain phenolic compounds, present in the flavedo and albedo tissues of navel oranges, which may contribute to the in inhibition of *P. citricarpa* development in citrus fruit.

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GENERAL DISCUSSION

Citrus exports are an important source of foreign exchange for South Africa and these exports are threatened by restrictive quarantine regulations linked to certain fruit pests and diseases. Fruit displaying symptoms of these diseases cannot be sold, both on the local and the international market. Citrus Black Spot (CBS) is a devastating disease caused by the fungus *Phyllosticta citricarpa* [(McAlapine) Aa] (Teleomorph: *Guignardia citricarpa* Kiely). This disease is causing unsightly lesions on the fruit, rendering them unappealing and unmarketable (ISPM, 2014). Citrus black spot is considered a phytosanitary disease, as classified by countries of the EU and the USA. Although the risk of *P. citricarpa* entering new areas is relatively low, every effort is made to prevent this fungus from entering areas where the disease has not been reported (Kiely, 1948; Carstens *et al*., 2012). This is ensured mainly through frequent checks of imported fruit shipments and strict quarantine regulations. Disease symptoms found on imported citrus fruit results in the rejection of an entire shipment, leading to financial losses for exporting countries (Carstens *et al.*, 2012).

Citrus black spot develops from ascospores and pycnidiospores which may be present in leaf litter and fruit lesions. Several types of lesions are associated with CBS, namely: hard spot, freckle spot, cracked spot, virulent spot and speckled blotch. Symptoms that may develop vary with climatic conditions, the type of citrus, as well as with fruit development in relation to disease development (Katzi, 1996; Kotzé, 2000).

Isolation of *P. citricarpa* from symptomatic fruit plays an integral part in identifying the pathogen and developing management strategies. One problematic area when isolating *P. citricarpa* lies in the accumulation of contaminants in the Petri dish, including other *Phyllosticta* species. This problem is closely related to the slow growth rate (24.9 mm week^{-1}) of *P. citricarpa in vitro*, resulting in less than 10% of plates used for isolation containing clearly visible (Baldassari *et al*., 2008). Radial growth rate measurements on growing media, have been shown to be a good means to determine fungal growth, even though only horizontal growth is measured and vertical growth or the number of spores produced by the fungus on the Petri dish are not taken into account (Loeck *et al*., 2004). Oatmeal Agar (OA) was the most efficient medium for growing *P. citricarpa* and because of its simple composition and visual transparency; radial measurements are easier and more precise (Baldassari *et al*., 2008).

The effect of flavedo and albedo phenolic extracts could not be evaluated since the fungus behaved in a similar way and as a result conclusions on whether fungal growth of *P. citricarpa* may be inhibited by phenolic compounds could not be made. There are no clear reasons that can be cited for inconsistent performance of the and their effect on *P. citricarpa*, although, phenolic compounds have been shown to inhibit pathogen growth at varying degrees of efficiency, with some fungi being completely inhibited by the presence of phenolic compounds and others being only partially inhibited (Zabka and Pavela, 2013; Oliviera *et al*., 2015).

Several reports have suggested that phenolic compounds protect plants from infection in several ways, although these results suggest that, under optimal growing conditions, the concentration of total phenolic compounds in navel flavedo and/or albedo tissues does not have an effect on the development of *P. citricarpa*. This may be host-dependent and may not apply to all other citrus species affected by this fungus. The rind of citrus fruit is an abundant source of phenolic compounds that occur in varying concentrations in the different tissues. This may contribute to the efficiency of total phenolics to inhibit or prevent pathogen attack (Bocco *et al*., 1998; Lattanzio *et al*., 2006).

An integrated pest management (IPM) approach is mainly used in citrus orchards to manage CBS, this includes cultural practices and fungicide applications. The induction of natural plant defences, such as ISR (induced systemic resistance) and SAR (systemic acquired resistance), have been explored as possible methods of control against many plant diseases but have not yet been investigated for *P. citricarpa*. Induced systemic resistance and SAR are mechanisms that may inhibit or limit pathogen attack through several processes that ultimately alter the concentration of phenolic compounds in the plant (Matern and Kneusel, 1988).

Results from this study contribute important preliminary information, particularly pointing toward a role that certain phenolic compounds, present in the flavedo and albedo tissues of navel oranges, may play in inhibiting *P. citricarpa* development in citrus fruit. Compounds such as the polymethyloxylated flavones and glycosylated flavanones may be found in the albedo and flavedo. These phenolic compounds exhibit a high biological and antimicrobial activity of any other phenolic compounds in citrus (Tatum *et al*., 1978; Benavente-Garcia *et al*., 1997; Bocco *et al*., 1998).

Future research building on this experiment should include methods to determine the vertical growth of fungi on growing media to be evaluated before the potential inhibitory effect of specific phenolics on growth of *P. citricarpa* can be determined. The presence of complex chemical processes that may include some phenolic compounds and other compounds produced by the tree working synergistically to prevent pathogen development should be further investigated. When citrus fruit is harvested, it undergoes several physiological changes, all of which may contribute to its degree of susceptibility to pathogen attack (Kotze, 1981; Iglesias *et al*., 2006). These processes and pathways may only allow the fungus to grow in the flavedo tissues when the fruit is still attached to the tree and may be inactivated when the fruit is harvested, hence the ability of the pathogen to develop into the albedo during postharvest when virulent spot symptoms are visible. The processes involved have not yet been described or well-explained and should be investigated (Kotze, 1981; Cardoso-Filho, 2003).

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Appendices

Appendix A:

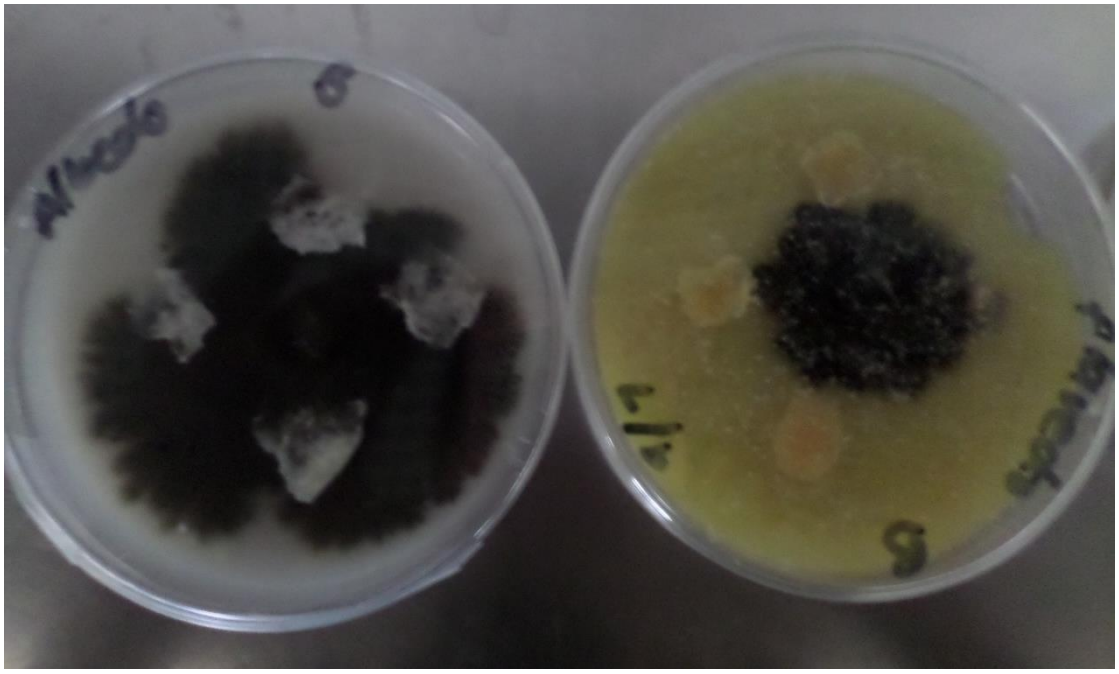

Fig. 1: Experimental setup in a Petri dish showing the control - containing only sterile navel tissue fragments - and Petri dishes containing *P. citricarpa* and sterile navel tissue fragments from the albedo and flavedo after 4 weeks of incubation

Appendix B: Wet mount of *P. citricarpa*

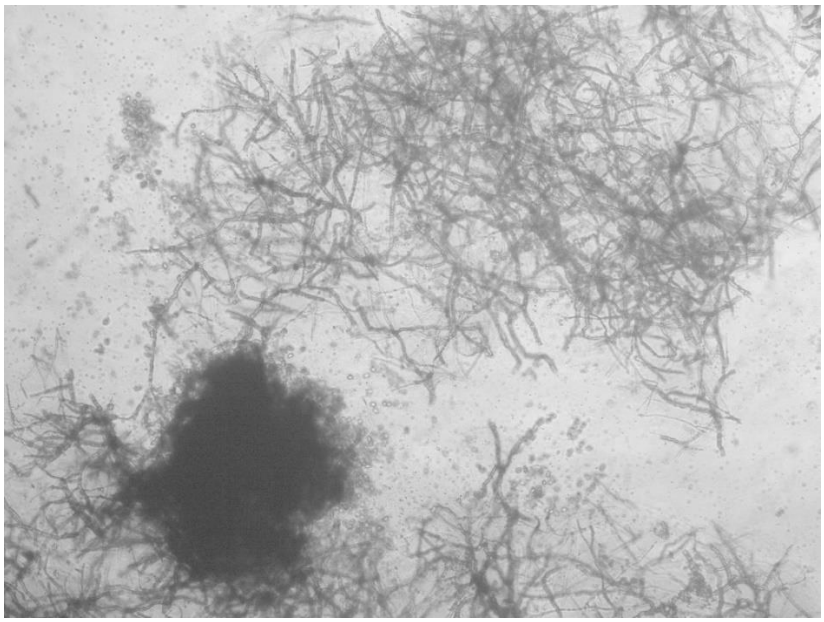

Fig. 2: Mycelia and spores of *P. citricarpa* under a light microscope (X40)

Appendix C: Leaves and fruit from which attempts to isolate *P. citricarpa* were made

Fig. 3: Leaves from *Citrus limon* L. tree showing typical CBS symptoms

Fig. 4: *Citrus limon* L. fruit showing typical CBS symptoms

Appendix D: *P. citricarpa* growth on OA amended with flavedo phenolics

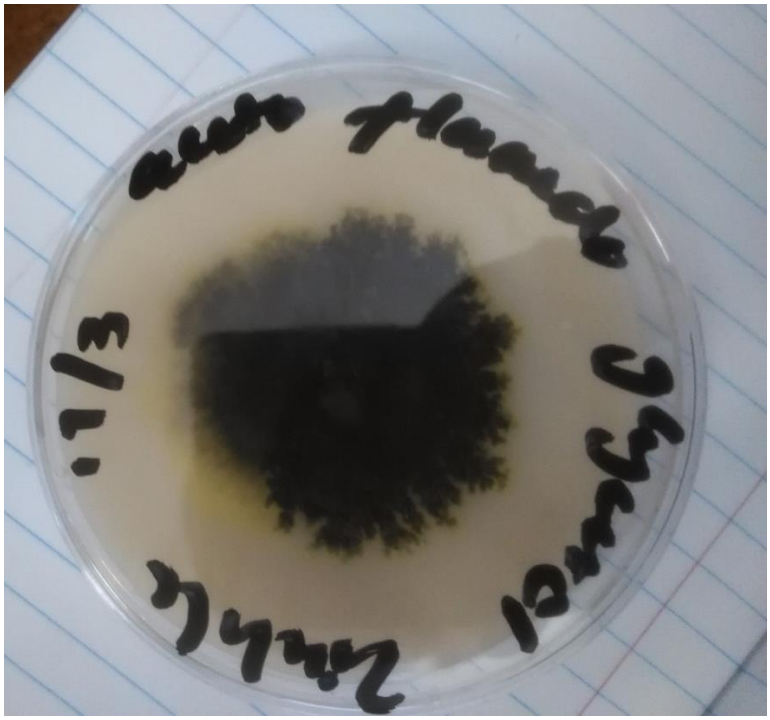

Fig. 5: *P. citricarpa* growth after 4 weeks on OA amended with phenols from flavedo tissues

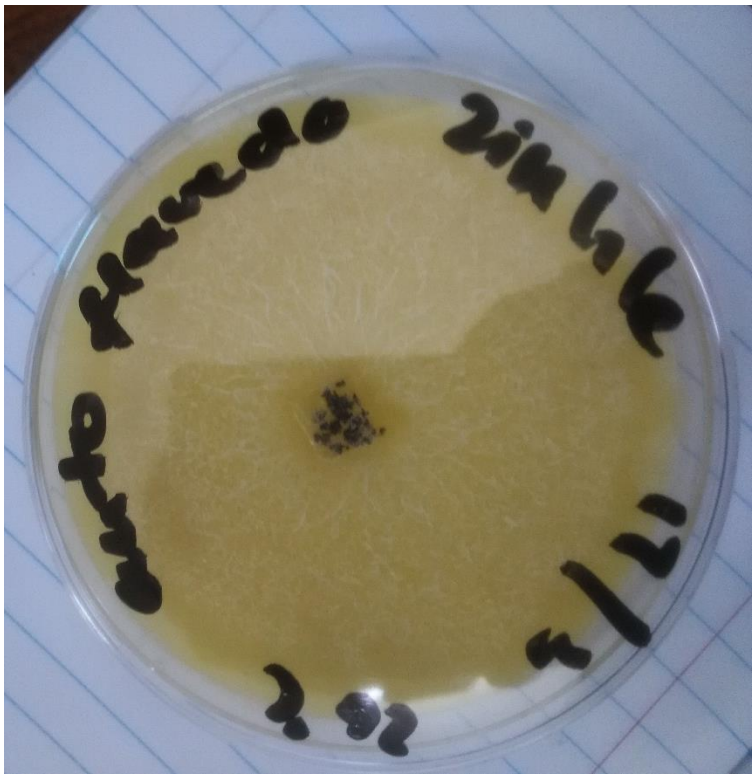

Fig. 6: *P. citricarpa* growth after 4 weeks on OA amended with phenols from flavedo tissues

Appendix E: *P. citricarpa* growth on OA amended with albedo phenolics

Fig. 7: *P. citricarpa* growth after 4 weeks on OA amended with phenols from albedo tissues

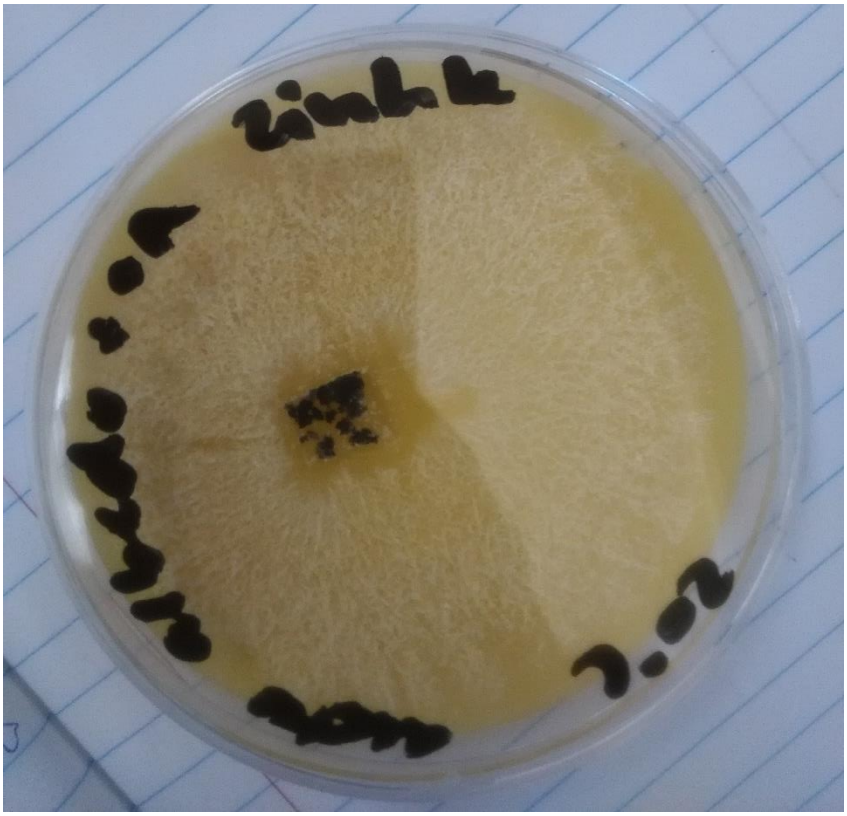

Fig. 7: *P. citricarpa* growth observed after 4 weeks on OA amended with phenols from albedo tissues