BASELINE SENSITIVITY OF *Guignardia citricarpa*, THE CAUSAL AGENT OF CITRUS BLACK SPOT TO AZOXYSTROBIN, PYRACLOSTROBIN AND FENBUCONAZOLE

By

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To my family and my fiancé, Victor

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Abstract of Thesis Presented to the Graduate School
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BASELINE SENSITIVITY OF *Guignardia citricarpa*, THE CAUSAL AGENT OF CITRUS BLACK SPOT TO AZOXYSTROBIN, PYRACLOSTROBIN AND FENBUCONAZOLE

By

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Citrus Black Spot (CBS), caused by *Guignardia citricarpa*, was first identified in Florida and the United States in March 2010. The economic impact of the disease is due to external blemishes on the fruit, making them unsuitable for the fresh market and to yield loss caused by premature fruit drop. Fungicide applications are the main control measures in other citrus production areas of the world. The purpose of this project was to evaluate the *in vitro* activity and baseline sensitivity of *G. citricarpa* isolates to azoxystrobin, pyraclostrobin and fenbuconazole. The effective concentration needed to reduce growth or germination by 50% (EC_{50}) was determined for fifty isolates from the two Florida counties where CBS is found. The effect of salicylhydroxamic acid (SHAM) on the inhibition of mycelial growth and conidial germination by azoxystrobin and pyraclostrobin was also assessed. The EC_{50} mean for mycelial growth for azoxystrobin was 0.021 µg/ml and the means for fenbuconazole and pyraclostrobin were significantly lower at 0.009 and 0.008 µg/ml, respectively (P= <0.0001). Similarly, the mean EC_{50} for azoxystrobin for conidial germination was 0.016 µg/ml and that for pyraclostrobin was significantly lower at 0.006 µg/ml (P= <0.0001). There was no effect of SHAM on inhibition of mycelial growth and conidia germination by the Q_oI fungicides. However,

SHAM slightly affected mycelium inhibition by pyraclostrobin. Results from this study will provide the baseline sensitivity to the fungicides tested and will help for future resistance monitoring of this newly introduced pathogen.

CHAPTER 1
INTRODUCTION

The major citrus-producing countries are Argentina, Australia, Brazil, China, Cuba, Egypt, India, Israel, Italy, Japan, Mexico, Morocco, South Africa, Spain, Turkey and the United States (Whiteside, 2000). In the U.S., Florida is the largest citrus producer with 70% of the total citrus production during the last decade (USDA, 2011). In the 2010-2011 season, Florida production was valued at 165.9 million dollars on 219,068 ha (USDA, 2011) with the majority of the fruit going to processing.

The majority of the commercial cultivars used as scions or rootstocks belong to the genus *Citrus* or are trifoliate orange (*Poncirus trifoliata* (L.) Raf.), which is used only as a rootstock (Timmer et al., 2000). In Florida, commercially grown citrus includes oranges, grapefruit, tangelos and tangerines (USDA, 2012).

Citrus black spot was exotic to Florida until April 2010, when it was found by the Florida Department of Agriculture and Consumer Services, Division of Plant Industry (DPI) (Schubert et al., 2012). Black spot is caused by the fungus *Guignardia citricarpa* Kiely, anamorph stage *Phyllosticta citricarpa* (McAlpine) van der Aa. The disease causes blemishes on the fruit rind and premature fruit drop, although the internal fruit quality remains unaffected. Lesions on the leaves are usually rare and do not affect the tree, but infected leaves on the orchard floor are an important source of inoculum (Timmer et al., 2003).

Most commercial citrus cultivars are susceptible to black spot including grapefruits, limes, mandarins, sweet oranges, and lemons; however sour oranges and Tahiti lime are asymptomatic (EPPO, 2009). The disease is favored in subtropical regions with high summer rainfall (Kotzé, 2000), and the pathogen is widespread

around the world (Sutton et al., 1998) although the disease has not been reported in regions with Mediterranean climates (Kotzé, 2000).

The pathogen produces two forms of reproductive structures. Ascospores are formed in leaf litter, whereas conidia (pycnidiospores) are found in fruit lesions, peduncles and leaves (Kotzé, 1981, 2000). Both ascospores and pycnidiospores have an important role establishing epidemics, but when the disease becomes established, and depending on summer rainfall patterns, ascospores are a more important source of inoculum (Kotzé, 1981). Citrus fruit remain susceptible to infection for four to five months after petal fall. After that period, fruit become resistant regardless of weather conditions or inoculum pressure (Kotzé, 2000). Leaves remain susceptible from development up to 10 months of age (Truter et al., 2007).

In other countries where the disease is established, black spot is mainly controlled with application of fungicides from different groups such as the benzimidazoles, strobilurins (Q_oI), dithiocarbamates, and copper products while fruit is actively growing (Agostini et al., 2006; Da Silva et al., 2009). On the other hand, cultural practices such as promotion of leaf litter decomposition (Bellotte et al., 2009), minimizing plant trash movement, avoidance of cultivars with off-season blooms, increasing air flow in the canopy, and using disease-free nursery stock have been promoted to reduce inoculum amount and spread (Dewdney et al., 2012; FCHRP working group, 2010).

Since only strobilurin fungicides and copper are registered for the control of citrus black spot in Florida, fungicide evaluation became necessary to determine minimally effective dosages and to monitor future shifts in pathogen sensitivity.

CHAPTER 2
LITERATURE REVIEW

Citrus Production in Florida

United States is one of the largest citrus producers in the world, and Florida has the greatest citrus production in the country (USDA, 2011). Since the introduction of citrus into St. Augustine, Florida in the 1500s, citrus production has spread to other states such as California, Texas and Arizona (USDA and APHIS, 2006).

Citrus varieties produced in Florida include oranges, grapefruit, tangelos and tangerines (USDA, 2012). Around 90% of the citrus production is destined for fruit processing and the remaining for the fresh market (Mossler, 2011). Most of the citrus growing areas are located on the ridge in central Florida and the flatwoods of the east coast and southwest Florida. Survey data of the 2008-2009 season indicated that the five Florida counties with the highest citrus production were in descending order: Polk, Highlands, Hendry, Desoto, and Hardee (USDA and NASS, 2009).

Freeze events during the 1980s promoted the implementation of different production practices such as using smaller trees and closer spacing, which increase the tree number per acre, and the use of new varieties. Thus, although the acreage has been decreasing, the total number of trees per acre has increased (Mossler, 2011). In 1988 the total commercial citrus acreage was 697,929 acres and it has decreased to 541,328 acres in 2011 (USDA, 2011).

The hurricanes in 2004 and 2005 damaged many trees and reduced citrus production in Florida. Also, these weather events contributed to the introduction and spread of citrus canker and huanglongbing which continue to threaten the citrus industry. Eradication attempts have been implemented to control the spread of citrus

canker and huanglongbing, however they were considered to be unfeasible (USDA and APHIS, 2006). Nevertheless, preventive measures adopted by growers contributed to increased citrus yields (USDA and APHIS, 2006).

For the 2003-2004 citrus season, Florida had a total production of 291.8 million boxes. However, by the following season, production declined to 169.3 million boxes; a 42% decrease (USDA, 2011). In the 2010-2011 season, Florida produced 166 million boxes of different citrus varieties, a 4% yield increase compared to the previous season (USDA, 2011), and it is estimated that for the 2011-2012 season there will be another increase to 170 million boxes (USDA, 2012). Despite the relative increase in Florida production, the global production of oranges dropped by 7% in 2011 compared to the previous year, and juice production fell by 9% for the same year due to smaller crops in Brazil, Mexico and the EU (USDA, 2012).

Citrus Black Spot

Citrus black spot (CBS) is caused by the ascomycete *Guignardia citricarpa* Kiely, anamorph *Phyllosticta citricarpa* (McAlpine) Van der Aa. Among *Citrus* species, susceptible hosts include grapefruit (*C. paradisici*), lemon (*C. limon*), lime (*C. aurantifolia*), mandarin (*C. reticulata*) and orange (*C. sinensis*); however sour orange (*C. aurantium*) is not susceptible (EPPO, 2009). ‘Tahiti’ lime (*C. latifolia*) has been classified as an “insensitive host”. Although viable ascospores have been isolated from ‘Tahiti’ lime leaves, *G. citricarpa* does not elicit disease symptoms even at high inoculum pressure under field conditions (Baldassari et al., 2007).

Black spot occurs in subtropical areas with summer rainfall. It affects the fruit rind and when it first appears in a new region, it is usually found on highly susceptible lemons. Symptomatic fruit are unacceptable for fresh fruit market, but they can be used

for processing (Kotzé, 1981). The disease reduces crop productivity due to premature fruit drop and it increases the cost of production (Baldassari et al., 2007).

The pathogen is found in many of the subtropical citrus production zones and is considered an economically important disease of citrus. The fungus was first described in New South Wales, Australia in 1895, causing significant losses on Valencia oranges (Sutton and Waterston, 1998). Later, crop losses up to 80% due to black spot were reported in South Africa in 1929 (EPPO, 2009). The disease has spread to different countries such as Argentina, Brazil, China (Hong Kong), Indonesia, Japan, Kenya, Mozambique, Philippines, Swaziland, Taiwan, Uruguay, Venezuela, Zambia, Zimbabwe (USDA and APHIS, 2010; Paul et al., 2005), and more recently the United States (Schubert et al., 2012). The disease, however, has not been reported in Mediterranean countries (Kotzé, 2000; Paul et al, 2005).

The spread of *G. citricarpa* to locations previously free of the disease could occur through the movement of infected plant materials, such as contaminated nursery stock, rather than the movement of infected fruit (EPPO, 2009). Also, the spread of the disease will depend on the effectiveness of quarantine measures and the actions taken after the first outbreak. Depending on the hosts and climatic conditions, the pathogen may take up to 5 to 30 years from the time the first symptoms are observed until it reaches epidemic rates (Kotzé, 1981). Once the disease is established in a region, it will not disappear; instead, epidemics will need to be managed using effective control measures (Kotzé, 1981).

Citrus Black Spot, a Quarantine Disease in Florida

Environmental conditions in Florida are favorable for the development of CBS (Kotzé, 1981). A predictive model used to determine the risk of CBS establishment after

pathogen introduction to the United States found that Florida conditions are highly favorable for the disease (Magarey et al., 2011). Other areas, such as California, have a low risk for disease development due to the lack of rainfall and prolonged leaf wetness periods that promote the disease (Magarey and Borchert, 2003; Magarey et al., 2011).

During a regular citrus grove survey on March 8 2010, a suspect sample of Valencia oranges from the Immokalee area was identified as citrus black spot. But, it was not until April 2010 that the U.S Department of Agriculture's Animal Plant Health Inspection Service (APHIS) confirmed the presence of *G. citricarpa* in Florida (APHIS, 2010).

Citrus black spot was introduced into Florida through unknown means (Schubert et al., 2012), but a risk assessment analysis conducted by APHIS identified some factors that could have led to the introduction of *G. citricarpa*. Since the disease is not present in neighboring countries such as Mexico, there is a low likelihood of introduction of the pathogen to the U.S. by natural spread (Holtz, 2010).

According to Kotzé (1981), windborne spores of this pathogen, as a source of long distance spread, are not as dangerous as infected plant materials. In view of the number of interceptions of contaminated leaves and unregulated non-commercial infected fruit over the last 23 years by the Plant Protection and Quarantine (PPQ), there is a medium to high likelihood that the pathogen was introduced to the U.S. through those pathways (Holtz, 2010). To prevent the spread of *G. citricarpa*, a federal order from APHIS established restrictions on the interstate and intrastate movement of regulated articles (APHIS 2012). Regulated articles were identified as citrus fruit, plant

parts such as leaves, budwood, and nursery stock or any other article that could possibly be a hazard for spread of CBS (APHIS, 2012).

Currently in Florida, two counties, Collier and Hendry, are confirmed to have black spot and sections have been designated as quarantine areas by APHIS (APHIS, 2012). Several conditions to restrict the interstate movement of regulated articles (citrus fruits and plant parts) were implemented. The restrictions include that the fruit must be washed, brushed, disinfested, treated and waxed at the packing house prior to shipment. Also, fruit should be free of leaves, stems or other regulated materials. For intrastate movement, vehicles transporting fruit should be covered at the cargo area with a tarpaulin. In addition, after the shipment, all possible contaminated objects such as field boxes, bins, and tarpaulins must be cleaned of debris; which must be heat treated, incinerated or buried in a landfill approved by APHIS (APHIS, 2012).

Besides the implementation of regulatory measures, control strategies must be undertaken. An economic assessment estimated that the cost for the control of CBS will be at least $220 million annually in the United States; however, due to preventive sprays of copper already use to control citrus canker, the actual cost may be not as high as the predicted value (USDA, 2002). In addition to the cost of control measures, it is predicted that in Florida there could be up to $847 million in losses due to the disease (Holtz, 2010).

Causal Organism

Guignardia citricarpa was identified in New South Wales by Kiely in 1948 (Kiely, 1948). For years, the anamorph was known as *Phoma citricarpa* McAlpine, but it was changed to *Phyllosticta citricarpa* (McAlpine) van der Aa (Kotzé, 2000). The spermatial stage is *Leptodothiorella* (Kotzé, 2000; Van der Aa, 1973), which forms readily in culture

(Wikee et al., 2011) and also appears on fallen leaves before the development of the pseudothecia (EPPO, 2009).

Pseudothecia of *G. citricarpa* are the most important source of inoculum and occur in leaf litter, but are never found on the fruit (Kotzé, 2000). The 100-175 µm dia. pseudothecia are erumpent, globose, often irregularly shaped, dark brown, and unilocular with a central ostiole. Asci are 8-spored, bitunicate, clavate to broadly ellipsoid, with slightly square apex and well-developed ocular chamber with dimensions of 40-65 x 12-15 µm. Ascospores (4.5-6.5 x 12.5-16 µm) are ellipsoid, aseptate, hyaline, sometimes slightly elongated, often guttulate, and their ends are obtuse with mucilaginous polar appendages (Hanlin, 1990; Kotzé, 2000; Van der Aa, 1973). Spermatia (5-8 x 0.5-1 µm) are hyaline, cylindrical to dumbbell-shaped with guttules at the end, and straight or slightly curved (Van der Aa, 1973).

Phyllosticta citricarpa pycnidia occur on leaves, fruit lesions, and fruit peduncles and are formed in abundance on dead leaves. Pycnidia are globose, dark brown to black, and 115-190 µm in diameter. Conidia are hyaline, one-celled, obovate or ellipsoid, aseptate, multiguttulate bearing a single apical appendage, and 5.5-7 x 8-10.5 µm (Kotzé, 2000; Van der Aa, 1973).

There has been confusion over the years about the identity of the fungus that causes black spot. *Guignardia citricarpa* was reported to be in countries where black spot had never been observed, as well as in 21 plant families (Everett and George, 2006; Kotzé, 1981). However, the confusion about the pathogen identity was partially clarified in 1964 (McOnie, 1964). Two morphologically similar *G. citricarpa* strains that infect citrus were identified (Kotzé, 1981, 2000; McOnie, 1964). The pathogenic strain

that caused black spot symptoms on citrus grew slowly in culture and produced a yellow pigment at the edges of the colonies when grown on oatmeal agar (OA). The other strain did not cause black spot symptoms on citrus, grew faster in culture, and did not produce yellow pigment on OA (Baldassari et al., 2006). With molecular techniques, the non-pathogenic strain was shown to be a ubiquitous endophyte with a wide host range, *G. mangiferae* (anamorph *P. capitalensis*) (Baayen et al., 2002; Baldassari et al., 2006). In order to accurately distinguish between *G. citricarpa* and *G. mangiferae*, specific primers have been developed (Bonants et al., 2003; Meyer et al., 2006; Peres et al., 2007; Van Gent-Pelzer et al., 2006).

The taxonomy of this non-pathogenic endophyte fungus is still in flux. A phylogenetic analysis carried out by Glienke et al. (2011) revealed that *P. capitalensis* is genetically distinct from *G. mangiferae*. Likewise, Wang et al. (2011) found that *P. capitalensis* isolates were distinct from *G. mangiferae*, concluding that *G. mangiferae* is not the teleomorph of *P. capitalensis*. On the other hand, morphological analysis and sequences of the internal transcribed spacer (ITS) region conducted by Okane et al. (2003) identified *G. endophyllicola* as the teleomorphic stage of the endophytic *P. capitalensis*. Recently, further *Phyllosticta* spp. have been found associated with citrus in Asia. Wang et al. (2011) and Wulandari et al. (2008) reported that *P. citriasiana* was isolated from tan spot symptoms on leaves and fruit peel of pomelos and *P. citrichinaensis* was isolated from pomelos, oranges, mandarins and lemons. The latter was considered a minor pathogen of citrus, with mild symptoms and minimal losses. Glienke et al. (2011) also described a new endophytic species, *P. citribraziliensis*, occurring on citrus in Brazil.

Disease Symptoms

Citrus black spot causes blemishes on the fruit rind and premature fruit drop although the internal quality remains unaffected. Lesions on the leaves do not affect the tree, but those infected leaves on the ground are an important source for inoculum (Timmer et al., 2003).

CBS symptoms on fruit are variable and have been categorized with different names. Hard spot or shot-hole spot is the most typical pre-harvest symptom for diagnosis of the disease. The lesions appear when the fruit starts to mature, at color break, on the side of the fruit that is most exposed to sunlight. The lesions are 3-10 mm diameter, circular, depressed, with brick red to black margins, and gray-white necrotic centers (Fig. 1A). On green fruit, the lesions are surrounded by a yellow halo (Bonants et al., 2003; Kotzé, 1981, 2000). Pycnidia may be found at the center of the lesion as slightly elevated black dots, but ascocarps are never formed in fruit lesions (Bonants et al., 2003; Kotzé, 1981). Even though hard spot is the most common symptom of black spot, the causal pathogen is isolated at low frequencies from these lesions (Kotzé, 2000).

Speckled blotch or false melanose is another symptom of black spot. The spots are numerous, small, 1-3 mm diameter, slightly depressed, tan, gray, reddish or dark brownish (Fig. 1B). Speckled blotch develops on maturing fruit, does not contain pycnidia, but in some cases can turn into hard spot as the season progresses (Bonants et al., 2003; Cooke et al., 2009; Kotzé, 1981).

Freckle spot or early virulent spot often appears when the fruit color has changed from green to orange. The lesions are irregularly shaped, slightly depressed, up to 7 mm long, and pycnidia may be present. Freckle spot can coalesce to form one big

lesion, which may turn into mature virulent spot during storage (Cooke et al., 2009; Kotzé, 1981, 2000). Virulent spot lesions are small, reddish, irregularly shaped, and are expressed on heavily infected mature fruit at the end of the season (Fig. 1C). Large numbers of pycnidia can develop in the sunken lesions under high humidity conditions. Virulent lesions can cause important postharvest losses (Cooke et al., 2009; Kotzé, 1981, 2000).

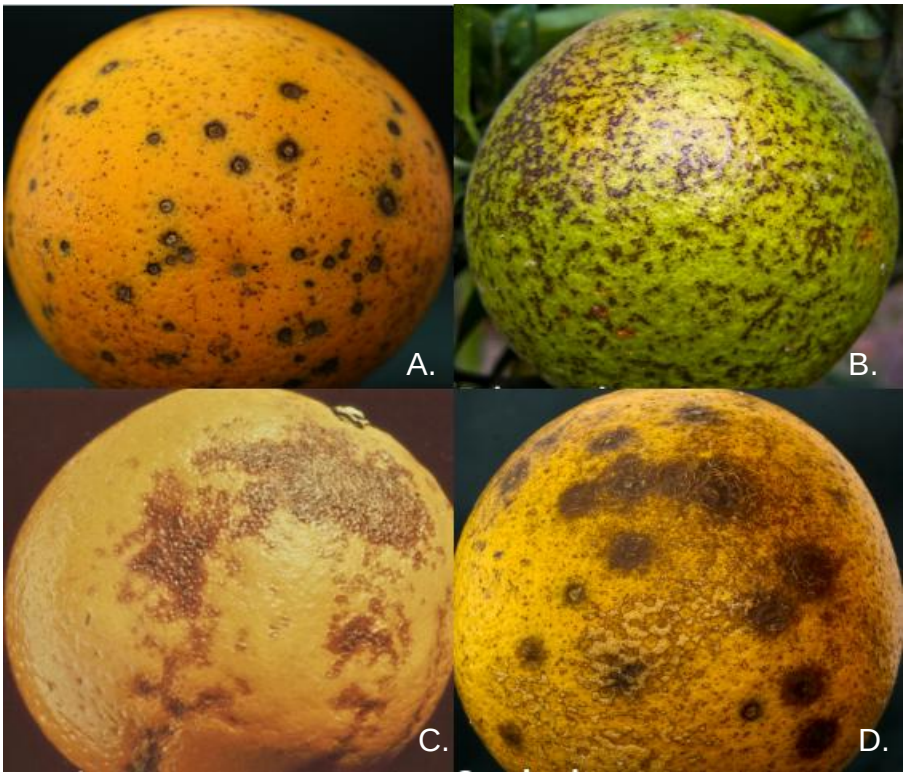

Figure 2-1. Citrus black spot symptoms. A. Hard spot; B. False melanose; C. Virulent spot; D. Cracked spot. Credit: University of Florida UF/IFAS citrus extension.

Finally, cracked spot occurs on green and mature fruit. Lesions are large, flat, dark brown with raised cracks on the surface (Fig. 1D). It is suspected that is caused by an interaction between the pathogen and rust mites (*Phyllocoptruta oleivora*) (Dewdney et al., 2010).

Leaf symptoms are rare, and most often appear on lemons. Fruit peduncles can also show symptoms. When symptoms are present on leaves, they are small, round, sunken and necrotic with gray centers, surrounded by a dark brown margin and yellow halo (Kotzé, 2000).

Epidemiology and Life Cycle

Inoculum availability, climatic conditions favorable for infection and susceptibility of the fruit are factors that influence in the epidemiology of CBS (Kotzé, 2000). Both ascospores and pycnidiospores have a role in establishing epidemics. In South Africa, accordingly to summer rainfall patterns, ascospores are an important source of inoculum (Kotzé, 1981). However, in Brazil, Spósito et al. (2008) found that conidia also have an important role in the disease epidemic.

Ascospores are formed in pseudothecia in the leaf litter on the orchard floor, approximately 50-180 days after leaves drop. Development and maturation of ascospores seem to be influenced by the frequency of wetting and drying cycles of the leaf litter, as well as prevailing temperatures (Kiely, 1948; Kotzé, 1981, 2000). Dead leaves decompose before the development of pseudothecia in cooler production areas (Kotzé, 2000). Lee and Huang (1973) found that moderate and evenly distributed rainfall favored pseudothecial development, whereas prolonged rain resulted in leaf decay and elimination of the pathogen. In Ghana, Brentu et al. (2012) reported pseudothecial and ascospore formation in the leaf litter after 30 to 50 days of wetting and drying periods.

Wind-borne ascospores are released during rainfall or irrigation events. The amount of rain has little effect on the number of ascospores released. The spores are discharged within the first hour of the rain and could continue for 12 hours or longer

(Kotzé, 1981). On the other hand, heavy showers could adversely affect the spore load in the air (Kotzé, 1981). In a Brazilian study, ascospore production was not found to be related to total rainfall or temperature, but loosely related to leaf wetness duration (Reis et al., 2006). According to Reis et al. (2006), as long as the leaves continue to be moist, even a small amount of rain will trigger the release of ascospores.

Pycnidiospores are not wind-borne, but those in leaf litter could reach susceptible fruit by rain splash (Kotzé, 1981, 2000). Truter et al. (2007) reported that *P. citricarpa* pycnidiospores were not able to infect or colonize detached green leaves or leaf litter of Eureka lemons indicating that infected fruit lying on the ground do not represent inoculum source for detached leaves. However, the authors pointed out that the level of pycnidiospores on leaf litter may depend on the level of infection of the young leaves while attached to the tree.

Pycnidiospores serve as a source of inoculum when dead twigs, out-of-season fruit or late-hanging fruit remain on the trees, possibly being washed down to still susceptible young fruit (Kotzé, 1981). In Florida, the cultivar Valencia is well known for producing two crops at the same time (Mossler, 2011), and those contaminated fruit could potentially spread pycnidiospores to susceptible tissue. However, conidia do not survive for long periods (Kotzé, 1981). Epidemiological studies conducted in Australia, established the importance of the ascospores as the main source of inoculum for pathogen spread and disease epidemics (Kiely, 1948). However, spatial pattern analysis in Brazilian groves determined that the spatial distribution of the disease was aggregated within trees with a maximum radius of 24.7 m, which indicates the limitation of the pathogen to disperse over long distances (Spósito et al., 2007). This aggregation

pattern observed in Brazil indirectly indicated that conidia also have an important effect as a source of inoculum within trees in this region (Spósito et al., 2008).

Citrus fruit remains susceptible to infection for four to five months after petal fall (Kotzé, 2000). In Brazil, similar fruit susceptibility periods have been observed (Reis et al., 2005). After that period, fruit becomes resistant regardless weather conditions or inoculum pressure (Kotzé, 2000). Leaves remain susceptible from development for up to 10 months (Truter et al., 2007).

For infection to occur, moisture is necessary for spore germination and appressorium formation (Fig. 2-2). A wetting period of 24-48 hours is required for the fungus to infect (Kotzé, 1981). From the appressorium, a thin penetration peg invades the cuticle and expands into a small mass of mycelium between the cuticle and the epidermal wall (Kotzé, 1981; McOnie, 1967). After infection is complete, the pathogen remains quiescent until the fruit become fully grown or mature when it grows further into the rind tissue producing black spot symptoms (Kotzé, 1981; McOnie, 1967).

Kotzé (1981) identified different factors that could affect symptom development: i. Temperature, rising temperatures stimulates symptom expression on mature fruit; ii. Light, high light exposure of fruit induces lesion development; iii. Drought, fruit from wilted trees had more black spot lesions than the fruit from trees that were not wilted; iv. Maturity of the fruit, the more mature a fruit become (changing from green to yellow), the higher the chances for the symptoms to appear; v. Tree vigor, CBS symptoms are more severe on fruit from older trees than fruit from vigorous young trees. In Ghana, Brentu et al. (2012) also found that disease incidence was lower in the young groves

surveyed than in older groves. Also, disease severity within a tree was not uniform; fruit on the side exposed to more sunlight had higher disease severity.

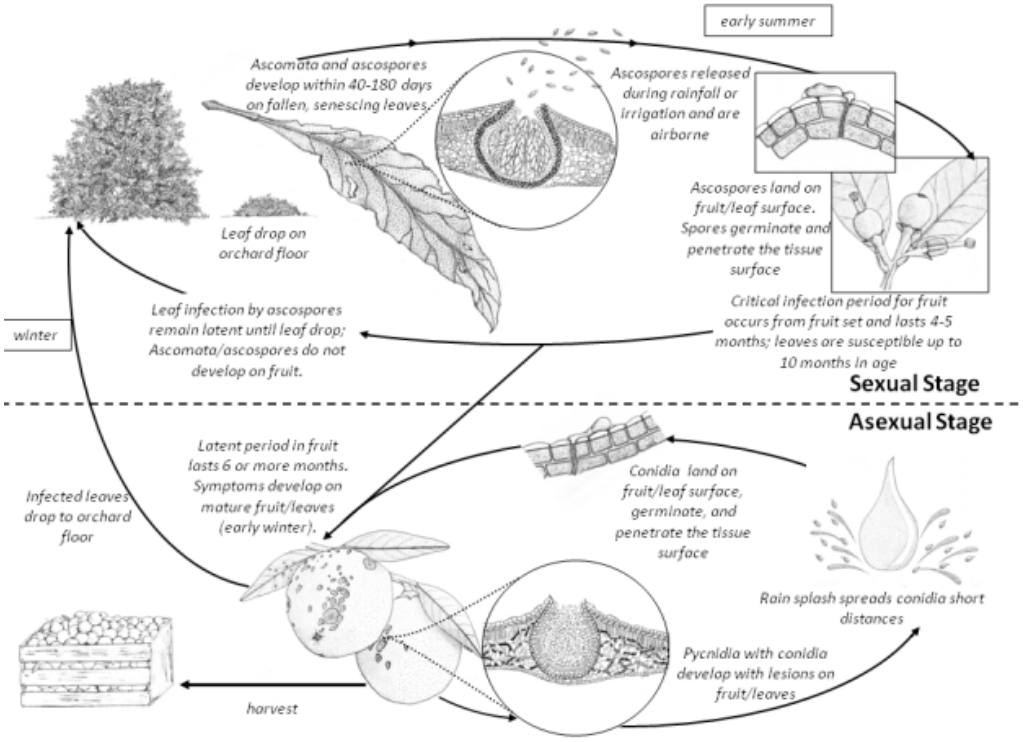

Figure 2-2. Life cycle of *Guignardia citricarpa*, the causal agent of citrus black spot. Figure by Hartzog, in: Holtz, 2010.

In vitro studies with *Phyllosticta* species conducted by Hoch et al. (2006) indicated that conidial attachment, germination and appressorium formation proceeded under hydrophobic surfaces, but not on nutrient agar, which is hydrophilic. It is thought that *Phyllosticta* species evolved the requirement to attach to hydrophobic surfaces because they are often found growing on hydrophobic plant tissues with waxy cuticle coatings (Hoch et al., 2006). Korf (1998) reported that conidial germination of *P. citricarpa in vitro* was increased by the addition of Valencia orange juice with a pH between 4.0 and 4.2. The extra stimulus required by conidia to germinate is thought to be provided by the juice nutrients. The same author also found that the optimal

temperature for conidial germination was 22^{o}C, and light was not important. Likewise, Mendes et al. (2005) found that the optimum temperature for conidial germination of *P. citricarpa* was between 18 and 28^{o}C, after 22 to 24 h of incubation. Meanwhile, for ascospores, Timossi et al. (2003) found that 24^{o}C was the optimum temperature for germination after 16 hours of incubation.

Disease Management

Understanding different factors such as the life cycle of a pathogen, environmental conditions conducive for disease, and the host characteristics are essential for the effective management of any disease (Maloy, 2005). Chemical and cultural practices currently used to control black spot are based on information generated from regions where the disease has been present for a long time.

Cultural Control

From understanding the role of ascospores and pycnidia in the life cycle of *G. citricarpa*, different cultural practices have been used in citrus orchards to reduce inoculum and to restrict pathogen spread. Sanitation practices in the orchard prior the harvesting time and after leaf drop may reduce or eradicate ascospores and pycnidiospores (Kotzé, 1981). In Brazil, Spósito (2004) evaluated the effect of ascospore suppression by removing the leaves from the orchard floor, as well as pycnidiospores suppression by early harvesting late matured fruit. The author reported reduction in disease severity by those treatments. Moreover, Bellotte (2009) evaluated leaf litter decomposition by using urea, calcium nitrate, dolomite lime, and two commercial products; all treatments provided reduction in disease severity by reducing ascospore inoculum.

Based on information on cultural practices in other areas, one recommended practice in Florida is to promote leaf litter decomposition. Leaf litter decomposition should start in mid-March, and can be promoted through different methods. One method is to increase irrigation frequency by using microsprinklers at least 5 times a week for a half hour per irrigation period for 1.5 months. A second method is the application of urea (209.6 kg/ha) or ammonium sulfate (628.8 kg/ha) to the leaf litter. Nitrate fertilizers, however, did not reduce the spore numbers of the citrus pathogen *Mycosphaerella citri*, the causal agent of greasy spot also found in the leaf litter. The third method is the application of dolomitic lime or calcium carbonate (2,495 kg/ha) to the leaf litter. All these methods have been shown to reduce the number of *M. citri* spores equally in the leaf litter, and it is expected that they can also reduce the ascospore numbers of *G. citricarpa* in the leaf litter (Mondal and Timmer, 2003; Mondal et al., 2007).

Other recommended cultural practice to restrict the spread of the pathogen includes minimizing plant trash movement within grove or among groves. While most citrus leaves do not show black spot symptoms, they could carry the ascospores, which are the main source of inoculum, and inappropriate movement of asymptomatic leaves or other trash could transport the pathogen to other sites (Dewdney et al., 2012; Florida CHRP working group, 2010).

Avoidance of citrus cultivars with off-season bloom and removal of declining trees is also recommended. Trees with different ages of fruit allow fruit-to-fruit infection via conidia, amplifying the disease (Dewdney et al., 2012; Florida CHRP working group, 2010). Furthermore, a good nutritional management program should be implemented

since stressed trees express more black spot symptoms (Florida CHRP working group, 2010; Kotzé, 1981).

To reduce the leaf wetness, it is recommended to increase the air flow in the canopy. Moreover, it is important to remove dead wood from the canopy because *G. citricarpa* can colonize and reproduce in dead twigs (Dewdney et al., 2012; Florida CHRP working group, 2010; Kotzé, 1981).

To avoid introduction of black spot within a grove, it is important to use planting stock from disease-free nurseries. In Florida, there is no nursery near known infected groves, but this may change as the distribution of the disease expands (Dewdney et al., 2012; Florida CHRP working group, 2010).

In Australia, where CBS has been present for many years, post-harvest fruit exposure to temperatures above 20^{o}C is avoided, since high temperatures can trigger disease expression (Cooke et al., 2009).

Chemical Control

Fungicide applications are essential for the control of black spot where the disease has been established. Protective and systemic products have been used to control CBS (Goes, 2002). From 1971 to 1982, single applications of benomyl to control the disease in South Africa were used until the pathogen became resistant to benomyl after 11 years of use (Herbert and Grech, 1985). Currently, in other areas, the disease is controlled with the application of fungicides from different groups such as benzimidazoles, strobilurins, dithiocarbamates and copper (Da Silva et al., 2009); however, only strobilurins and copper are registered for black spot control in Florida.

Under Florida conditions, monthly applications of copper or strobilurin fungicides (azoxystrobin, pyraclostrobin or trifloxystrobin) are recommended from early May to

mid-September to control black spot. Those products have shown to be effective against the disease in other regions of the world (Dewdney et al., 2012; Fogliata et al., 2011; Goes, 2002; Miles et al., 2004). Only four strobilurin applications are allowed per season. Thus, it is recommended to reserve strobilurin applications for periods when copper phytotoxicity may occur (temperatures exceeding 34^{o}C), especially when applied on fruit for the fresh market. Two consecutive sprays of strobilurin fungicides should be avoided to manage the development of pathogen resistance. Application in nurseries should also be avoided since these could lead to selection of resistant strains that could be distributed to groves (Dewdney, 2010; Dewdney et al., 2012; Florida CHRP working group, 2010).

The best application method of those fungicides is through the use of air-blast sprayers, using a volume of 2338 L/ha for application to ensure full coverage of fruit and leaves (Dewdney et al., 2010; Florida CHRP working group, 2010).

As part of an integrated disease management, it has been considered important to establish spore trapping and to monitor environmental conditions (rainfall, dew periods and temperature) to determine the time and intensity of ascospore release to better time the application of protective fungicides (Kotzé, 2000; USDA, 2002).

Strobilurin Fungicides

Quinone outside inhibitors (QoI), also known as strobilurins, are an important class of fungicides for agriculture. They were first marketed in 1996 and by 2002, there were six commercially available strobilurin fungicides (Bartlett et al., 2002). By 1999, strobilurins represented 10% of the global fungicide market with sales of $415 million and they are registered for use on 84 different crops in 72 countries (Bartlett et al., 2002).

The natural fungicidal derivatives of β-methoxyacrylic acid, a secondary metabolite, are strobilurin A, oudemansin A, and myxothiazol A, and they are produced by Basidiomycete wood-rotting fungi such as *Strobilurus tenacellus* (Bartlett et al., 2002).

The mode of action of strobilurin fungicides is based on the inhibition of mitochondrial respiration by binding at the Q_o site of the cytochrome *b*. Cytochrome *b* is located in the bc_1 complex (complex III) in the inner mitochondrial membrane of fungi and other eukaryotes. When binding to the Q_o site takes place, electron transfer between cytochrome *b* and cytochrome c_1 is blocked, disrupting production of pathogen ATP (Bartlett et al., 2002; Gisi and Sierotzki, 2008).

For true fungi, spore germination, and for fungi like-organisms, zoospore motility, are stages that are particularly sensitive to strobilurins since these stages are highly energy demanding and this mode of action disrupts energy production (Bartlett et al., 2002).

Several plant pathogens can avoid the toxic effects of Q_oI fungicides by the expression of alternative oxidase pathway, to sustain ATP synthesis (Jin et al., 2009). The alternative oxidase pathway takes place in the inner mitochondrial membrane (Vanlerberghe and McIntosh, 1997) and it can be inhibited by salicylhydroxamic acid (SHAM) and *n*-propyl gallate (Schonbaum et al., 1971; Siedow and Bickett, 1981). For this reason, SHAM is usually added to Q_oI fungicides when tested *in vitro* (Duan et al., 2012). The addition of SHAM to azoxystrobin to *in vitro* tests with *Sclerotinia sclerotiorum* allowed pathogen inhibition, whereas there was none when azoxystrobin was used alone (Duan et al., 2012). On the other hand, the mixture of SHAM and

azoxystrobin in *in vitro* tests with *Colletotrichum capsici*, *Botrytis cinerea*, *Rhizoctonia solani*, and *Magnaporte grisea* showed a synergistic effect on mycelium inhibition; however, as time passed, mycelium respiration did rise and SHAM did not reduce the oxygen consumption (Jin et al., 2009). It is thought that alternative respiration does not have an important role during infections in planta, possibly due to host flavones that interfere with the activation of this pathway (Vincelli and Dixon 2002), but it maintains viability of the fungus *in vitro*, thus, it should be blocked during *in vitro* studies (Duan et al., 2012).

All commercial formulations of strobilurin fungicides have broad spectrum activity against the four major groups of plant pathogenic fungi, but the level of control varies according to the type of strobilurin used. Studies of toxicity in this group of fungicides indicates that they represent minimal risk to human health, as well as to the environment since they are readily degraded through adsorption, microbial degradation and photolysis (Bartlett et al., 2002).

The first report of resistance to Q_oI fungicides was in 1998 in wheat powdery mildew (*Blumeria graminis* f.sp. *tritici*). In 1999, barley powdery mildew (*B. graminis* f.sp. *hordei*) also developed resistance to these fungicides (Heaney et al., 2000). In those cases, resistance was associated with a single-point mutation in the cytochrome *b* gene which leads to a change from glycine (G) to alanine (A) at amino acid residue 143 (G143A) (Gisi et al., 2000; Heaney et al., 2000). It was later confirmed with other fungal plant pathogens that this point mutation was responsible for the loss of disease control when Q_oI fungicides were used as the sole product (Gisi and Sierotzki, 2008). A second amino acid substitution that has been shown to reduce sensitivity to Q_oI fungicides is

the replacement of phenylalanine with leucine at position 129 (F129L) (Pasche et al., 2004). A third amino acid substitution from glycine to arginine at the position 137 (G137R) was detected recently in *Pyrenophora tritici-repentis*, conferring to the pathogen a reduced sensitivity to the Q_oI fungicides (Siertozki, 2007).

Managing the build-up of pathogen resistance to Q_oI fungicides is an important matter, especially for the citrus industry in Florida, since the availability of effective alternatives is limited for rotational purposes, and inappropriate use would reduce the number of products for black spot. For this reason, it is important to follow the guidelines established by the Fungicide Resistance Action Committee (FRAC) for the use of Q_oI fungicides (FRAC, 2010).

Azoxystrobin

Azoxystrobin is one of the strobilurin fungicides labeled for use on citrus; it was first marketed in 1996 by Syngenta Crop Protection. It has a broad spectrum of activity, can be used in a wide range of crops and could increase yield. Azoxystrobin can be taken up into the leaf cells and also can move to new growing parts of the plant through systemic xylem movement (Bartlett et al., 2002). It can be considered as a protective and curative fungicide and can be used as a foliar, seed or soil treatments (Schutte et al., 2003).

Field evaluations of azoxystrobin carried out by Miles et al. (2004) in Queensland, Australia, demonstrated that azoxystrobin was as effective as or more so than the industry standard copper/mancozeb for controlling citrus black spot and reduced fruit rind damage compared to the standard products.

Schutte et al. (2003) also reported up to 100% control of black spot in South Africa when azoxystrobin was used in tank mixtures with mancozeb and mineral oil.

However, *in vitro* studies on mycelium inhibition of *G. citricarpa* indicated that even at high concentrations of azoxystrobin, the pathogen could not be completely inhibited, although sporulation rate was reduced up to 100% (Possiede et al., 2009).

Pyraclostrobin

Pyraclostrobin is one of the newest strobilurin fungicides on the market. It was first marketed in 2002 by BASF Corporation. It has broad spectrum activity and can be used on a wide range of crops (Bartlett et al., 2002).

Pyraclostrobin is not a xylem systemic fungicide. *In vitro* studies conducted by Karadimos et al. (2005) using pyraclostrobin to control *Cercospora beticola* on sugar beet suggested that this strobilurin fungicide has some translaminar activity which helps it to penetrate the leaf tissue and be deposited on the cuticle of the opposite leaf surface, inhibiting spore germination, spore production and mycelium growth. Similar results were obtained by Ammermann et al. (2000), which strongly suggest that pyraclostrobin has protectant, curative and translaminar activity, affecting different developmental stages of the fungus (Stierl et al., 2000).

Pyraclostrobin has shown good black spot control in the field. Rodriguez et al. (2010) demonstrated up to 88% disease control when pyraclostrobin was applied twice in a season. Almeida (2009) also reported good protective activity of pyraclostrobin even at a high inoculum pressure in Brazil. Fogliata et al. (2011) compared the efficacy of three different strobilurins with copper and mancozeb in the control of CBS on lemons in Argentina. Two applications of azoxystrobin, pyraclostrobin and trifloxystrobin in a season provided up to 96% control of the disease and there was no difference in the efficacy of the strobilurins. These results suggest that the strobilurin fungicides provide good control of black spot even under environmental conditions favorable to the

disease, but the high risk of pathogen resistance to these fungicides should limit their usage (Fishel, 2012).

Triazole Fungicides

The fungicides belonging to the triazole group are also known as the demethylation inhibitors (DMI). Among other fungicides in this group are the imidazoles, piperazines, pyridines and pyrimidines (FRAC, 2012). DMI fungicides inhibit demethylation at the 14-α carbon of lanosterol or 24-methylene dihydrolanosterol which are the substrates for the cytochrome P450-dependent 14α-demethylase in the biosynthesis of fungal sterols, for example, ergosterol (Gisi et al., 2000). Most fungi are able to synthesize ergosterol as their main sterol (Mercer, 1991). The absence of ergosterol and the increase of other compounds promote fungal cell wall disorganization, inducing disruption of the membrane (Zambolim et al., 2007).

Sterol-biosynthesis inhibiting fungicides were developed and registered in the 1970s for many crops (Mercer, 1991). These fungicides have local systemic activity in the apoplast and have protective and curative activity against a wide spectrum of foliar, root and seedling diseases, and for instance they can be applied as foliar, seed or soil treatments (Agrios, 2005; Bushong and Timmer, 2000).

There is a medium risk for the development of pathogen resistance to the DMI fungicides (FRAC, 2012). A single-point mutation on the *CYP51* gene causing an amino acid change from tyrosine (Y) to phenylalanine (F) in the 136 position (Y136F) was responsible for conferring resistance to *Erysiphe graminis* and *Uncinula necator* to the DMIs (Gisi et al., 2000). Moreover, five different mutations (G129A, Y132H, S405F, G464S, and R467K) at the *CYP51* gene of *Candida albicans* conferred resistance to the azole fungicide group (Sanglard et al., 1998). Although there are differences in the

spectrum of activity of the DMIs, cross-resistance is expressed among all DMI compounds that are active against the same pathogen (FRAC, 2012; Gisi et al., 1997). For this reason, the FRAC guidelines should be taken in to consideration for resistance management of these fungicides (FRAC, 2012; Zambolim et al., 2007).

In vitro studies on the control of CBS in South Africa using fungicides from the DMI group showed that difenoconazole considerably reduced mycelial growth of the pathogen (Korf, 1998); however, in another study using imazalil, mycelial growth could not be completely inhibited even at high doses of this fungicide (Deising et al., 2007).

Overall, fungicides of this group are considered as non-toxic for birds and bees, but appropriate disposal of the product must be followed to not harm the environment (Fishel, 2011).

Fenbuconazole

Fenbuconazole is a triazole fungicide that was first introduced in 1988 (Russell, 2005). The fungicide has protective activity against a broad spectrum of pathogens of many crops (Bushong and Timmer, 2000; Russell, 2005).

Although fenbuconazole is registered for citrus in Florida, it is not labeled for the control of black spot. Fenbuconazole has been reported to be effective for the control of different fungal pathogens in citrus. Field experiments showed that fenbuconazole provided good control of *Elsinoë fawcettii*, the causal agent of citrus scab (Timmer and Zitko, 1997), as well as controlling *M. citri* (Mondal and Timmer, 2006). Nevertheless, the effectiveness of the fungicide in controlling greasy spot was reduced when the applications were conducted after inoculation indicating that fenbuconazole had better activity when applied preventively (Mondal and Timmer, 2006). Holb and Schnabel (2006) found that protective applications of fenbuconazole were significantly more

effective in controlling mycelial growth and disease development of *Monilinia fructicola* than curative applications. Regardless of the good control of some citrus pathogens, fenbuconazole has been ineffective against melanose caused by *Diaporthe citri* and Alternaria brown spot caused by *Alternaria alternata* (Bushong and Timmer, 2000; Timmer and Zitko, 1997).

Objective

The objective of this project is to evaluate strobilurin and triazole fungicides for *in vitro* activity and to determine the baseline sensitivity of *Guignardia citricarpa* isolates from Florida.

CHAPTER 3
BASELINE SENSITIVITY OF CITRUS BLACK SPOT ISOLATES TO AZOXYSTROBIN, PYRACLOSTROBIN AND FENBUCONAZOLE.

Citrus black spot (CBS), caused by *Guignardia citricarpa* Kiely, anamorph stage *Phyllosticta citricarpa* (McAlpine) Van der Aa, was an exotic disease to Florida. In April 2010, the presence of the disease in Florida was confirmed by the U.S Department of Agriculture's Animal Plant Health Inspection Service (APHIS, 2010; Schubert et al., 2012). Most commercially grown citrus species including grapefruit, tangerines, sweet oranges, and lemons are susceptible to CBS. However, sour orange has been shown to be resistant (EPPO, 2009). Citrus black spot causes extensive blemishes on the fruit rind affecting the fruit appeal for the fresh market, although the internal quality remains unaffected. On the other hand, premature fruit drop may occur, reducing crop yield (Baldassari et al., 2006; Timmer et al., 2003). If not controlled in Florida, an estimated $847 million dollars in losses could occur due to the disease (Holtz, 2010).

Control of black spot is mainly based on preventive applications of fungicides during the period of fruit susceptibility (Schutte et al., 2003); nevertheless, cultural practices have been implemented in Florida to reduce inoculum and pathogen spread (Dewdney et al., 2012; Florida CHRP working group, 2010). In other areas where CBS is present, the disease is controlled with fungicides from different groups such as the benzimidazoles, strobilurins (Q_oI), dithiocarbamates, and copper products (Da Silva et al., 2009; Schutte et al., 2003). Currently in Florida, only strobilurin and copper products are registered for the control of black spot. Monthly applications of copper and strobilurins (azoxystrobin, pyraclostrobin or trifloxystrobin) are recommended from early May to mid-September; but there is a label limit of four strobilurin applications in a season. For this reason, it is suggested to reserve strobilurin fungicides for times when

there is concern about copper phytotoxicity (temperatures exceeding 34^{o}C), especially when applied for fresh fruit (Dewdney, 2010; Dewdney et al., 2012; Florida CHRP working group, 2010).

Strobilurin fungicides block electron transport at the Quinol-oxidizing site of the cytochrome *b* complex (complex III), disrupting ATP production (Bartlett et al., 2002; Gisi and Sierotzki, 2007). Spore germination is the fungal stage that is particularly sensitive to strobilurins (Bartlett et al., 2002). The mode of action of this group of fungicides is highly specific and many different pathogens have lost sensitivity to Q_oI fungicides due to a single point of mutation that leads to a change from glycine (G) to alanine (A) at amino acid residue 143 in the cytochrome *b* gene (G143A) (Gisi and Sierotzki, 2007; Gisi et al., 2000; Heaney et al., 2000). A second amino acid substitution that has been shown to reduce sensitivity to Q_oI fungicides is the replacement of phenylalanine with leucine at position 129 (F129L) (Pasche et al., 2004). A third amino acid substitution from glycine to arginine at the position 137 (G137R) was detected recently in *Pyrenophora tritici-repentis*, conferring to the pathogen, a reduced sensitivity to Q_oI fungicides (Siertozki, 2007).

Fenbuconazole, which belongs to the triazole group of fungicides, has been used in Florida since 1999 for the control of greasy spot (caused by *Mycosphaerella citri*) on grapefruit (Mossler, 2011) and is also effective against citrus scab (caused by *Elsinoë fawcettii*) (Timmer and Zitko, 1997). Triazoles are known as demethylation inhibitors (DMI) and act by inhibiting the biosynthesis of fungal sterols, such as ergosterol (Gisi et al., 2000). Resistance to DMIs fungicides has been reported. Five different mutations (G129A, Y132H, S405F, G464S, and R467K) at the *CYP51* gene of *Candida albicans*

conferred resistance to the azole fungicide group (Sanglard et al., 1998). Moreover, a single point of mutation in the *CYP51* gene, leading to an amino acid change from tyrosine to phenylalanine at position 136 (Y136F) conferred resistance to *Erysiphe graminis* and *Uncinula necator* (Gisi et al., 2000).

Since black spot control mainly relies on fungicide applications, it is necessary to determine the baseline sensitivity of the pathogen to monitor for future shifts in population sensitivity. Azoxystrobin and pyraclostrobin have been reported to provide good control of black spot in the field as well as in *in vitro* studies (Almeida, 2009; Fogliata et al., 2011; Miles et al., 2004; Rodriguez et al., 2010; Schutte et al., 2003). Fenbuconazole, however, has not been tested for control of black spot. Due to the site-specific mode of action of strobilurin and DMI fungicides, the potential of resistance development should not be ignored. Although *G. citricarpa* resistance to strobilurins or DMIs has not been reported, *in vitro* studies determined that mycelium growth was not completely inhibited even at high concentrations of azoxystrobin (Possiede et al., 2009). On the other hand, the same author pointed out that the variation in sensitivity to the fungicide is possibly related to genetic variability of *G. citricarpa* isolates. The baseline sensitivity of other Florida citrus pathogens such as *Colletotrichum acutatum*, *Alternaria alternata*, *Elsinoë fawcettii*, *Diaporthe citri* and *Mycosphaerella citri* to azoxystrobin, pyraclostrobin and fenbuconazole has been determined (Mondal et al., 2005). Most isolates from the different pathogens were sensitive or tolerant to the fungicides tested with the exception *of A. alternata* to azoxystrobin (Mondal et al., 2005). More recently, resistance to this fungicide has been reported (Vega et al., 2012)

The evaluation of azoxystrobin, pyraclostrobin and fenbuconazole for *in vitro* activity will help to determine the baseline sensitivity of *G. citricarpa* isolates from Florida and to monitor future shifts in sensitivity of this recently introduced pathogen.

Materials and Methods

Fungal Isolates and Culture

Fifty isolates of *G. citricarpa* from the two Florida counties where CBS is present (Table 3-1), were evaluated for their *in vitro* sensitivity to the fungicides azoxystrobin, pyraclostrobin and fenbuconazole. Briefly, symptomatic fruit were washed, surface disinfested in a 5% NaOCl solution, rinsed in sterile deionized water (SDW) and air dried for 2-3 h. The lesions were excised, placed in 50% ethanol for 30 sec, then 5% NaOCl solution and then rinsed with SDW for 7 min. The lesions were thoroughly rinsed and dried in a laminar air-flow hood. Sections of the lesions were placed on carrot agar (CA; Peres et al. 2007) and incubated at room temperature with 12 h of light. If pycnidia were selected for isolation, instead of laying individual pycnidia on CA, they were placed on moistened sterile filter paper and incubated overnight. Pycnidia were selected under the stereomicroscope and individually placed on CA. All isolations were incubated for 5-6 days. Isolates with typical morphology were placed on the indicator media oatmeal agar and observed for yellow halo production (Baayen et al. 2002). Each isolate was also subjected to PCR identification with the primer sets NP-Br-ITS-Gc and NP-Br-ITS-Gm as described by Peres et al. (2007). The isolates were single-spored before the commencement of the assays. To obtain single-spore isolates, a 10^5 conidia/ml suspension was made and a 20 µl aliquot was spread onto potato dextrose agar (PDA). Plates were incubated for two days and germinated conidia were picked from the media

surface with a flame-sterilized needle under a stereomicroscope. The conidia were placed onto fresh PDA plates and incubated for a week.

For long-term storage, all isolates were kept on sterile filter paper in sealed plastic containers containing $CaSO_4$ desiccant at -20^{o}C. For mycelium and conidium production, *G. citricarpa* isolates were transferred to half-strength potato dextrose agar (½ PDA) and grown for 14 days at 25^{o}C.

Table 3-1. List of isolates, location and source of isolates evaluated for baseline sensitivity. Isolations were made from Valencia fruit in 2010 and 2011.

Isolate no.	Location County	Symptom type or structure
11-27[b]	Collier	Pycnidia
11-28[a,c]	Collier	Pycnidia
11-29[a,c]	Collier	Pycnidia
11-30	Collier	Pycnidia
11-31	Collier	Pycnidia
11-32[a,b,c]	Collier	Pycnidia
11-33[b]	Collier	Pycnidia
11-34[a,b,c]	Collier	Pycnidia
11-35	Collier	Pycnidia
11-36[a,c]	Collier	Pycnidia
11-120[b]	Collier	Freckle spot
11-121	Collier	Freckle spot
11-122[b]	Collier	Freckle spot
11-123[a,b,c]	Collier	Freckle spot
11-124	Collier	Freckle spot
11-125	Collier	Freckle spot
11-126[a,b,c]	Collier	Freckle spot
11-127[a,b,c]	Collier	Freckle spot
11-128[a,c]	Collier	Freckle spot
11-129[a,c]	Collier	Freckle spot
11-133	Collier	Pycnidia
11-134	Collier	Pycnidia
11-135	Collier	Pycnidia
11-136	Collier	Pycnidia
11-137[b]	Collier	Pycnidia
11-138[b]	Collier	Pycnidia

Table 3-1. Continued.

Isolate no.	Location County	Symptom type or structure
11-139	Collier	Pycnidia
11-140	Collier	Pycnidia
11-141	Collier	Pycnidia
11-142	Collier	Pycnidia
11-150[a]	Hendry	Hard spot
11-151[b]	Hendry	Hard spot
11-152[a]	Hendry	Hard spot
11-153	Hendry	Hard spot
11-154	Hendry	Hard spot
11-155	Hendry	Hard spot
11-156[b]	Hendry	Hard spot
11-157	Hendry	Hard spot
11-158	Hendry	Hard spot
11-159[b]	Hendry	Hard spot
11-160[a,b]	Hendry	Hard spot
11-161[b]	Hendry	Hard spot
11-162[b]	Hendry	Hard spot
11-163[a,b]	Hendry	Hard spot
11-164[a]	Hendry	Hard spot
11-165	Hendry	Hard spot
11-166[b]	Hendry	Hard spot
11-167	Hendry	Hard spot
11-168[b]	Hendry	Hard spot
11-169	Hendry	Hard spot

[a] Isolates used to determine the effect of SHAM at 10 and 100 µg/ml on mycelial growth inhibition and spore germination inhibition .
[b] Isolates used to test the effect of different SHAM concentrations.
[c] Isolates used to test azoxystrobin technical grade vs. commercial grade.

Mycelium Growth Inhibition Assay

Commercial formulations of the following fungicides were used: azoxystrobin (Abound®, Syngenta Crop Protection), pyraclostrobin (Headline® SC, BASF Corporation) and fenbuconazole (Enable® 2F, Dow AgroSciences). These fungicides were diluted in SDW to prepare stock solutions of 100 and 1 mg of active ingredient/ml. From 1 mg/ml stock, 0, 3.5, 35 and 350 µl and from 100 mg/ml stock, 35 and 350 µl

were added to molten half-strength PDA (3500 ml) after cooling to 55^{o}C to obtain final concentrations of 0, 0.001, 0.01, 0.1, 1, and 10 µg of active ingredient per ml.

Twenty ml of amended PDA was poured into 100-mm diameter petri dishes using a sterile bottle top dispenser (Fisherbrand). Three-mm diameter mycelium plugs from the actively growing area of the fungal colony were placed at the center of each plate. Three replicates were used for each fungicide concentration.

Plates were incubated for 14 days at 25^{o}C under continuous light, and colony diameter was determined for each of the 50 isolates as the average of two perpendicular measurements. The diameter of the mycelium plug was subtracted from the average colony diameter for each replicate.

The percent inhibition of the fungicide amended plates was calculated relative to the growth of the non-amended control for each isolate. Each experiment was conducted twice.

Spore Germination Inhibition Assay

Pycnidiospore production was done as described by Kuo and Hoch (1996); however, some modifications were implemented. Isolates of *G. citricarpa* were cultured on ½ PDA for 14 days. Then, conidia were washed off using 4 ml of sterile water with 0.02% Tween 20. The suspension was transferred to four micro-centrifuge tubes, each with 1 ml of the suspension and centrifuged at 5000 rpm for 5 min. The supernatant was discarded and the pellet resuspended with sterile water. Once the pellets from the four tubes were combined for each isolate, they were centrifuged again at 5000 rpm for 5 min. This step was repeated twice. Finally, the conidial concentration was adjusted at 10^{6} spores/ml using a hemacytometer.

A germination medium consisting of 2% of Valencia orange juice (pH 4.0) was prepared to stimulate conidial germination (Korf, 1998). Germination was assessed in hydrophobic slides (Fisher Scientific) and each well contained a volume of 10 μl. Ten-fold serial dilutions of strobilurin fungicides ranging from 0.001 to 1 μg/ml were prepared prior their addition to the wells. Each well received 7.5 μl of 2% Valencia juice, 1.25 μl of the corresponding fungicide concentration, and 1.25 μl of the adjusted conidial suspension. Control wells contained 1.25 μl of SDW instead of the fungicide. Hence, the suspension in each well had a 3:1 ratio of Valencia orange juice to medium.

Hydrophobic slides were placed into a humidified petri dish to prevent the medium from desiccation (Kuo and Hoch, 1996; Noronha, 2002). All slides were kept in a humid chamber for 20 h. After 20 h of incubation, a cover slip was placed on each slide and 100 conidia were observed under the microscope at 400x to determine the percent germinated conidia. A conidium was considered germinated if the germ tube was equal or longer than the length of the conidium. There were three replicates for each of the 50 isolates and the experiment was conducted twice. The percent spore inhibition was calculated for each isolate-strobilurin fungicide-experiment combination.

Effect of Strobilurin Fungicides and SHAM on Mycelium Growth

Fifteen *G. citricarpa* isolates were tested to determine whether salicylhydroxamic acid (SHAM) affected the response of fungal growth to azoxystrobin and pyraclostrobin. SHAM was dissolved in methanol at 0.1 mg/ml. The amount of methanol in the media was 0.1% (vol/vol). Half-strength PDA (900 ml) was amended with 9 and 90 mg of SHAM to obtain final concentrations of 10 and 100 μg/ml.

The effect of SHAM at 10 and 100 μg/ml on fungal growth was evaluated in combination with final fungicide concentrations of 0, 0.001, 0.01, 0.1, 1, and 10 μg/ml. Percent growth inhibition was based on comparison with the SHAM plates with no fungicide. Inoculation method and measurements were done as described for mycelium inhibition assay, with three replications per concentration, and the experiment was conducted twice.

Effect of Strobilurin Fungicides and SHAM on Spore Germination

The same isolates used in the previous experiment were used to evaluate the effect of SHAM on spore germination inhibition. Different SHAM concentrations were tested prior to the addition of strobilurin fungicides. A stock suspension of SHAM at 0.1 mg/ml was serially diluted to obtain final concentrations in each well of 10, 25, 50 and 100 μg/ml of SHAM. The content of methanol in each well was 0.1% vol/vol. Each well received 7.5 μl of 2% Valencia orange juice, 1.25 μl of the adjusted spore suspension, and 1.25 μl of the corresponding SHAM concentration. Control wells received 1.25 μl of SDW instead of SHAM. Thus, the suspension in each well had a 3:1 ratio of Valencia orange juice to medium.

The effect of SHAM at 10 μg/ml on spore germination was evaluated in combination with final strobilurin fungicides concentrations of 0, 0.001, 0.01, 0.1, and 1 μg/ml. In order to maintain a 3:1 ratio of Valencia Juice to medium and SHAM, there were modifications in the amount of medium added to each well. Therefore, each well received 7.5 μl of 2% orange juice, 0.83 μl of each fungicide concentration, 0.83 μl of SHAM, and 0.83 μl of the adjusted conidial suspension. Control wells received 0.83 μl of SDW instead of fungicide.

After the slides were loaded with the suspension, the same procedure as described in the spore inhibition assay was followed. There were three replications per isolate-concentration and the experiment was conducted twice. Percent inhibition was determined for each isolate-fungicide-experiment combination.

Effect of SHAM on Mycelium Inhibition

The effect of different concentrations of SHAM was tested on mycelium growth of twenty isolates of *G. citricarpa* without the addition of strobiliurin fungicides.

SHAM was diluted at 0.1 mg/ml in methanol at 0.1% vol/vol of media. Half-strength PDA (1200 ml) was amended with 12, 30, 60 and 120 mg of SHAM to obtain final concentrations of 10, 25, 50 and 100 µg/ml. There were three replications per concentration and the experiment was done twice.

Inoculations and measurements were done as described for the mycelium inhibition assay. Percent inhibition was calculated relative to the non-amended SHAM plates and was subjected to Analysis of Variance and mean separation using the Least Significant Difference (LSD).

Azoxystrobin Technical vs. Commercial Grade and the Effect of the Different Grades and SHAM on Mycelium Inhibition

To test whether or not there was a difference between commercial formulation of azoxystrobin (Abound®, Syngenta Crop Protection) or technical grade active ingredient, ten isolates of *G. citricarpa* were tested against the two forms of the chemical. Azoxystrobin commercial and technical grade stock solutions of 100 µg of active ingredient per ml were diluted in water or acetone, respectively, and added to molten ½ PDA at 0, 0.001, 0.01, 0.1, 1 and 10 µg/ml.

In addition, the effect of SHAM at 100 µg/ml, added to commercial and technical grade azoxystrobin, was also tested on mycelium growth inhibition. The dilution method was done as described for the effect of SHAM on mycelium growth assay.

For both tests, there were three replications per isolate-fungicide concentration and each experiment was repeated twice. Inoculation method, measurements, and calculation of percent inhibition was done as described for mycelium inhibition assay.

EC_{50} Calculation and Statistical Analysis

For mycelium inhibition assays, the effective concentration to reduce growth by 50% (EC_{50}) was determined by fitting a four parameter logistic (sigmoidal) function when azoxystrobin was used and a three parameter function when pyraclostrobin and fenbuconazole were used. On the other hand, for spore inhibition assays, EC_{50} was determined by fitting a three parameter sigmoidal function for both strobilurins.

The effect of fungicide, isolate, experiment, and all the two-way interactions were investigated by an analysis of variance. Treatment means were separated using the Least Significant Difference (LSD) with PROC GLM (SAS 9.3, SAS Institute, Carry, NC).

Results

Mycelium Growth Inhibition Assay

To test for the homogeneity of variance, the standardized residuals were plotted against predicted values. In addition, the normality of the data distribution was tested with a univariate analysis. Since variances were equal, data from different experiments were pooled to calculate the mean EC_{50} for each fungicide-isolate combination.

The EC_{50} of *G. citricarpa* isolates to azoxystrobin, pyraclostrobin, and fenbuconazole was determined and the analysis of variance showed that the fungicide

effect was highly significant (P <0.0001). No interactions between experiment, fungicide, and isolate were found (Table 3-2).

The mean EC_{50} value of the 50 isolates for azoxystrobin was significantly higher (P <0.0001) than the EC_{50} values for fenbuconazole and pyraclostrobin (Table 3-3). There was no statistical difference between the mean EC_{50} values for fenbuconazole and pyraclostrobin (Table 3-3). For azoxystrobin, 86% of the isolates had an EC_{50} between 0.02 to 0.03 µg/ml (Fig. 3-1). For pyraclostrobin, however, the EC_{50} values were more evenly distributed over the range, and 48% of the isolates were between 0.003 to 0.006 µg/ml (Fig. 3-2). For fenbuconazole, 98% of the isolates had EC_{50} values between 0.007 and 0.01 µg/ml (Fig. 3-3).

Azoxystrobin inhibited mycelial growth up to 75% at 1 µg/ml, but only 66% at 10 µg/ml (Fig. 3-4). This reduction in growth inhibition at the highest concentration was not observed either with pyraclostrobin or fenbuconazole. At 10 µg/ml, growth inhibition was 90% and 97% for pyraclostrobin (Fig. 3-5) and fenbuconazole (Fig. 3-6), respectively.

Spore Germination Inhibition Assay

Preliminary results from the spore germination test using 0, 0.001, 0.01, 0.1, 1 and 10 µg of azoxystrobin and pyraclostrobin per ml indicated that 10 µg/ml completely inhibited spore germination. Thus, the highest fungicide concentration, 10 µg/ml, was eliminated from all spore inhibition assays.

A significant effect for fungicide, isolate, and fungicide by isolate interaction were shown by ANOVA (Table 3-4). For azoxystrobin, 64% of the isolates had an EC_{50} between 0.02 and 0.03 µg/ml (Fig. 3-7); whereas 66% of the isolates had an EC_{50} between 0.003 and 0.006 µg/ml for pyraclostrobin (Fig. 3-8).

Conidial germination was inhibited up to 94% and 91% at 1 µg/ml by azoxystrobin and pyraclostrobin, respectively (Figs. 3-9 and 3-10). No shift in the spore inhibition was observed at the highest concentration of azoxystrobin as occurred in the mycelium inhibition assay.

A separate analysis of variance was conducted to look at the differences in the fungicide and isolate interactions. The mean azoxystrobin EC_{50} for the isolate 11-155 (EC_{50}= 0.030 µg/ml) was significantly higher than for isolates 11-159 (EC_{50}= 0.002 µg/ml) and 11-156 (EC_{50}= 0.001 µg/ml), but were not different to the other 47 isolates (range EC_{50}= 0.003 to 0.03 µg/ml) ($P < 0.0001$). The mean pyraclostrobin EC_{50} value for the isolate 11-162 (EC_{50}= 0.024 µg/ml) was significantly higher than for the other 49 isolates evaluated (range EC_{50}= 0.001 to 0.01 µg/ml) ($P = 0.0072$) (data not shown).

Effect of Strobilurin Fungicides and SHAM on Mycelium Growth

The addition of SHAM at 10 and 100 µg/ml to media amended with azoxystrobin had no effect on the mycelial growth EC_{50} values of the 15 *G. citricarpa* isolates. However, the addition of SHAM at the same concentrations to pyraclostrobin had a significant effect ($P = 0.017$ and 0.006), respectively, on the EC_{50} for mycelial growth (Table 3-5 and 3-6). No other factors or interactions between factors were significant.

At the highest concentration of azoxystrobin, the growth inhibition was 67%, but the addition of SHAM at 10 and 100 µg/ml increased growth inhibition to 83%. But, when SHAM was added at 100 µg/ml with the lowest rate of azoxystrobin, the growth inhibition was reversed since more mycelial growth was observed on those plates than on the SHAM plates with no fungicide (Fig. 3-11). Although SHAM increased growth inhibition at 10 µg/ml for azoxystrobin, pairwise comparison indicated that there was no

difference in the EC_{50} values and for this reason SHAM was not used to determine the baseline sensitivity of the 50 *G. citricarpa* isolates.

With the addition of SHAM to pyraclostrobin, a similar pattern of growth inhibition was observed as with pyraclostrobin alone (Fig. 3-12). The addition of SHAM at 10 µg/ml to the lowest concentration of pyraclostrobin inhibited growth more than SHAM at 100 µg/ml or pyraclostrobin alone. However, the EC_{50} values from both SHAM concentrations were the same, and pairwise comparison indicated that the addition of SHAM to pyraclostrobin had an effect in the EC_{50} values (Table 3-6).

Effect of Strobilurin Fungicides and SHAM on Spore Germination

Different SHAM concentrations were tested prior to the addition of the strobilurin fungicides. Complete spore germination inhibition was observed using only SHAM at 25, 50 and 100 µg/ml. Crystals formed on the hydrophobic slides when SHAM was added at 100 µg/ml. Apparently, there is a reaction between the acid in the orange juice and the high concentration of SHAM which induces crystal formation.

SHAM at 10 µg/ml was tested for activity on spore germination inhibition when added to azoxystrobin and pyraclostrobin. An ANOVA indicated that SHAM had no effect on the sensitivity of conidial germination when added to strobilurins (Table 3-7). The isolate had a significant effect (P= 0.0007) in the experiment with azoxystrobin; however interactions between factors were not significant for either fungicide. The mean EC_{50} value for the isolates tested with azoxystrobin only was 0.015 µg/ml and with SHAM was 0.016 µg/ml. When pyraclostrobin was used alone, the mean EC_{50} value was 0.006 µg/ml and the EC_{50} mean with SHAM was 0.005 µg/ml (Table 3-8). The percent spore inhibition when azoxystrobin or pyraclostrobin was used alone was very similar to the inhibition when SHAM was added to the fungicides (Figs. 3-13 and 3-14).

Without SHAM, the maximum spore germination inhibition was 93% for azoxystrobin and 92% for pyraclostrobin. With SHAM at 10 µg/ml, the maximum spore germination inhibition was 95% and 97% for azoxystrobin and pyraclostrobin, respectively (Figs. 3-13 and 3-14).

Effect of SHAM on Mycelium Inhibition

Analysis of variance of growth inhibition by SHAM at 10, 25, 50, and 100 µg/ml indicated that there was a significant difference among SHAM concentrations ($P < 0.0001$). The main effects of experiment and isolate were not significant and no significant interactions between factors were detected (data not shown). The maximum mycelial growth inhibition was obtained with SHAM at 25 µg/ml, inhibiting growth by 18.4% (Fig. 3-15).

Azoxystrobin Technical vs. Commercial Grade and the Effect of the Different Grades and SHAM on Mycelium Inhibition

No significant effect between the two azoxystrobin grades was demonstrated by ANOVA. Also, no significant effect of the experiment or isolate was found and interactions between factors were not significant (Tables 3-9 and 3-10).

The percent mycelium inhibition by both azoxystrobin grades was similar at the different concentrations. At 1 µg/ml of commercial grade, there was a 74% inhibition, but when the concentration increased to 10 µg/ml, inhibition was reduced to 67%. The same trend was also observed when using technical grade; at 1 µg/ml, there was 76% inhibition of mycelial growth, but at 10 µg/ml the inhibition was reduced to 69% (Fig. 3-16).

The effect of SHAM at 100 µg/ml added to the different azoxystrobin grades was also tested. There was no effect of the addition of SHAM to technical or commercial

grade as determined by ANOVA (P= 0.2006) (Table 3-10). However, pairwise comparison of EC_{50} values from technical grade with or without SHAM were significant (P= 0.0001), indicating that although there was no difference in the addition of SHAM when comparing the two azoxystrobin grades, the addition of SHAM did affect the EC_{50} value of azoxystrobin technical grade.

The mycelial growth inhibition by azoxystrobin commercial grade at 1 µg/ml with SHAM was 88%, but at 10 µg/ml, the inhibition was reduced slightly to 81%. The growth inhibition by technical grade at 1 µg/ml with SHAM was 83% and at 10 µg/ml, contrary to the commercial grade, the inhibition was higher, 91% (Fig. 3-17). Despite of the effect of SHAM on the growth inhibition when the maximum rate of azoxystrobin technical grade was used, it had no significant effect on the EC_{50} values of either grade of azoxystrobin.

Discussion

Citrus black spot, an emerging disease in Florida, is mainly controlled with applications of strobilurin and copper fungicides. Establishment of the baseline sensitivity of *G. citricarpa* isolates never exposed to strobilurins or DMIs in FL, which are currently used and potentially effective fungicides, will help to monitor future shifts in pathogen sensitivity. Azoxystrobin, pyraclostrobin and fenbuconazole are currently used on Florida citrus; however, fenbuconazole use is limited to diseases other than black spot. Pathogens are prone to the development of resistance to strobilurins and DMI fungicides; hence the importance of this project.

In our study, we established the baseline sensitivity of *G. citricarpa* isolates to azoxystrobin, pyraclostrobin and fenbuconazole. Baseline sensitivity derived from mycelium inhibition of other citrus pathogens to azoxystrobin were higher than *G.*

citricarpa when compared to our results; the mean EC_{50} of *E. fawcettii* was 0.06 µg/ml, the mean EC_{50} for *C. acutatum* was 0.40 µg/ml, the *D. citri* EC_{50} mean was 0.08 µg/ml, and the EC_{50} mean for *M. citri* was 1.62 µg/ml (Mondal et al., 2005). Vega et al. (2012) found for Q_oI sensitive conidia of *A. alternata* a mean EC_{50} of 0.44 µg/ml for azoxystrobin. Although Mondal et al. (2005) reported EC_{50} values higher than 100 µg/ml from *A. alternata* mycelium, whereas the values from sensitive isolates conidia were much lower, indicating that mycelium of this pathogen is insensitive to azoxystrobin (Mondal et al., 2005; Vega et al., 2012). In addition, the baseline from mycelium of isolates of *Plasmopara viticola* showed a large range of EC_{50} values, from 0.04 to 0.78 µg/ml (Wong and Wilcox, 2000). The most similar baseline EC_{50}'s to azoxystrobin was from sensitive isolates of *C. graminicola*, which had a range of EC_{50} values from 0.05 to 0.1 µg/ml (Avila-Adame et al., 2003). *In vitro* activity of *G. citricarpa* to azoxystrobin was also evaluated in Brazil; even at higher doses of azoxystrobin (10 µg/ml), mycelium growth could not be totally inhibited and some isolates had a decrease in mycelium inhibition when compared to the lowest concentration of the fungicide (1 µg/ml) (Possiede et al., 2009). Similar results were obtained in this study, where a reduction in inhibition was observed at 10 µg/ml with all the isolates tested. On the other hand, the same authors reported complete sporulation inhibition by azoxystrobin, suggesting that spore production may be more sensitive to the effect of strobilurins.

Few reports on *in vitro* activity of pyraclostrobin are available. In this study, isolates of *G. citricarpa* were highly sensitive to pyraclostrobin. Mondal et al. (2005) established the baseline sensitivity of five fungal citrus pathogens and their EC_{50} values to pyraclostrobin were higher when compared to our results. Similarly, *G. citricarpa*

isolates were highly sensitive to fenbuconazole. Results, similar to ours, from the sensitivity of *Monilinia oxycocci* isolates to fenbuconazole found that the pathogen EC_{50} values ranged from 0.0001 to 0.01 µg/ml (McManus et al., 1999). Baseline from other DMI fungicide such as cyproconazole, showed that sensitive isolates of *M. graminicola* had EC_{50} values between 0.01 to 0.05 µg/ml (Gisi et al., 2000), which are higher than to our baseline values. Likewise, never exposed isolates of *Uncinula necator* to other DMI fungicides had higher EC_{50} values; ranging from 0.03 to 0.09 µg/ml (Erickson and Wilcox, 1997). Development of resistance to this group of fungicides had been reported, and it is considered to be the result of a slow, stepwise loss of control (FRAC, 2005). Although resistance to DMI is associated with multiple mutations at the target gene, 14α-demethylase, high levels of resistance are observed after a stepwise adaptation (polygenic resistance) (FRAC, 2005; Gisi et al., 2000).

Spore germination is a particularly sensitive fungal stage to strobilurins (Bartlett et al., 2002). In this study, the EC_{50} values from conidia of *G. citricarpa* to azoxystrobin were lower than EC_{50} values reported from conidia germination of *Erysiphe graminis* f.sp. *tritici* on cereals, where the EC_{50} ranged from 0.022 to 0.235 µg/ml (Chin et al., 2000). Our results showed that the baseline from mycelial growth was higher than for spore germination, implying that *G. citricarpa* conidia are more sensitive to Q_oI fungicides. In other studies, spore germination has also been shown to be more sensitive to pyraclostrobin. EC_{50} values of pyraclostrobin for *Uncinula necator* were similar to our results, ranging from 0.0016 to 0.010 µg/ml with a mean of 0.0044 µg/ml (Wong and Wilcox, 2002). Despite belonging to the same group of fungicides, *G. citricarpa* was more sensitive to pyraclostrobin than to azoxystrobin for mycelium and

spore inhibition. In another study with *Alternaria solani*, pyraclostrobin had greater spore germination inhibition than azoxystrobin (Pasche et al., 2004). Vega et al. (2012) also reported that conidia of *A. alternata* were more sensitive to pyraclostrobin than to azoxystrobin.

Some fungal plant pathogens evade the toxic effects of Q_oI fungicides by the alternative respiration pathway, which allows the generation of ATP without electron transport through complex III, and thus, allows the fungus to survive even at high doses of fungicide (Olaya and Köller, 1999; Ziogas et al., 1997). It has been reported that residual growth of *M. grisea* sustained by alternative respiration was involved in the spontaneous emergence of Q_oI resistant-cytochrome *b* target site mutants (Avila-Adame and Köller, 2003b). Nevertheless, for several fungal species this rescue mechanism had little effect in the field when Q_oI fungicides were used to control the disease (Olaya and Köller, 1999; Ziogas et al., 1997). It has been proposed that for those fungal species, plant antioxidants such as flavones present in the host can silence this mechanism during infection by quenching the reactive oxygen (Avila-Adame and Köller, 2003a). Therefore, differences between *in vitro* and *in vivo* for the same studied pathogen are likely to be found (Olaya and Köller, 1999). For this reason, the alternative respiration should be blocked for some pathogens, by using salicylhydroxamic acid (SHAM) during *in vitro* experiments.

The response of *G. citricarpa* isolates to different concentrations of SHAM was variable between mycelium growth and spore germination. SHAM at 25 µg/ml showed the highest mycelium inhibition; meanwhile, higher concentrations completely inhibited spore germination. No other studies where SHAM at low doses inhibited pathogen

mycelium or germination were found. Nevertheless, in another study, sub-lethal doses of the fungicide mefenoxam enhanced *Pythium* damping-off disease; which was related to the proposed concept of “hormesis” (Garzón et al., 2011). Hormesis is a physiological process where factors that trigger homeostatic disruptions at high doses produce adaptive responses at low doses and can result in metabolic stimulation (Calabrese and Baldwin, 2002). Although in our experiment low doses of SHAM increased the inhibition of mycelium, it may be possible that adaptive responses of *G. citricarpa* to high doses of SHAM stimulate pathogen growth, but inhibited growth at low doses. More research would be needed to confirm these results as well as the hypothesis.

In the current study, SHAM did not affect the activity of azoxystrobin. Neither mycelium growth nor spore germination was significantly affected by the addition of SHAM to the media. Similar results were obtained with the citrus pathogen *A. alternata*, where the addition of SHAM did not increase the activity to azoxystrobin (Mondal et al., 2005). Nevertheless, Vega et al. (2012) found for the conidia of the same pathogen that SHAM did affect growth in an isolate-dependent manner. The sensitivity of *C. graminicola* and *Penicillium digitatum* also showed no significant synergistic effect of SHAM on their sensitivity to azoxystrobin (Avila-Adame et al., 2003; Kanetis et al., 2008). Our results also indicated that the addition of SHAM to pyraclostrobin did affect the activity of mycelium growth although the EC_{50} values were similar. But, spore germination was not affected by SHAM. Comparable to our results, the addition of SHAM to pyraclostrobin significantly reduced the colony diameter of the citrus pathogen *D. citri*; even though EC_{50} values could not be calculated due to reversed inhibition at low doses of the fungicide (Mondal et al., 2005). From our results, there was no effect of

SHAM on the EC_{50} values, indirectly indicating that *G. citricarpa* is not using the alternative respiration pathway.

Interestingly, in our study, 10 µg/ml azoxystrobin showed lower mycelium growth inhibition than at 1 or 0.1 µg/ml. Thus, to evaluate whether or not the grade of the active ingredient was influencing these results, technical grade was compared to commercial grade. The results indicated that there were no differences between the two grades; therefore, the same pattern was exhibited for both. The addition of SHAM slightly increased the inhibition at 10 µg/ml; however it did not affect their EC_{50} values. Although there is no evidence that *G. citricarpa* is using alternative respiration, the pathogen may be activating this pathway specifically at 10 µg/ml of azoxystrobin and SHAM is blocking this pathway or else the mycelium is not taking up the fungicide well at higher concentrations and SHAM increases the permeability.

Overall, *G. citricarpa* isolates were very sensitive to azoxystrobin, pyraclostrobin and fenbuconazole. In other regions with citrus black spot, azoxystrobin and pyraclostrobin were tested for field efficacy with positive results (Almeida, 2009; Fogliata et al., 2011; Miles et al., 2004; Rodriguez et al., 2010; Schutte et al., 2003). In Florida citrus, fenbuconazole is already used to control citrus scab, greasy spot, and sooty mold (Mondal and Timmer, 2003, 2005; Timmer and Zitko, 1997). In this study, we present evidence that fenbuconazole is highly effective inhibiting *G. citricarpa* growth *in vitro*; hence the potential use of this fungicide in the field must be evaluated.

When chemical control measures fail, it may be due to selection pressure for pathogen resistance. The high specificity of the mode of action of fungicides, together with sexual recombination that occurs in some fungal pathogens promote the selection

of resistant isolates after prolonged periods of use (Brent and Hollomon, 1998; Jutsum et al., 1998). Through the years, several pathogens have become insensitive to strobilurin and DMI fungicides (FRAC, 2012; Gisi et al., 2000; Heaney et al., 2000; Rosenzweig et al., 2007; Vega et al., 2012; Zambolim et al., 2007) and there is a high risk of *G. citricarpa* developing resistance. Consequently, resistance management practices are important for the continued use of strobilurins and DMI fungicides. The baseline sensitivity established in this study will help to monitor future shifts in Florida populations of *G. citricarpa* to assure the continued effectiveness of the spray programs that are currently recommended. The efficacy of fenbuconazole to inhibit *G. citricarpa in vitro* was shown. The next step will be to demonstrate through field trials that it is a good option for black spot management and a viable rotational product.

Table 3-2. Analysis of variance of the effective concentration of fungicides to inhibit mycelial growth by 50% (EC_{50}) of fifty *Guignardia citricarpa* isolates.

Source of variation	df	SS	MS	F value	P value
Experiment (E)	1	0.0001032	0.0001032	1.03	0.3115
Fungicide (F)[a]	2	0.011019	0.005510	55.22	<.0001[b]
Isolate (I)	49	0.006079	0.000124	1.24	0.1800
E x F	2	0.000233	0.000117	1.17	0.3149
E x I	49	0.004195	0.000086	0.86	0.7203
F x I	98	0.013431	0.000137	1.37	0.0589

[a] Azoxystrobin, pyraclostrobin and fenbuconazole.
[b] Significant effects are underlined.

Table 3-3. Mean effective concentration of fungicides to inhibit mycelial growth and spore germination by 50% (EC_{50}) of fifty *Guignardia citricarpa* isolates.

Fungicide	Mycelium inhibition	Spore inhibition
	EC_{50} µg/ml[a]	
Azoxystrobin	(0.01-0.08)[b] 0.021 a[c]A[d]	(0.001-0.03) 0.016 aB
Fenbuconazole	(0.006-0.01) 0.009 b	n/a [e]
Pyraclostrobin	(0.002-0.03) 0.008 bA	(0.001-0.02) 0.006 bB

[a] Average of two independent experiments.
[b] Minimum and maximum mean EC_{50} value of mycelium and spore inhibition for each fungicide.
[c] Mean separation within column followed by the same lower case letter are not significantly different according to *t* test (LSD) ($P \leq 0.05$).
[d] Mean separation within rows followed by the same capital letter are not significantly different according to *t* test (LSD) ($P \leq 0.05$).
[e] Fenbuconazole was not tested in the spore inhibition assay.

Table 3-4. Analysis of variance of the effective concentration of azoxystrobin and pyraclostrobin to inhibit spore germination by 50% (EC_{50}) of fifty *Guignardia citricarpa* isolates.

Source of variation	df	SS	MS	F value	P value
Experiment (E)	1	0.000070	0.000070	2.62	0.1117
Fungicide (F)[a]	1	0.005375	0.005375	202.70	<.0001[b]
Isolate (I)	49	0.003955	0.000081	3.04	<.0001
E x F	1	0.000089	0.000089	3.34	0.0738
E x I	49	0.001063	0.000022	0.82	0.7581
F x I	49	0.003198	0.000065	2.46	0.0010

[a] Azoxystrobin and pyraclostrobin.
[b] Significant effects are underlined.

Table 3-5. Analysis of variance of the effective concentration of azoxystrobin and pyraclostrobin amended with 10 and 100 µg/ml of SHAM on the inhibition of mycelial growth by 50% (EC_{50}) of fifteen *Guignardia citricarpa* isolates.

Source of variation	Azo-SHAM 10[a]	Azo-SHAM 100	Pyra-SHAM 10	Pyra-SHAM 100
	P values[b]			
Experiment (E)	0.9990	0.7159	0.2747	0.1750
SHAM (S)	0.7136	0.0726	<u>0.0171</u>[c]	<u>0.0063</u>
Isolate (I)	0.2596	0.7842	0.0824	0.1355
E x S	0.9771	0.7383	0.6400	0.8935
E x I	0.1320	0.2526	0.2018	0.5521
S x I	0.3197	0.7236	0.6543	0.1103

[a] Fungicides azoxystrobin (Azo) and pyraclostrobin (Pyra) were amended with 10 and 100 µg/ml of SHAM.
[b] P values for comparison of the EC_{50} values of SHAM amended and nonamended was determined from *F* test.
[c] Significant effects are underlined.

Table 3-6. Mean effective concentration of azoxystrobin and pyraclostrobin amended with 10 and 100 µg/ml of SHAM to inhibit mycelial growth by 50% (EC_{50}) of fifteen *Guignardia citricarpa* isolates.

	No SHAM	SHAM 10	SHAM 100
		EC_{50} µg/ml	
Azoxystrobin	0.017 a[a]	0.016 a	0.013 a
Pyraclostrobin	0.008 a	0.005 b	0.005 b

[a] Mean separation within rows followed by the same letter are not significantly different according to *t* test (LSD) (P≤ 0.05).

Table 3-7. Analysis of variance of the effective concentration of azoxystrobin and pyraclostrobin with or without SHAM at 10 µg/ml to inhibit spore germination by 50% (EC_{50}) of fifteen *Guignardia citricarpa* isolates.

	Azo-SHAM 10[a]	Pyra-SHAM 10
Source of variation	P values[b]	
Experiment (E)	0.1759	0.2455
SHAM (S)	0.3849	0.5887
Isolate (I)	<u>0.0007</u>[c]	0.1239
E x S	0.1885	0.5132
E x I	0.1667	0.7403
S x I	0.5737	0.8411

[a] Fungicides azoxystrobin (Azo) and pyraclostrobin (Pyra) were amended with 10 µg/ml of SHAM.
[b] P values for comparison of the EC_{50} values of SHAM amended and unamended was determined from *F* test.
[c] Significant effects are underlined.

Table 3-8. Mean effective concentration of azoxystrobin and pyraclostrobin with or without SHAM at 10 µg/ml to inhibit spore germination by 50% (EC_{50}) of fifteen *Guignardia citricarpa* isolates.

	No SHAM	SHAM 10
	EC_{50} µg/ml	
Azoxystrobin	0.015 a[a]	0.016 a
Pyraclostrobin	0.006 a	0.005 a

[a] Mean separation within rows followed by the same letter are not significantly different according to *t* test (LSD) (P≤ 0.05).

Table 3-9. Analysis of variance of the effective concentration to inhibit mycelial growth by 50% (EC_{50}) when using azoxystrobin technical grade vs. commercial grade with ten *Guignardia citricarpa* isolates.

Source of variation	df	SS	MS	F value	P value
Experiment (E)	1	0.000062	0.000062	3.25	0.1049
Fungicide (F)[a]	1	0.000000	0.000000	0.00	0.9939
Isolate (I)	9	0.000513	0.000057	2.97	0.0603
E x F	1	0.000075	0.000075	3.92	0.0791
E x I	9	0.000428	0.000048	2.48	0.0961
F x I	9	0.000138	0.000015	0.80	0.6274

[a] Fungicides azoxystrobin technical grade and azoxystrobin commercial grade (Abound) were tested for their activity on mycelium inhibition.

Table 3-10. Mean effective concentration to inhibit mycelial growth by 50% (EC_{50}) when using azoxystrobin technical grade vs. commercial grade with and without SHAM and ten *Guignardia citricarpa* isolates.

	Azoxystrobin technical	Azoxystrobin commercial
	EC_{50} µg/ml	
No SHAM	0.017 a[a]A[b]	0.016 aA
SHAM 100	0.013 bA	0.014 aA

[a] Mean separation within columns followed by the same lower case letter are not significantly different according to *t* test (LSD) (P≤ 0.05).
[b] Mean separation within rows followed by the same capital letter are not significantly different according to *t* test (LSD) (P≤ 0.05).

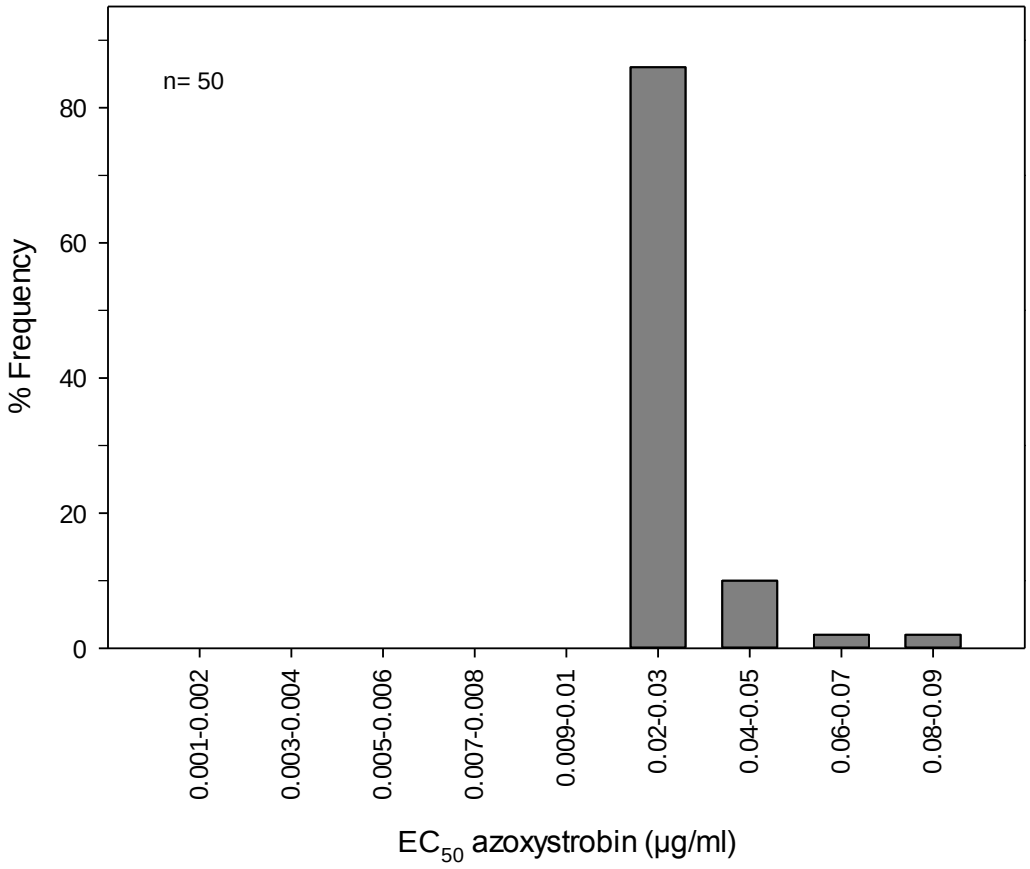

Figure 3-1. Frequency distribution of the effective concentration of azoxystrobin to reduce mycelial growth by 50% (EC_{50}) of *Guignardia citricarpa* isolates.

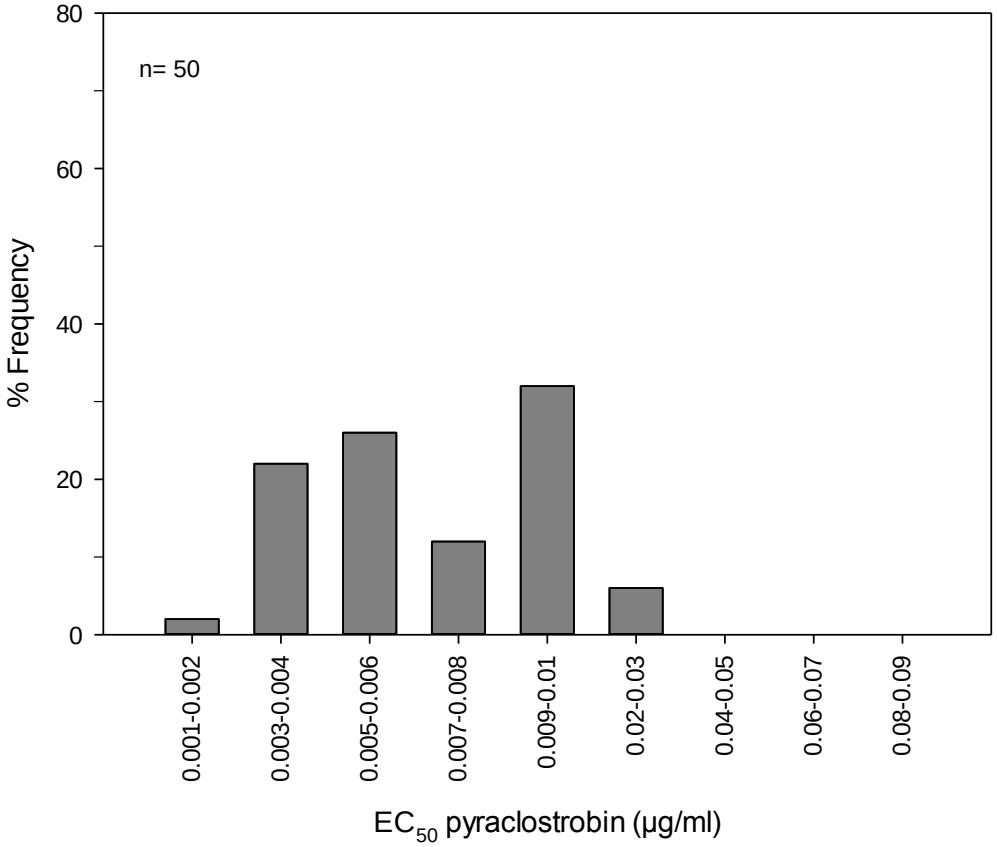

Figure 3-2. Frequency distribution of the effective concentration of pyraclostrobin to reduce mycelial growth by 50% (EC_{50}) of *Guignardia citricarpa* isolates.

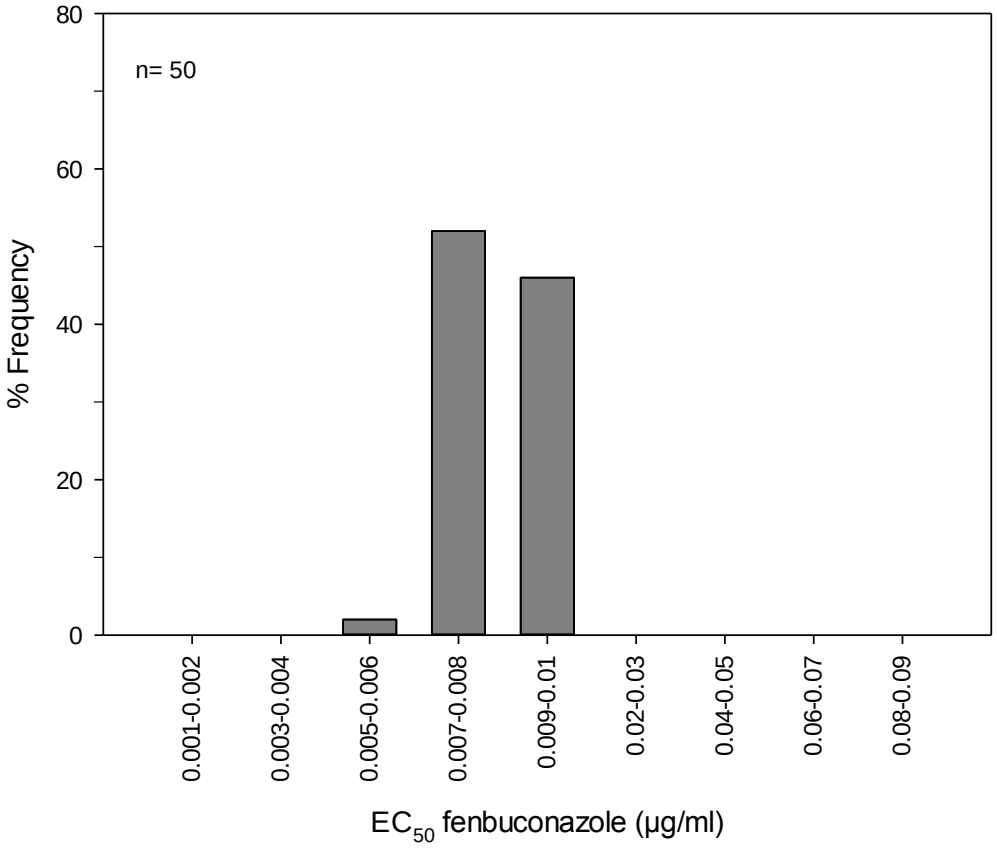

Figure 3-3.Frequency distribution of the effective concentration of fenbuconazole to reduce mycelial growth by 50% (EC_{50}) of *Guignardia citricarpa* isolates.

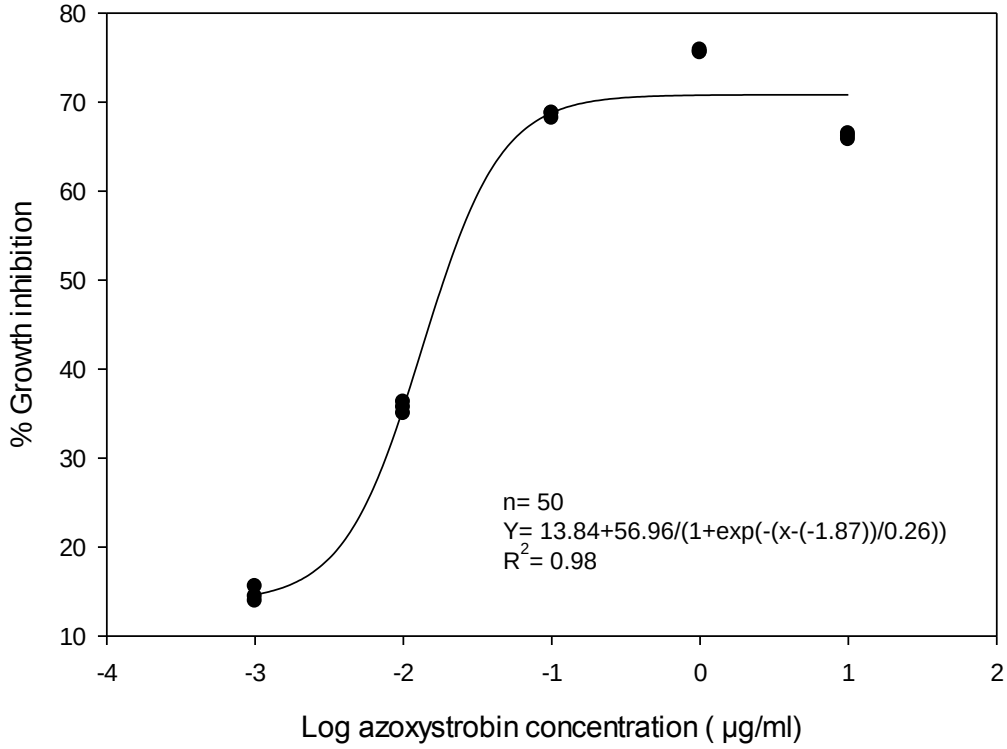

Figure 3-4. Inhibition of mycelial growth of *Guignardia citricarpa* by different concentrations of azoxystrobin. Points represent the average of two experiments and three replicates for each concentration.

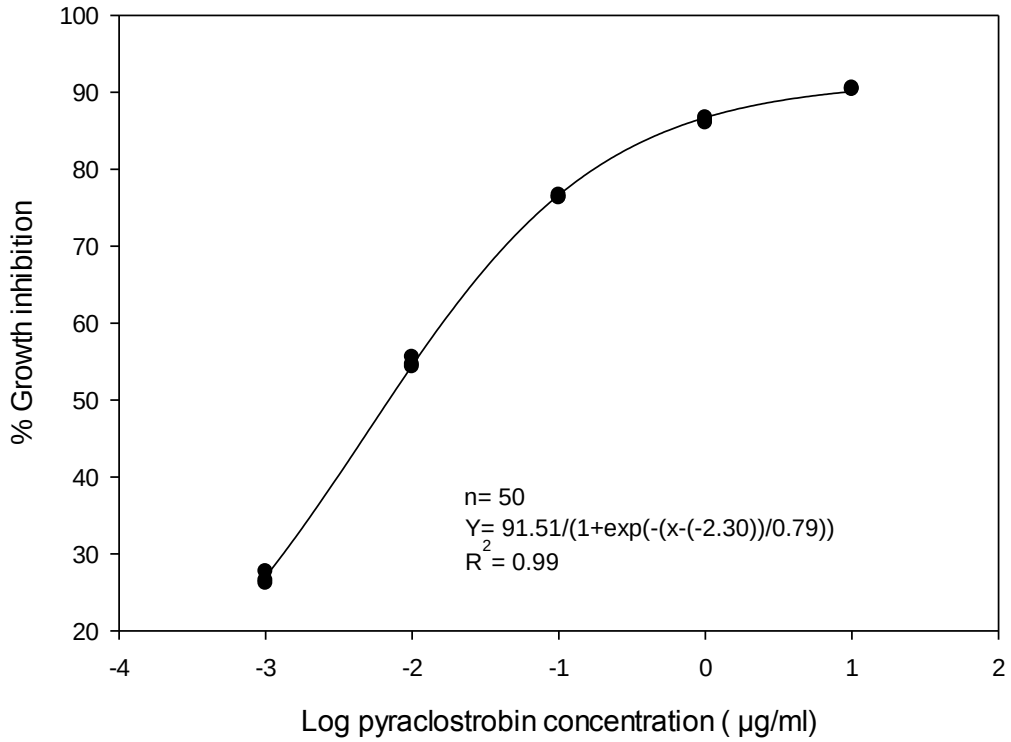

Figure 3-5. Inhibition of mycelial growth of *Guignardia citricarpa* by different concentrations of pyraclostrobin. Points represent the average of two experiments and three replicates for each concentration.

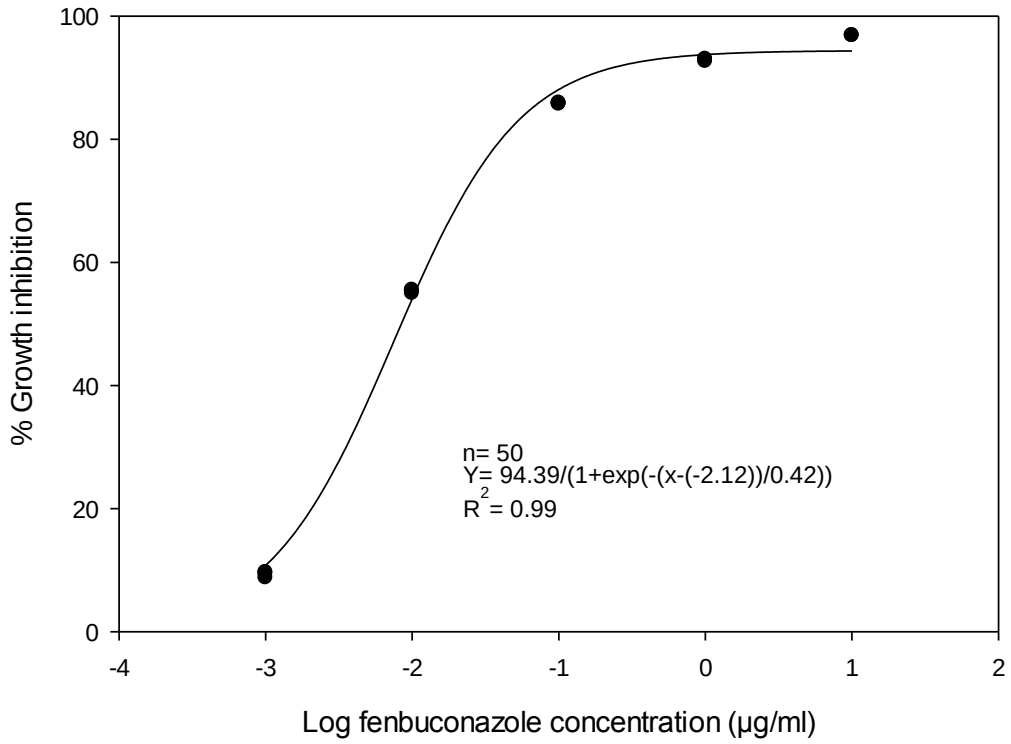

Figure 3-6. Inhibition of mycelial growth of *Guignardia citricarpa* by different concentrations of fenbuconazole. Points represent the average of two experiments and three replicates for each concentration.

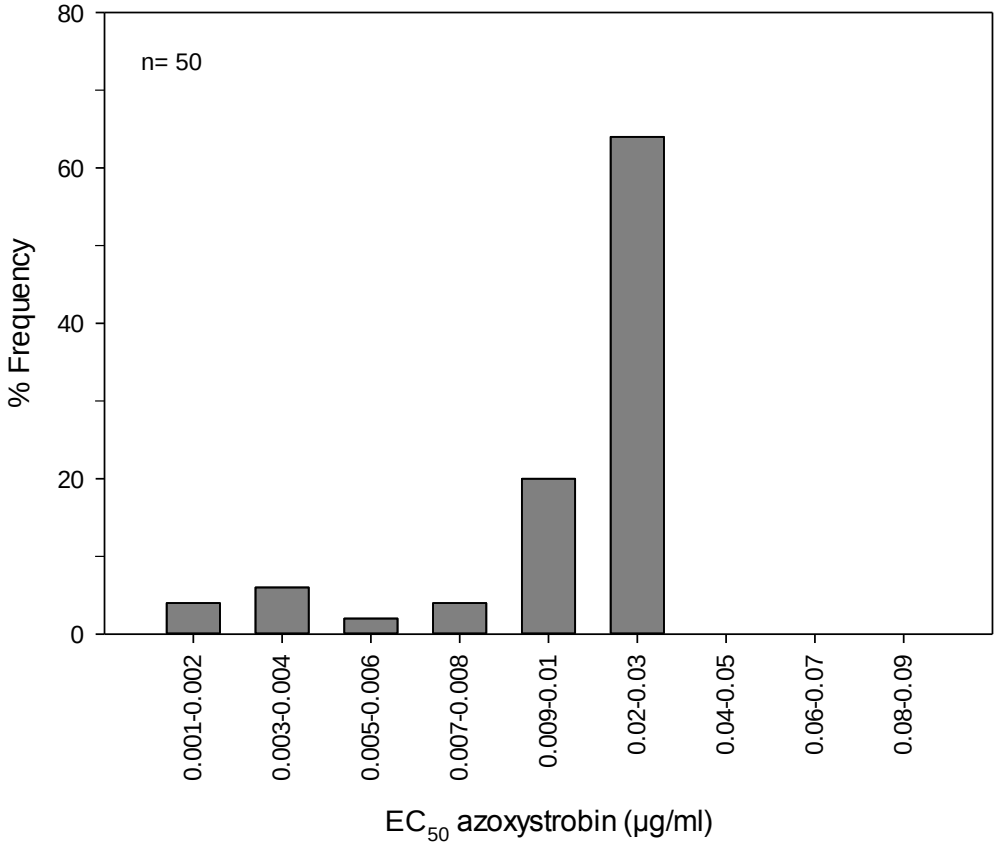

Figure 3-7. Frequency distribution of the effective concentration of azoxystrobin to reduce spore germination by 50% (EC_{50}) of *Guignardia citricarpa* isolates.

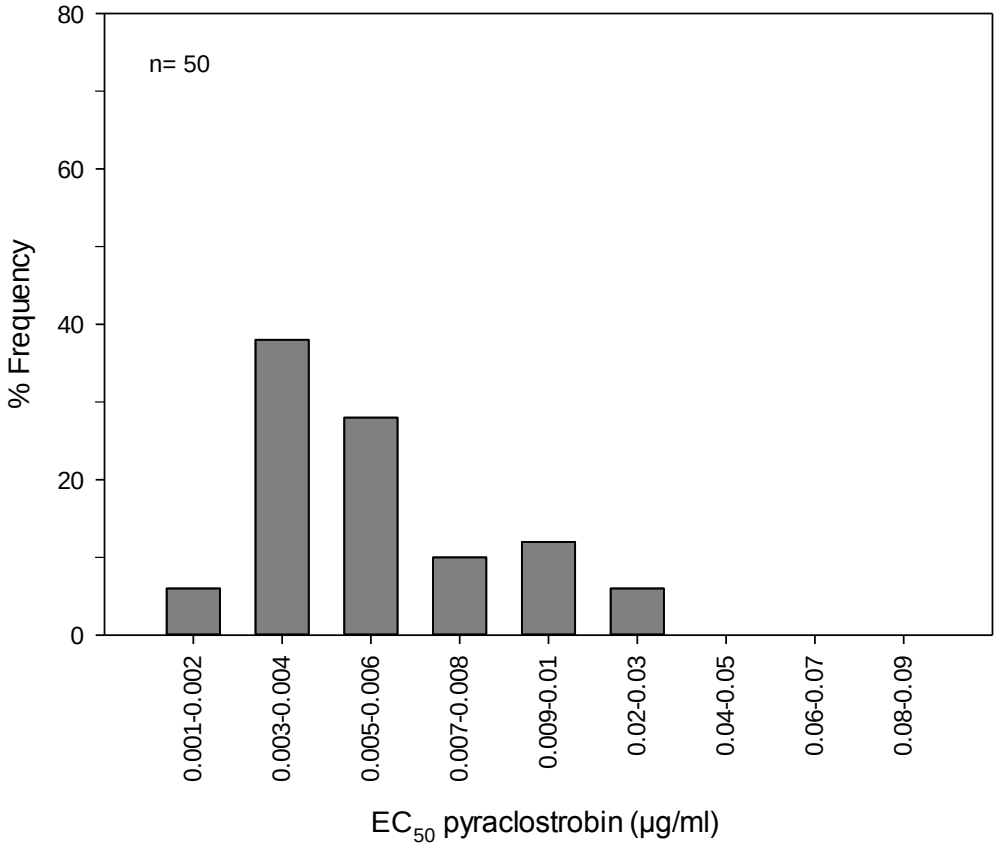

Figure 3-8. Frequency distribution of the effective concentration of pyraclostrobin to reduce spore germination by 50% (EC_{50}) of *Guignardia citricarpa* isolates.

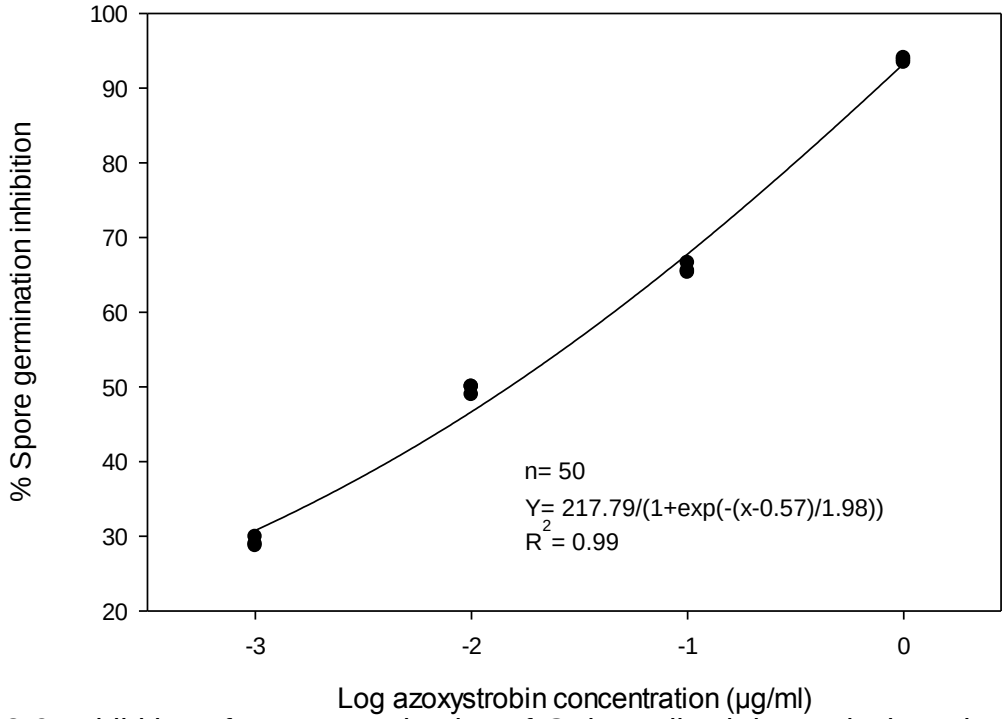

Figure 3-9. Inhibition of spore germination of *Guignardia citricarpa* isolates by different concentrations of azoxystrobin. Points represent the average of two experiments and three replicates for each concentration.

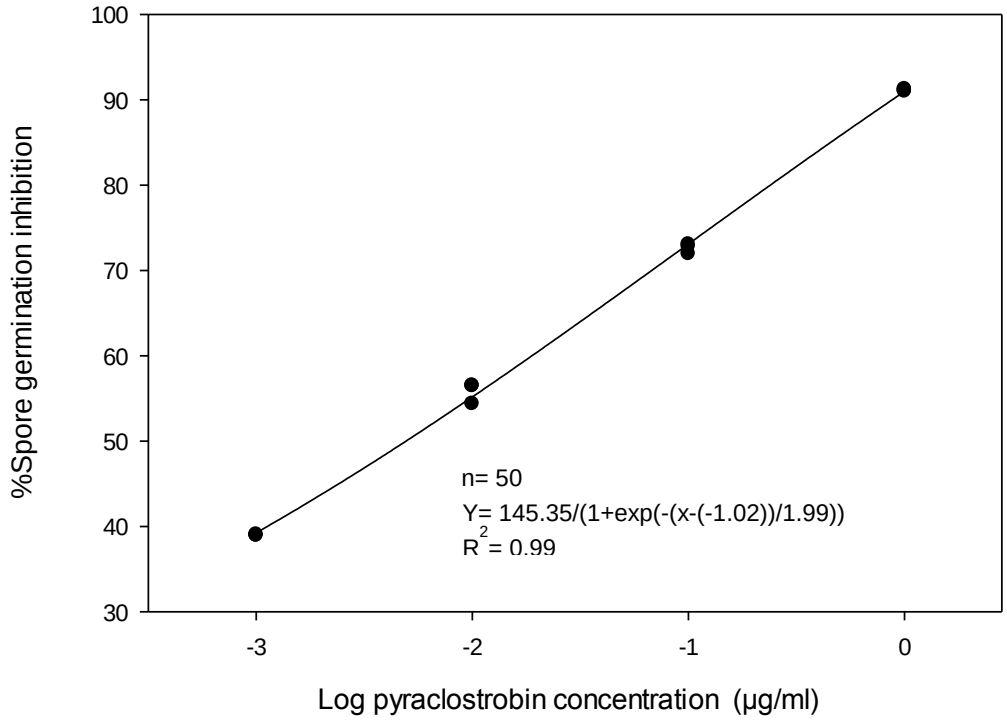

Figure 3-10. Inhibition of spore germination of *Guignardia citricarpa* isolates by different concentrations of pyraclostrobin. Points represent the average of two experiments and three replicates for each concentration.

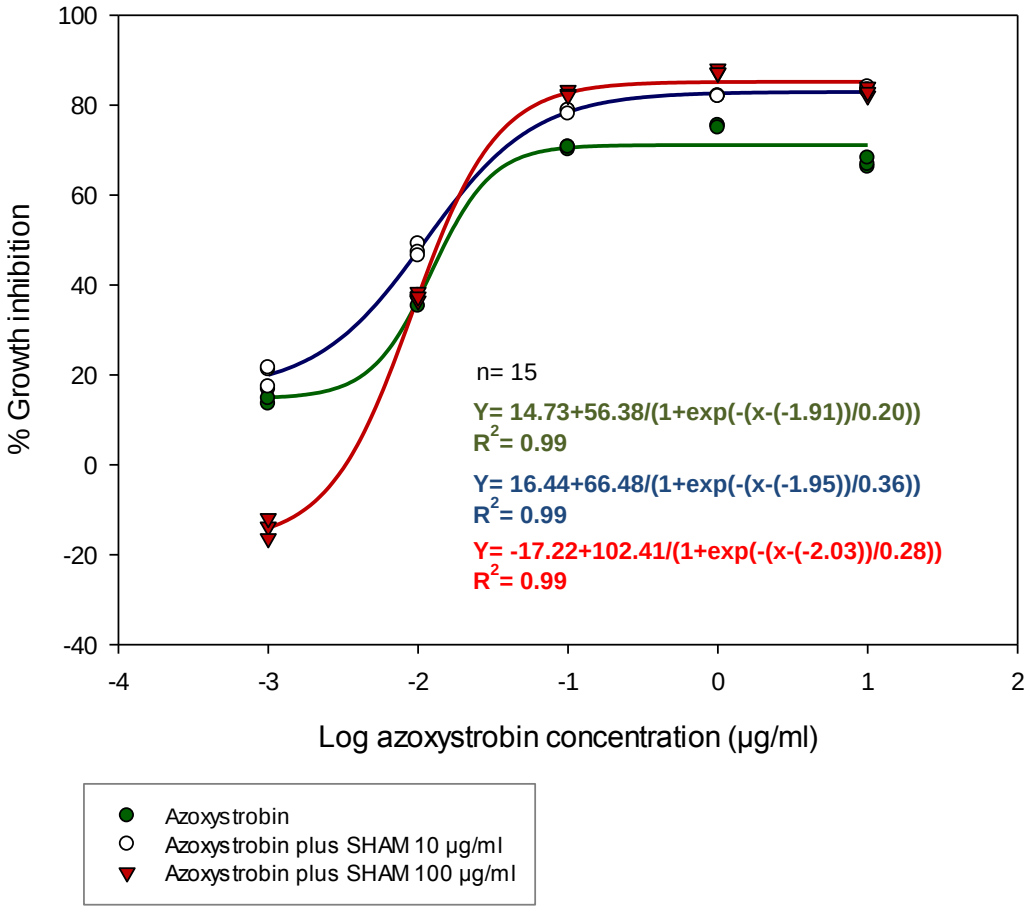

Figure 3-11. Effect of salicylhydroxamic acid (SHAM) on the activity of azoxystrobin on mycelial growth of *Guignardia citricarpa* isolates. Points represent the average of two experiments and three replicates for each concentration.

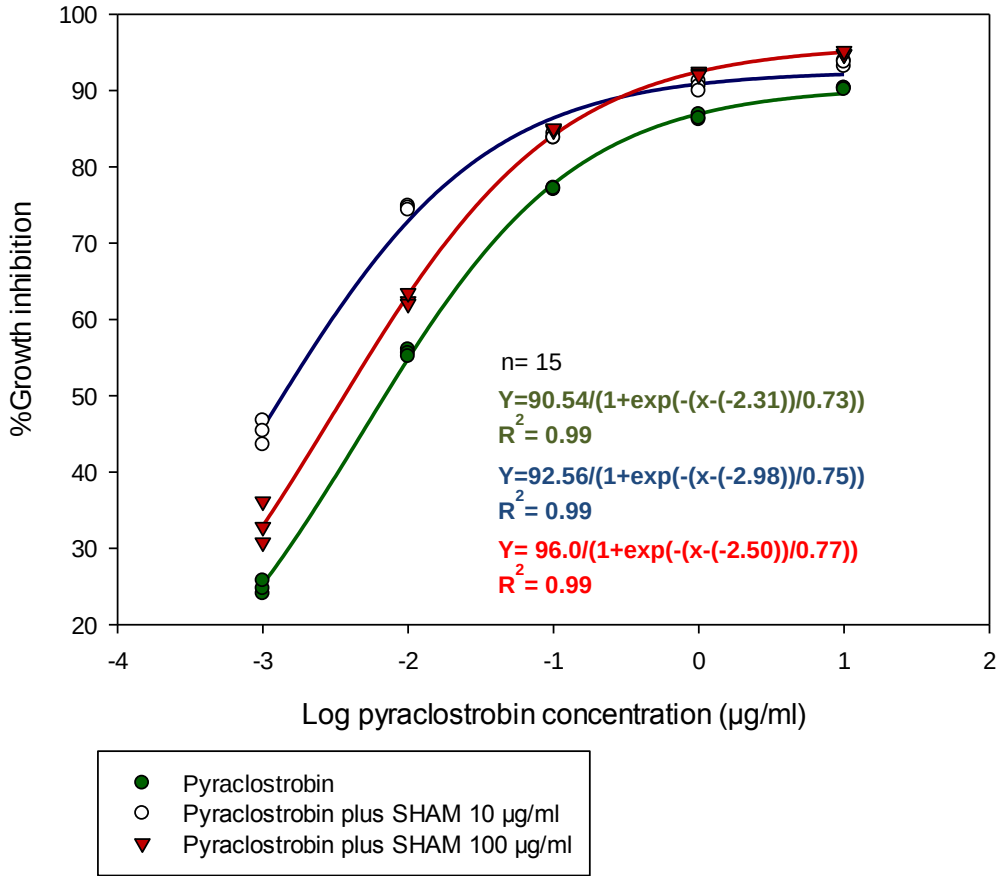

Figure 3-12. Effect of salicylhydroxamic acid (SHAM) on the activity of pyraclostrobin on mycelial growth of *Guignardia citricarpa* isolates. Points represent the average of two experiments and three replicates for each concentration.

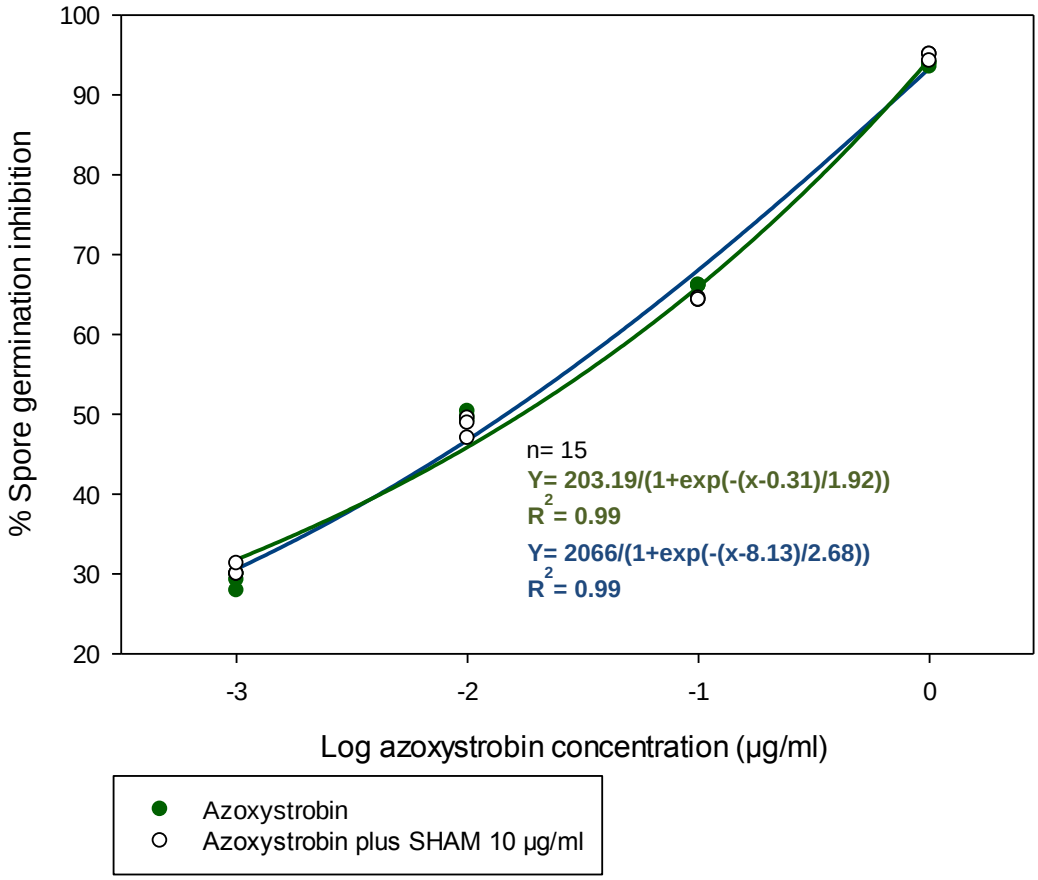

Figure 3-13. Effect of salicylhydroxamic acid (SHAM) on the activity of azoxystrobin on spore germination of *Guignardia citricarpa* isolates. Points represent the average of two experiments and three replicates for each concentration.

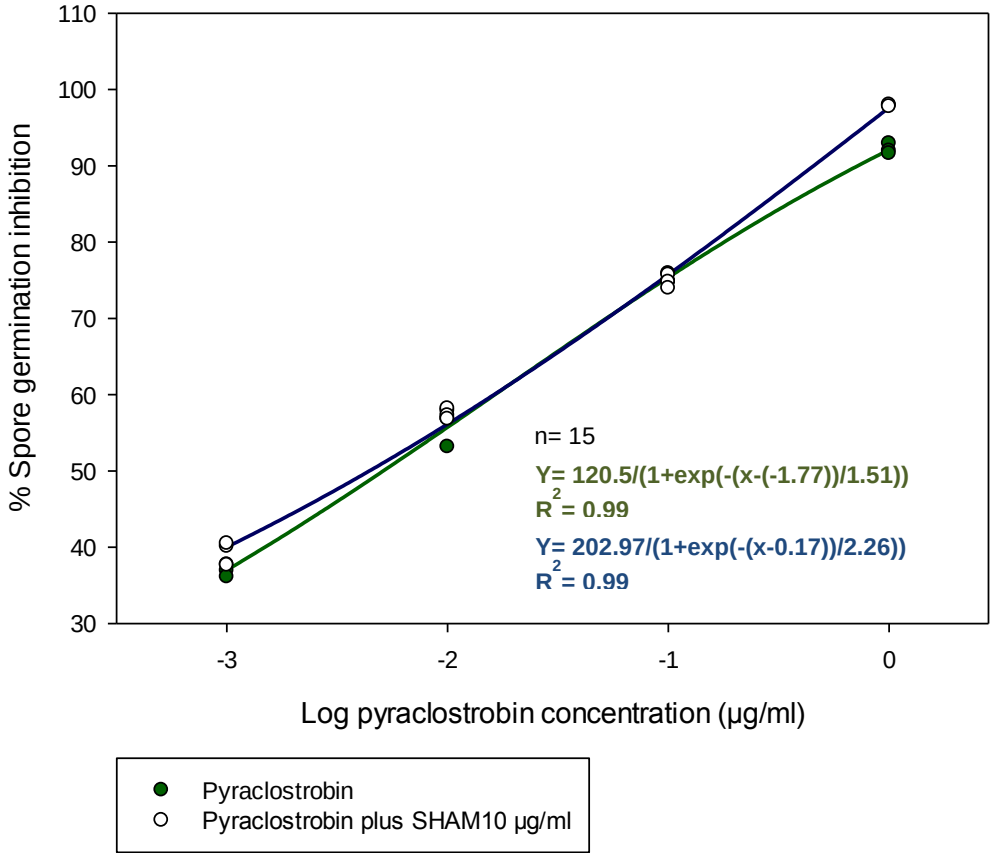

Figure 3-14. Effect of salicylhydroxamic acid (SHAM) on the activity of pyraclostrobin on spore germination of *Guignardia citricarpa* isolates. Points represent the average of two experiments and three replicates for each concentration.

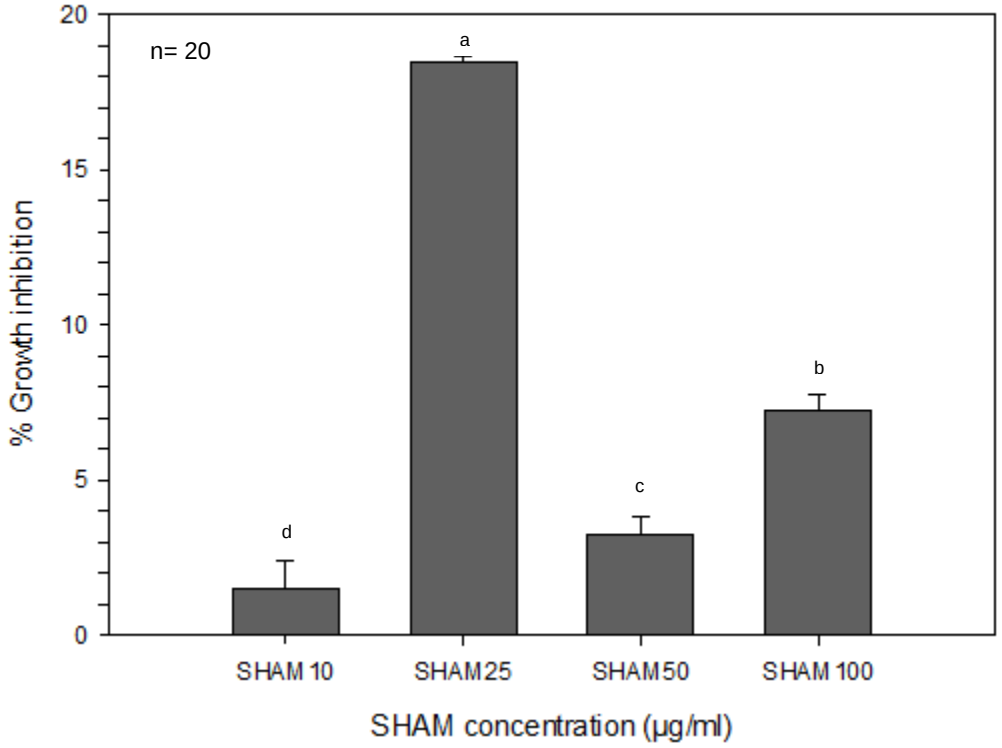

Figure 3-15. Inhibition of mycelial growth by different SHAM concentrations. Bars with the same letter are not significantly different according to *t* test (LSD) (P≤ 0.05). The error bars represent the standard error.

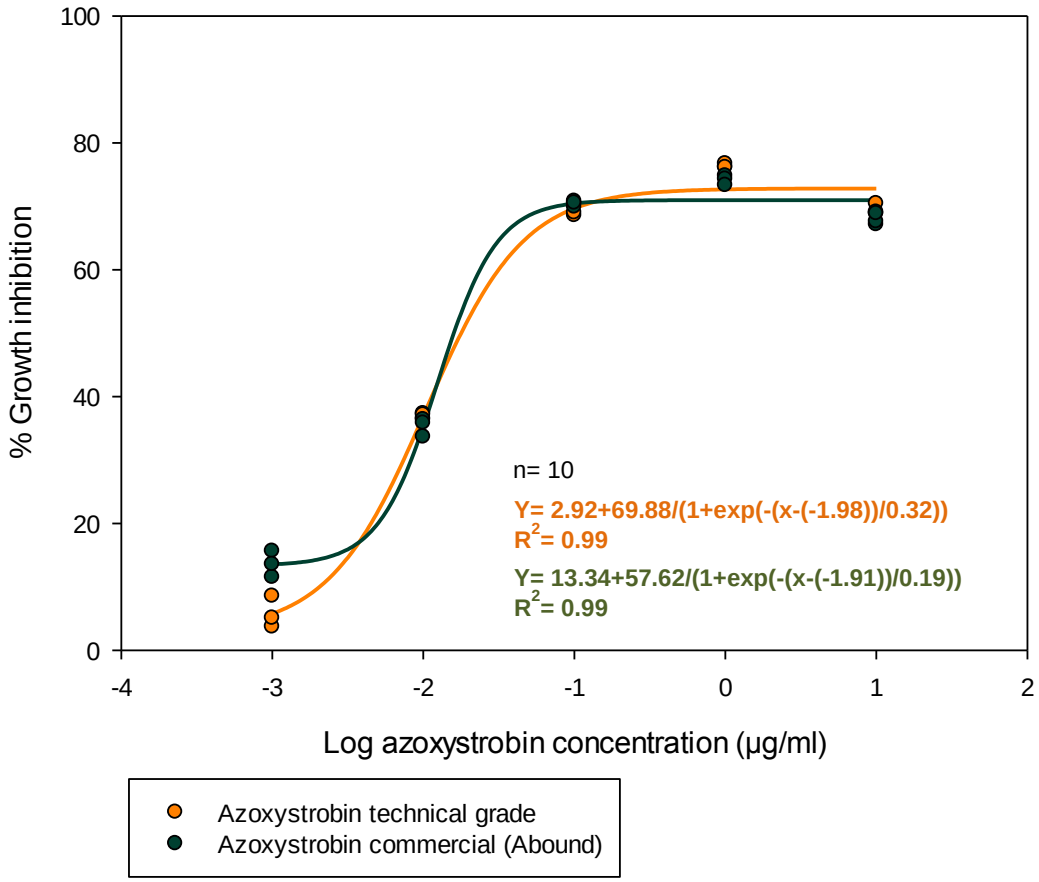

Figure 3-16. Inhibition of mycelium growth of *Guignardia citricarpa* by different concentrations of azoxystrobin technical grade and commercial grade. Points represent the average of two experiments and three replicates for each concentration.

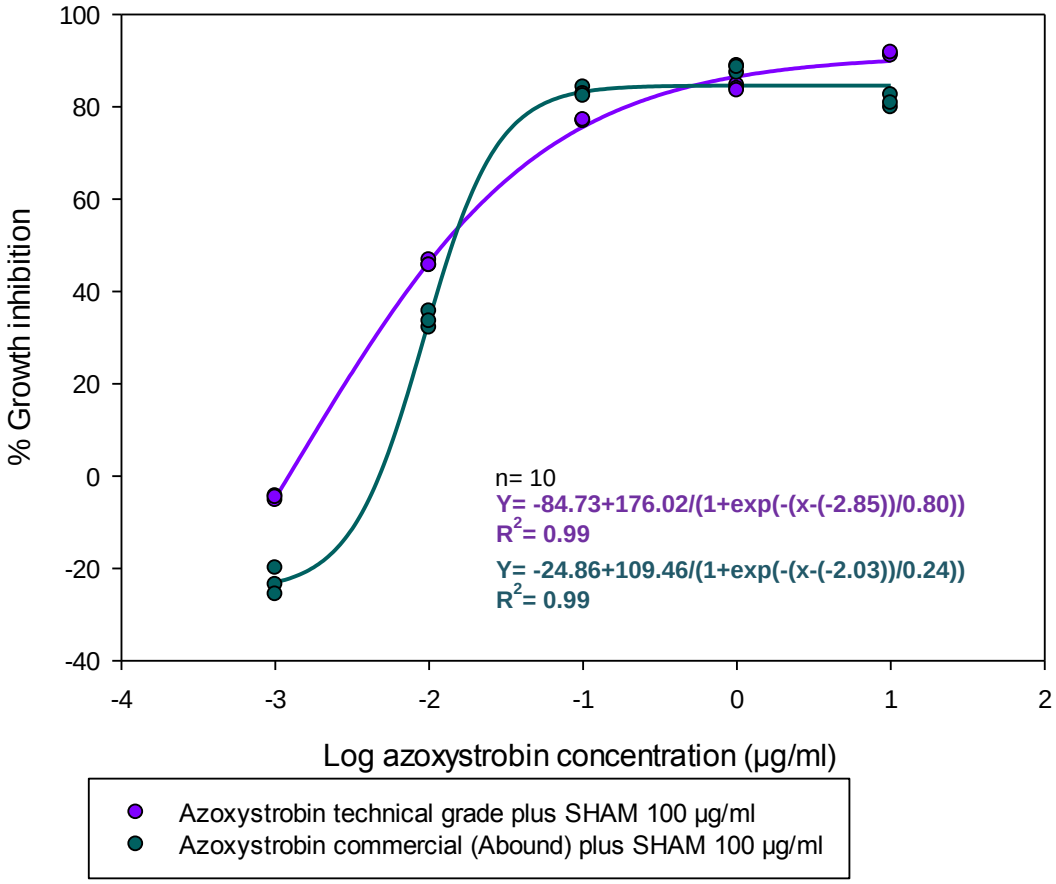

Figure 3-17. Effect of salicylhydroxamic acid (SHAM) on the activity of technical and commercial grades of azoxystrobin on mycelial growth of *Guignardia citricarpa* isolates. Points represent the average of two experiments and three replicates for each concentration.

CHAPTER 4
CONCLUSIONS

Since the discovery of citrus black spot in Florida, quarantine measures were established to prevent the spread of the disease. In addition, cultural and chemical control practices were recommended based on information from other areas where CBS is found. The Q_oI fungicides azoxystrobin and pyraclostrobin are registered in Florida for the control of black spot. The DMI fungicide fenbuconazole is also registered for use on Florida citrus, but only for greasy spot and scab. Since pathogens have a history of resistance to these fungicides, the establishment of baseline sensitivity to currently used and potentially effective fungicides becomes an important mechanism to detect population resistance.

In this study, we evaluated the sensitivity of *G. citricarpa* isolates to azoxystrobin, pyraclostrobin and fenbuconazole. These fungicides provided good intrinsic activity against black spot. From the baseline values, pyraclostrobin and fenbuconazole inhibited mycelial growth by 50% at a lower concentration than did azoxystrobin. Moreover, spore germination was more sensitive than mycelium growth to the Q_oI fungicides, which corresponds to the mode of action of this group of fungicides. Also, pyraclostrobin was more effective than azoxystrobin on inhibiting spore germination.

Alternative respiration, a pathway used by many fungi to avoid disruption of the mitochondrial electron transport chain, such as activated by Q_oI fungicides, can be blocked by using SHAM *in vitro*. No evidence was found that *G. citricarpa* isolates activated this pathway since the EC_{50} values where SHAM was not used did not differ significantly from those where SHAM was used, for both azoxystrobin and

pyraclostrobin. At the same time, low doses of SHAM directly inhibited mycelial growth and spore germination.

Interestingly, mycelial growth was increased at 10 µg/ml compared to 1 and 0.1 µg/ml of azoxystrobin. For this reason, commercial and technical azoxystrobin grades were evaluated, but the growth was not different at the higher concentration by either grade. SHAM was tested in combination with the fungicides as well, and no significant differences in the EC_{50} values were observed. One possible reason of the increased mycelium growth at the maximum rate of azoxystrobin is that the fungus activates a low level of alternative respiration at 10 µg/ml and SHAM is blocking it, or SHAM is modifying the medium permeability enhancing the fungicide take up by the mycelium. More research will be necessary to determine if this hypothesis is correct.

Since black spot is an emerging disease, detection of resistant isolates to the fungicides tested was not expected. However, if precautionary measures are not taken, after prolonged used, the development of *G. citricarpa* resistance to strobilurins is likely. Results from this study will help to monitor population shifts in sensitivity to these fungicides which would contribute to the long-term effectiveness of currently used spray programs in Florida. At the same time, this study confirmed the potential use of fenbuconazole to control black spot; hence, field experiments should be conducted to establish its efficacy.

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BIOGRAPHICAL SKETCH

Martha Hincapie was born in Santa Marta, Colombia. She graduated from “Instituto la Milagrosa” high school with an accounting specialization in 2003. In 2004, she won a scholarship from Dole Fruit Company to conduct agronomic studies at EARTH University in Costa Rica. During her bachelor’s studies, she did an internship at the “Fundação Mokiti Okada in Brazil, where she became interested in the field of plant pathology. After her graduation in 2007, she started an internship at Dr. Natalia Peres’ strawberry pathology lab for two years. Then, she began as a plant pathology graduate student. Her master’s research consisted in the establishment of baseline sensitivity of *Guignardia citricarpa*, the causal agent of citrus black spot, to azoxystrobin, pyraclostrobin, and fenbuconazole.