THE POTENTIAL INOCULUM SOURCES, *IN VITRO* GERMINATION, AND LEAF INFECTION BY *Phyllosticta citricarpa*, THE CITRUS BLACK SPOT PATHOGEN

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2016
To my lovely wife, Yi-Tien Lu and my family for always being with me
ACKNOWLEDGMENTS

It has been a long journey since the first day I decided to come to the U.S. to pursue my dream of getting a Ph.D. degree. This journey was full of surprise and excitement, but meanwhile accompanied with failure and discouragement. Without the help from many people, I definitely cannot have gotten this far. First of all, I would like to sincerely thank my major advisor Dr. Megan M. Dewdney for her guidance, motivation, patience, and tolerance over the past 6 years. Her mentoring always provides insightful suggestions that step-by-step guided me toward my career goal as a research scientist. Without such support, all of my work would have not been possible. I would also like to thank Dr. Jeffrey A. Rollins for his accommodation in support of my research for 2 years when I first came to Gainesville. His guidance and encouragement have been immensely helpful. I always remember what he said: “Focus on science and science will pay you back and show you a way to your goal.” Additionally, I would like to thank my other committee members, Dr. Natalia A. Peres, Dr. Michelle D. Danyluk, and Dr. Timothy S. Schubert for their support and valuable inputs into my research projects.

I would like to thank to all members working in Dr. Dewdney’s lab: Dr. Katia Ferreira Rodrigues, Dr. Byron Patricio Vega Jiménez, Tracy N. Hobbs, Jenna Lastinger, Naweena Thapa, Ke Zhang, Katrina R. Nicoletta, Collin Lewis-Solomon, Etelvina Aguilar, and Dr. Sachindra N. Mondal for their help and giving me such a nice and supportive working environment during this time. I would also like to express my sincere gratitude to Dr. Evan G. Johnson for always being so patient to answer any questions I have and advise me whenever I feel lost or depressed. I also thank Dr. Jose C. Huguet-Tapia for his help in bioinformatic analysis and Daniel L. Stanton for the help in the microscopic work.

Plant Pathology Department has been like a big happy family to me. All faculty, staff, and students are always so friendly and helpful. Among them, I would like to especially thank
Dr. Rosemary Loria, Dr. Matthew E. Smith, Heather Capobianco, Jessica Ulloa, Lauretta Rahmes, Dr. Xiaofei Liang, Dr. Jianan Wang, Erin Wood, Amanda Strayer, Sujan Timilsina, Juliana Pereira, James Orrock, Mayara Murata, and Yucheng Zhang. I am so glad to meet all of these good friends in the past few years.

I also thank my friends in Taiwan: Dr. Kuan-Chun Chen, Dr. Hao-Wen Cheng, Dr. Li-Yuan Wang, Dr. Yuh-Kun Chen, Dr. Wen-Hsin Chung, Yu-Han Huang, and Tai-Yuan Chen for having me in their mind.

Most importantly, I am extremely grateful for my wife, Yi-Tien Lu who is always the first person standing by me and has been patiently supportive with her unwavering love and faith in me. In the end, I sincerely thank my parents and the whole family for their support and love.
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Phylosticta citricarpa, the citrus black spot pathogen, was first identified in Florida in March 2010 and subsequently has become established. P. citricarpa causes the reduction of fruit yield and marketability. Both ascospores and conidia of P. citricarpa have been known to greatly contribute to the disease epidemiology, but information regarding the conditions required for production of both types of inocula and how conidial germination and infection of P. citricarpa occurs is lacking. Therefore, the overall objective of this research was to investigate the conditions essential for inoculum production and the infection process by P. citricarpa. The mating-type (MAT) loci of P. citricarpa and P. capitalensis were first identified via draft genome sequencing and characterized at the structural and sequence levels. P. citricarpa was determined to have an idiomorphic, heterothallic MAT locus structure, whereas P. capitalensis was found to have a single MAT locus consistent with a homothallic mating system. Furthermore, the interaction between P. citricarpa and the common twig-colonizing pathogen Diaporthe citri on dead twigs was studied. Preliminary results suggest that D. citri is a dominant fungus on dead twigs in the presence of equal amounts or similar ratios of inocula of both species on twigs. To aid the quantification of D. citri on twig samples, a quantitative polymerase chain reaction (qPCR) assay for D. citri was developed. Following the investigation of the relative humidity
(RH) effect on *P. citricarpa* development on twigs, a possible humidity threshold between 72 and 82% RH for fungal development was identified. As to the effects of citrus juices on conidial germination and appressorium formation of *P. citricarpa*, all tested juices, especially 'Valencia', favored germination and appressorium formation (> 85%, *P* < 0.05), whereas sterile water rarely stimulated germination (< 1%). The 'Valencia' juice effect was concentration- and pH-dependent, and the maximum rate was reached in 1.5% juice with pH of 3.4. Further analysis of critical juice components using synthetic juice revealed that sugars, salts, citric acid, and thiamine combined were most important for germination and appressorium formation (> 80%, *P* > 0.05). In addition, a paraffin embedding and sectioning method was demonstrated to be a useful tool to investigate the leaf infection process by *P. citricarpa*, although thus far only few samples have been processed successfully. Overall, findings from this research will provide useful information on conditions required for inoculum production and the infection process by *P. citricarpa*, which, in turn, will help improve disease control programs.
Citrus

Citrus belongs to the genus *Citrus* within the family Rutaceae and is one of the most economically important fruit crops worldwide. Citrus has been grown in many tropical and subtropical countries with 135 million tons produced in 2013 (Davies and Albrigo 1994; FAOSTAT 2015). Among the citrus-producing countries, China is the leading citrus producer in the world, followed by Brazil, the United States of America (US), India, and Mexico (FAOSTAT 2015). Other counties such as Spain, Egypt, Nigeria, Turkey, Argentina, Italy, Iran, South Africa, and Pakistan also significantly contribute to the citrus production of the world (FAOSTAT 2015). Various types of citrus have been developed, among which sweet orange, grapefruit, mandarin, lemon, and lime are five major citrus groups with commercial importance (Davies and Albrigo 1994; FAOSTAT 2015). According to the report made by FAOSTAT (2015), sweet orange (*C. sinensis*) is the most widely distributed citrus crop in the world, accounting for 53.8% of the global production in 2013, followed by mandarin (*C. reticulata*) (21.2%), lemon (*C. limon*)/lime (*C. aurantifolia*) (11.1%), and grapefruit (*C. paradisi*) (6.1%). In addition to those citrus crops with marketable fruit, trifoliate orange (*C. trifoliata*) and its hybrids are commonly used as rootstocks around the world.

**Citrus Production in Florida**

Florida, California, Texas, and Arizona are the four major citrus producers in the US (USDA-NASS 2016). With the hot and humid climate, Florida has been the leading producer of citrus in the US, accounting for 5 million tons or 55.9% of the total citrus production in the 2014-2015 season, followed by California (40.6%), Texas (2.6%), and Arizona (0.9%) (USDA-NASS 2015). In Florida, Polk, Hendry, Highlands, DeSoto, and Hardee Counties are the top five
counties for citrus production in the 2013-2014 season according to the Florida Agricultural Statistics Service (2015). Many different types of citrus are produced in Florida and sweet orange is the most common type in the state which consists of 84.3% of total citrus production in the 2013-2014 season (Florida Agricultural Statistics Service 2015). While most of the citrus produced in Florida is destined for juice processing, other citrus varieties such as grapefruit and mandarin are also produced for fresh consumption (Florida Agricultural Statistics Service 2015; USDA-NASS 2015).

Plant pathogens, especially Huanglongbing (HLB, or citrus greening) and citrus canker, have been problematic for citrus growers in Florida. In addition, weather events such as freezes and hurricanes can extensively damage citrus orchards and contribute to the spread of pathogens into disease-free areas. Altogether, plant pathogens and weather events are the major limiting factors for citrus production in Florida. According to the forecast of Florida orange production in the 2015-2016 season made by USDA-NASS (2016), it is predicted that 76 million boxes will be picked, the worst season since the 1963-1964 season, representing a 29% decline in orange production from the previous season. Given that the citrus industry in Florida contributes an important part of the local agricultural economy, supporting more than 75,800 jobs with an estimated economic impact of approximately $9 billion (Hodges and Spreen 2012), it will be necessary to address the current problems in order to sustain the citrus production in Florida.

**Citrus Black Spot**

**Economic Importance**

Citrus black spot caused by *Phylllosticta citricarpa* (McAlpine) van der Aa (teleomorph: *Guignardia citricarpa* Kiely) is a common fungal disease in many humid sub-tropical citrus producing areas. This disease affects all commercially-grown citrus cultivars; sweet orange, mandarin, grapefruit, and lemon, among which lemon is the most susceptible (Baldassari et al.
Although sour orange (*C. aurantium*) and Tahiti lime (*C. latifolia*) do not produce citrus black spot symptoms, *P. citricarpa* can none-the-less be isolated from asymptomatic host tissue (Baldassari et al. 2008; Kotzé 1981). *P. citricarpa* causes fruit surface blemishes and thus decrease the fruit value for the fresh fruit market even though the internal fruit quality is not affected (Kotzé 1981). When citrus groves are severely infected by citrus black spot, fruit yield declines due to premature fruit drop (Kiely 1949; Kotzé 1981; Spósito et al. 2011), which is a particular concern for juice processors. Furthermore, *P. citricarpa* is currently listed as an A1 quarantine pest that is currently absent from the European and Mediterranean Plant Protection Organization (EPPO) region. The European Union (EU) quarantine measures for citrus black spot have considerably slowed or prevented citrus export to the EU from countries with the disease (EFSA 2014).

**Distribution**

*P. citricarpa* was first described to cause substantial yield reduction in 'Valencia' orange orchards in New South Wales, Australia (Benson 1895). Afterward, significant crop losses due to this disease was reported, up to 80%, in South Africa in 1929 (CABI/EPPO 1997; Kotzé 1981). Since then, citrus black spot has been spread to many humid sub-tropical citrus-producing areas, including parts of Southeast Asia, Australia, South Africa, South America, and most recently North America (Florida) (Kotzé, 1981; Paul et al., 2005; Schubert et al., 2012). However, this disease has never been reported from Mediterranean citrus-producing countries where *P. citricarpa* is considered as a quarantine pathogen (Paul et al., 2005; Yonow et al., 2013).

**Citrus Black Spot in Florida**

The hot and humid environmental conditions in Florida are expected to favor the development of citrus black spot (Kotzé, 1981). This expectation is further explored with a predictive model, showing that the climatic conditions in Florida highly favor the disease.
development (Magarey et al. 2011). Other areas in the US, such as California, are predicted to be at low risk of disease development, which is due to the lack of sufficient rainfall to provide prolonged leaf wetness periods for disease development and inoculum production (Magarey et al. 2011).

In March 2010, citrus black spot-like symptoms were found on 'Valencia' orange trees from the Immokalee area in southwest Florida (Schubert et al. 2012; USDA-APHIS 2010b). These samples were subsequently verified as citrus black spot using pure culture isolation and the polymerase chain reaction (PCR) technique with species-specific primers (Schubert et al. 2012). How the disease was introduced into Florida remains mystery. According to the risk assessment conducted by USDA-APHIS (2010b), some possible introduction pathways were identified, including the natural spread by windborne spores and the movement of contaminated plant materials. Given that citrus black spot is not present in neighboring countries like Mexico, the introduction of this disease through natural spread is less likely (USDA-APHIS 2010b). Instead, the introduction of citrus black spot into the US through contaminated plant materials is another legitimate pathway worth consideration since a number of unregulated and non-commercial infected fruit were intercepted between 1985 and 2007 (USDA-APHIS 2010b).

**Morphology**

The pseudothecium of *P. citricarpa* is formed sub-epidermally only in decomposing leaf litter (Kiely 1948). The pseudothecium (125 to 135 µm in diameter) is a globose, dark brown to black, often solitary thick-walled structure, but sometimes can be found in groups of two or three (Kiely 1948). An ostiole (14 to 16 µm in diameter) of the pseudothecium is only present when mature. The asci (50 to 85 µm × 12 to 15 µm) formed within the pseudothecia are clavate or cylindrical with eight ascospores at maturity (Kiely 1948). Ascospores (8.0 to 17.5 µm × 3.3 to
8.0 μm) are hyaline to greyish and are curved and aseptate with a large central guttule (Kiely 1948). Mucoid cap-like appendages are found on both ends of ascospores (Kiely 1948), typical of the genus *Guignardia*.

The pycnidia of *P. citricarpa* can be produced on lesions of symptomatic fruit, dead twigs, and in decomposing leaf litter (Spósito et al. 2011; van de Aa 1973; Whiteside 1967). Pycnidia (up to 250 μm in diameter) are globose or pyriform and dark brown to black with a thick wall of 20 to 50 μm and a single ostiole of 10 to 15 μm in diameter (Glienke et al. 2011). Conidiophores (10 to 20 μm × 4 to 7 μm) developed in the pycnidia are cylindrical, hyaline, and coated with a mucoid layer (Glienke et al. 2011). Conidiogenous cells are also cylindrical, hyaline, and 7 to 12 μm × 3 to 4 μm. Pycnidiospores (conidia) (7.1 to 7.8 μm × 10.3 to 11.8 μm) are ellipsoid to obovoid, hyaline, aseptate, and smooth-walled with a narrow flat base (Glienke et al. 2011; Schubert et al. 2012). They are also enclosed within a thin mucilaginous sheath (1 to 2 μm) with a hyaline and mucoid apical appendage that tapers toward an acute apex (Glienke et al. 2011; Schubert et al. 2012).

Spermatia of *P. citricarpa* are usually produced simultaneously with the formation of pycnidia and conidia both in pure culture and on host tissue (Baayen et al. 2002; Kiely 1948; van der Aa 1973). Their fruiting bodies are similar to pycnidia in size and shape (Kiely 1948). Spermatia (7 to 10 μm × 1.8 to 2.5 μm) are dumbbell-shaped, straight to slightly curved, and hyaline (Baayen et al. 2002; Kiely 1948; van der Aa 1973).

The culture morphology of *P. citricarpa* on artificial medium depends on the external nutritional sources. For instance, *P. citricarpa* cultures grown on potato dextrose agar (PDA) are dark brown to black (Baayen et al. 2002; van der Aa 1973). Conidiomata appear as black masses and begin to produce conidia after incubation for 8 days on PDA (Baayen et al. 2002; van der Aa
1973). On oatmeal agar (OA), *P. citricarpa* produces a distinct yellow pigment, which has been used as a quick screening method to distinguish *P. citricarpa* from other *Phyllosticta* species (Baayen et al. 2002; Baldassari et al. 2008).

**Disease Symptoms**

Citrus black spot can cause multiple, rind-limited fruit symptoms, which is influenced by climatic conditions and the stage of fruit maturity, including hard spot, false melanose, freckle spot/early virulent spot, cracked spot, and virulent spot (de Goes et al. 2000; Kiely 1948; Kotzé 1981) (Fig. 1-1).

Hard spot is the most diagnostic pre-harvest symptom in the field and characterized as nearly circular dark brown lesions (3 to 10 mm in diameter) that are depressed with a gray central crater with a red to brown margin (Kiely 1948; Kotzé 1981; Schubert et al. 2012) (Fig. 1-1A). Hard spot often appears on the side of the fruit that has been most exposed to sunlight when fruit starts ripening (Kotzé 1981). Pycnidia often are present within the gray center as slightly raised black dots (Kotzé 1981).

False melanose consists of numerous, tiny, slightly raised, dark brown to black lesions (1 to 3 mm in diameter) that typically appear on green fruit and are devoid of pycnidia (Kotzé 1981) (Fig. 1-1B). Sometimes a tear-streaked pattern is observed possibly due to the flow of spore-containing water over the fruit surface (Agostini et al. 2006). False melanose can coalesce and develop into hard spot with the progress of the season (Kotzé 1981).

Freckle spot or early virulent spot is characterized as small, irregular, reddish to light brownish, depressed lesions (up to 7 mm in diameter) that usually occur on mature and post-harvest fruit (Kotzé 1981) (Fig. 1-1C). Freckle spot can develop into hard spot or coalesce to form virulent spot later in the season or during storage (Kotzé 1981). Pycnidia may be observed within the lesions, particularly under high humidity conditions (Schubert et al. 2012). The
appearance of freckle spot on citrus fruit usually represents severe fruit infection by citrus black spot in the orchard.

Cracked spot is superficial, slightly raised, cracked lesions with irregular diffuse margins (up to 15 mm in diameter) (Schubert et al. 2012) (Fig. 1-1D). Cracked spot does not contain pycnidia but sometimes may have hard spot within the lesion (Schubert et al. 2012). Cracked spot lesions have only been observed in the Americas and are reported to be associated with the citrus rust mites (de Goes et al. 2000).

Virulent spot is characterized as irregularly shaped, sunken, reddish lesions; this is the most severe symptom of citrus black spot (Kiely 1948; Kotzé 1981) (Fig. 1-1E). These lesions often coalesce to form big, brown to black lesions that can cover a large portion of the fruit surface with a leathery texture and may contain many pycnidia (Kiely 1948; Kotzé 1981). Virulent spot occurs on fully mature, heavily infected fruit toward the end of the season and can cause serious post-harvest damage (Kiely 1948; Kotzé 1981).

Despite the occurrence of multiple fruit symptoms, leaf symptoms are rare but observed most frequently on lemons (Kiely 1949). Leaf symptoms are small, round, necrotic spots with a gray to brown center and are usually surrounded by a black to reddish margin and a yellow halo (Kiely 1949) (Fig. 1-2). Hard spot-like symptoms on twigs are not commonly observed in the citrus-producing areas in the world, except on lemons in South Africa (Truter 2010). However, inoculum produced on dead twigs can be an important source of infection within the tree canopy, as was reported in Brazil and Zimbabwe (Spósito et al. 2011; Whiteside 1967). Twig symptoms are small, round, sunken spots (0.5 to 2 mm in diameter) with a gray to light brown center and a brown to black margin (Truter 2010). Pycnidia may also be produced inside these lesions (Truter 2010).
Inoculum Sources

While the citrus black inoculum spot can come from either ascospores or pycnidiospores, epidemiological studies outside of North America indicate the most important inoculum for the spread of citrus black spot is ascospores that are produced solely in decomposing leaf litter (Kotzé 1981; Reis et al. 2006; Spósito et al. 2008) (Fig. 1-3). Pseudothecia of *P. citricarpa* can form in leaf litter approximately 50 to 180 days after leaf abscission under alternating wetting and drying cycles (Kotzé 1981; Reis et al. 2006; Spósito et al. 2008). During leaf wetting events, ascospores are forcibly ejected up to 1.2 cm from mature pseudothecia and carried over some distances (< 24.7 m) by wind currents to susceptible plant tissue (Huang and Chang 1972; Lee and Huang 1973; McOnie 1964b; Spósito et al. 2007). Peak ascospore releases usually occur in late spring from April through early September with favorable conditions for infection from May to September in the Northern hemisphere climate (Fourie et al. 2013; Kotzé 1981). Rainfall has been reported to be essential for ascospore discharge, which occurs within an hour of leaf wetting and can continue for 12 h or longer (Lee and Huang 1973; McOnie 1964b; Reis et al. 2006). However, too much rainfall will adversely affect the pseudothecial formation and ascospore discharge, due to the rapid decomposition of leaf tissue (Kotzé 1981; Lee and Huang 1973). In Brazil, Reis et al. (2006) found that ascospore discharge in orchards was only loosely correlated with leaf wetness duration but not with temperature or total rainfall, suggesting that the ascospore discharge can be initiated even by a small amount of rain. In addition, the pattern of hourly spore trapping in Florida does not match the pattern of rainfall, indicating that the relationship between ascospore discharge of *Phyllosticta* species and rainfall is not consistent in Florida and may be more complicated than what was reported previously (M. M. Dewdney, personal communication).
The *in vitro* production of *P. citricarpa* ascospores has not been observed (Baayen et al. 2002; Baldassari et al. 2008; McOnie 1964a). Despite a single report from Moran Lemir et al. (2000) that showed that an artificial lemon extract and malt extract (LEMA) medium was able to stimulate ascospore production of *P. citricarpa* after 8 days incubation at 27°C and subsequent 21 days incubation at 6°C, this result has not been repeated by other groups (Baldassari et al. 2008), including our own. Whether the conditions for ascospore production by *P. citricarpa* have not been appropriate or whether *P. citricarpa* requires a compatible mating type partner to achieve sexual reproduction remains unknown.

Conidia of *P. citricarpa* are considered a source of secondary inoculum and produced on lesions of symptomatic fruit, dead twigs, and in leaf litter (Kotzé, 1981; Spósito et al. 2011; Whiteside 1967) (Fig. 1-3). Mature conidia surrounded by a mucilaginous substance emerge from the ostiole and are disseminated by rain splash to nearby susceptible fruit and leaves, a process which can occur multiple times per season from November to March in the Northern hemisphere (Kotzé, 1981). *P. citricarpa* conidia have been documented to play an important role in the disease spread within tree canopies in Brazil and Zimbabwe (previously called Rhodesia) where infections are attributed to conidial inoculum from late hanging or out-of-season symptomatic fruit and dead twigs even in the presence of ascosporic inoculum (Spósito et al. 2011; Whiteside 1967). However, according to studies originally conducted in Australia and South Africa, ascospores can be solely responsible for the disease epidemic even in the presence of both inoculum sources in the orchards (Kiely 1948; Kotzé 1981; Spósito et al. 2008; Spósito et al. 2011), which is due to the limited rainfall in the summer, the restricted flowering period, and the early harvest of citrus fruit prior to the beginning of the new fruit set.
Conidial production of *P. citricarpa* on artificial medium is affected by light and temperature (Brodrick and Rabie 1970). It was found that a significantly higher number of conidia was produced under continuous light than under alternating light/dark conditions or continuous darkness (Brodrick and Rabie 1970). Moreover, the temperature effect on conidial production appeared to be medium-dependent as incubation at 27°C significantly stimulated more conidial production on flavedo pieces of citrus fruit than at 20°C, although the reverse was also true for conidial production on PDA (Brodrick and Rabie 1970). The number of conidia produced on PDA was significantly higher after incubation at 20°C under continuous light for 15 days than for 10 or 20 days, which may be because most of conidia were immature after 10 days incubation and at 20 days conidia were not easily released due to the gelatinous matrix formed within the pycnidia (Brodrick and Rabie 1970).

**Germination**

Since the *in vitro* production of *P. citricarpa* ascospores has not been successful to date, only few studies report the investigation of the germination processes of ascospores. In addition, since those studies were conducted before the advent of molecular identification of *Phyllosticta* species, it is uncertain whether the collected ascospores were from *P. citricarpa* or *P. capitalensis*. Kiely (1948) reported that ascospores required more than 24 h to germinate on PDA at 25°C and 4 days to reach over 90% germination. However, McOnie (1967) found that the germination efficiency of ascospores on lemon leaves was inconsistent, ranging from 14 to 91% after 24 h incubation and was not enhanced by a longer incubation period of 48 h. The difference in efficiency of ascospore germination between these two studies is likely attributed to the preference of surface wettability, as was observed for conidial germination of *Phyllosticta* species (Shaw et al. 2006).
Ascospore germination of *P. citricarpa* began with the onset of germ tube emergence (Kiely 1948), followed by the migration of cytoplasmic contents into the germ tube. After 48 h, the cytoplasmic contents had migrated from the ascospores into the melanized appressoria, and a septum was formed to separate the appressorium from the germ tube (Kiely 1948). As a result, the cell walls of ascospores and germ tubes became scarcely discernible. The size of appressoria varied greatly and seemed to be inversely proportional to the length of germ tubes as the relatively smaller appressoria (3 to 8 μm in diameter) were produced with the average length of germ tubes being 78 μm (Kiely 1948). McOnie (1967) also observed that ascospores produced on artificial medium varied more greatly in size and shape than those produced on leaf or fruit surface. The initiation of appressorium germination of *P. citricarpa* appeared to be related to the location where they were formed, as appressoria only germinated on the surface of the medium but not within the medium, suggesting that the supply of oxygen during the appressorium germination may be critical (Kiely 1948).

Conidial germination of *P. citricarpa* was found to occur neither on artificial media such as water agar or PDA nor in sterile water on a hydrophobic surface (Darnell-Smith 1918; Kiely 1948; Korf 1998), implying the requirement of additional stimuli for conidial germination. Darnell-Smith (1918) firstly reported that the orange peel extract has the capacity to stimulate conidial germination of freshly discharged *P. citricarpa* conidia in 12 h. Kiely (1948) further tested the capabilities of extracts of various fruit tissues at different ages for germination and proposed that some organic acids might play an important role in stimulating conidial germination. Afterwards, he tested an assortment of organic acids (citric acid, tartaric acid, lactic acid, and oxalic acid) for stimulating germination and found that 0.1 to 0.5% citric acid greatly improved conidial germination to over 80%. However, such a high germination rate of *P.*
*citricarpa* conidia in citric acid solutions has not been repeated by other laboratories (Korf 1998), including our own. Furthermore, Korf (1998) reported that the addition of 2% 'Valencia' juice into the conidial suspension of *P. citricarpa* drastically increased the germination rate to approximately 60% after 48 h incubation at 22°C. These results indicate that other factors besides citric acid may also be involved in initiation of conidial germination.

The germination processes of *P. citricarpa* conidia are similar to those of ascospores (Kiely 1948; Korf 1998), but two and sometimes three germ tubes can be observed during ascospore germination, a phenomenon never been observed in the case of conidial germination (Kiely 1948).

**The Latent Period**

The long latency following infection is an important characteristic of citrus black spot (Kotzé 1981; McOnie 1967). This latent period begins after the successful penetration via a thin penetration peg through the cuticle and formation of a mycelial mass between the cuticle and the epidermal cells (McOnie 1967). When fruit is fully developed and reaches maturity, the latent period is terminated, triggering the onset of symptom development (Kotzé 1981). While the underlying mechanisms of the termination of citrus black spot latency are not clear, Kotzé (1981) highlighted several factors that could be influential in the senescent state of host tissue and contribute to the disease development: (i) Temperature. Rising temperatures significantly stimulate symptom development on mature fruit as more fruit lesions were observed at 27°C than at 20°C; (ii) Light. The exposure of fruit to high light intensities stimulates symptom development as more fruit symptoms appeared on the side of the fruit with light exposure, particularly in the upper halves of old trees; (iii) Drought. Drought affects symptom development as more lesions were induced on fruit from wilted trees than from non-wilted trees; (iv) Fruit maturity. More fruit symptoms will appear when fruit is fully developed and reaches maturity.
particularly with the fruit color changes from green to yellow or orange; (v) Tree vigor. Tree condition and age affects symptom development as fruit symptoms are less severe and sometimes even not observed on younger, more vigorous trees. Brentu et al. (2012) also reported a similar observation in Ghana where the younger citrus orchards had lower disease severity than older ones.

**Citrus Black Spot Management**

The spread of *P. citricarpa* to disease-free areas can occur through either the movement of pathogen-contaminated plant materials such as nursery stocks or its airborne ascospores (CABI/EPPO 1997; Kotzé 1981). Once arrived, the spread of the pathogen will rely greatly on the effectiveness of quarantine measures and the actions taken by local growers. Disease epidemics may be peak in 5 to 30 years from the first observation of symptoms in the field depending on the climatic conditions and available hosts (Kotzé 1981). Once the disease becomes severe, it will have to be managed by effective control practices (Kotzé 1981).

**Quarantine Measure**

To curb the spread of citrus black spot in Florida, the Animal and Plant Health Inspection Service (APHIS) issued a federal order designating 25 sections in Collier County and eight sections in Hendry County as quarantine areas (USDA-APHIS 2010a). Currently, the quarantine areas have been expanded to 91 sections in Collier County, 133 sections in Hendry County, six sections in Lee County, and one section in Polk County due to the additional detection of citrus black spot during ongoing field surveys (USDA-APHIS 2015). Moreover, this order restricts the interstate and intrastate movement of regulated articles, including citrus fruit, plant materials (leaves, budwood, nursery stock, and debris), and any other articles that could potentially become hazards for the spread of the disease (USDA-APHIS 2012). The shipping of citrus fruit from regulated areas is allowed only after fruit has been subjected to the mandated treatments at
a certified packing house from the grove, including inspection, grading, washing, brushing, disinfecting, and waxing (USDA-APHIS 2012). In addition, bulk loads of harvested citrus fruit must be free of regulated plant materials such as leaves or stems, and the cargo area of transport vehicles must be covered with a tarpaulin when transporting fruit for packing or processing (USDA-APHIS 2012). After shipments are completely unloaded, all possible contaminated objects such as trailers, tarpaulins, field boxes, and bins must be cleaned of plant debris and sanitized. Debris subsequently must be heat treated, incinerated, or buried at a landfill or other APHIS-approved disposal sites (USDA-APHIS 2012).

**Cultural Control**

Multiple cultural practices intended for reducing inoculum sources of citrus black spot in the field have been recommended (Dewdney et al. 2016). Regardless of the cause, declining trees in an orchard should be removed since they are often more susceptible to the disease and tend to have more dead twigs, leaf drop, and off-season bloom as a symptom of stress (Dewdney et al. 2016). Regular sanitary pruning should also be applied to decrease the number of dead twigs in the tree canopy and increase the canopy air flow to reduce the leaf wetness periods required for successful fungal infection (Dewdney et al. 2016; Kotzé 1981).

Removing the leaf litter from the orchard floor is considered an important control measure for citrus black spot (Spósito 2004; Spósito et al. 2011). In addition, accelerating leaf litter decomposition using urea, calcium nitrate, dolomite lime, or Soil-Set® + Compostaid® has been documented to aid disease control under field conditions (Bellotte et al. 2009). In Florida, while the field evaluation of compounds for accelerating leaf litter decomposition in relation to the control of citrus black spot is ongoing, three approaches have been recommended according to the effectiveness in reducing the ascospore inoculum of *Mycosphaerella citri*, the causal agent of greasy spot (Mondal and Timmer 2003; Mondal et al. 2007). The first approach is to increase
the frequency of microsprinkler irrigation to at least five times a week for half an hour per period for 1.5 months (Dewdney et al. 2016). The second approach is to treat leaf litter with urea (187 lb/acre) or ammonium sulfate (561 lb/acre) (Dewdney et al. 2016). The last approach is to treat leaf litter with dolomitic lime or calcium carbonate (2226 lb/acre) (Dewdney et al. 2016). All approaches above are equally effective in reducing the ascospore inoculum of *M. citri*, but dolomitic lime or irrigation methods should not be used simultaneously with the high nitrogen treatments due to the opposite modes of action (Dewdney et al. 2016).

Late-hanging fruit and overlapping fruit crops can be another important source of inoculum, particularly in 'Valencia' blocks. If at all possible, the old crop should be removed before the new fruit set in the following season (Dewdney et al. 2016). Minimizing the movement of plant debris within and among citrus groves is also essential since the main inoculum (ascospores) of citrus black spot is produced in leaf litter which is very easy to be inadvertently dispersed from one location to another (Dewdney et al. 2016). As with all diseases, clean nursery stock should be used for planting to prevent the introduction of citrus black spot to disease-free areas (Dewdney et al. 2016). Currently, while no registered commercial nurseries in Florida are close to the known infected groves, the situation may change due to the gradual expansion of the distribution of citrus black spot (Dewdney et al. 2016).

**Chemical Control**

Routine fungicide applications are the major strategy for the control of citrus black spot. Copper, as one of the main protective fungicides, has given satisfactory results in controlling the disease (Kiely 1950; Kotzé 1963). However, some of disadvantages of copper applications such as phytotoxicity to the plant, rind stippling, and toxic accumulation in soil must be taken into account. Alternatives to copper fungicides are essential for the long term control of the disease (Schutte et al. 1997). Previously, the application of benomyl to control citrus black spot in South
Africa was a successful but short-lived measure as benomyl-resistant isolates were identified in the field after 11 years of use (Herbert and Grech 1985). Years later, Schutte et al. (2003) reported that 99% reduction of citrus black spot incidence was achieved by two applications of azoxystrobin (QoI fungicide, quinone outside inhibitor) mixed with mancozeb and mineral oil applied during the summer in the groves of South Africa where benomyl resistance had developed. In another field trial in Australia, applications of QoI fungicides, particularly azoxystrobin were found to be as effective in controlling citrus black spot as copper with the additional benefits of lower phytotoxicity and rind stippling (Miles et al. 2004). In addition, Possiede et al. (2009) reported that the use of a fungicide mixture of azoxystrobin and benomyl obtained a great success in controlling citrus diseases, including citrus black spot.

In Florida, a field trial conducted in a grove where citrus black spot has become established showed that applications of different groups of fungicides and products registered for controlling citrus diseases in Florida did not demonstrate significant differences in the disease control of citrus black spot (Roberts et al. 2012). These findings may be attributed to the unrepresentative sample sets, the uneven presence of inoculum in the grove, or fruit drop caused by other diseases rather than citrus black spot such as huanglongbing and/or citrus canker (Roberts et al. 2012). None-the-less, preliminary results of a recent field trial for selected fungicide regimes, including Kocide 3000 rotated with Quadris Top 2.71SC, Enable 2F plus 5% citrus oil rotated with Kocide 3000, and Pristine 38WG rotated with Kocide 3000 showed that, while not significant, all fungicide applications reduced the fruit drop and the number of fruit with lesions (Roberts and Dewdney 2015). Further studies will be required to determine the efficacy of these fungicides in controlling citrus black spot in Florida.
Currently, different groups of fungicides have been recommended for disease control in Florida, including copper, QoI, DMI (demethylation inhibitor), and SDHI (succinate dehydrogenase inhibitor) fungicides (Dewdney et al. 2016). However, since only four applications of QoI fungicides are allowed per season, QoI fungicides should be reserved when fruit phytotoxicity caused by copper is an issue, particularly in hot summer (temperatures > 34°C) (Dewdney et al. 2016). In addition, two consecutive applications of QoI fungicides should be avoided to reduce the possibility of the occurrence of pathogen resistance (Dewdney et al. 2016).

**Research Objectives**

Citrus black spot is an emerging disease in Florida and can cause substantial economic loss to the local citrus industry. Both ascospores and conidia have been known to greatly contribute to the disease spread in citrus-producing areas of the world where this disease has been established. Given the epidemiology of the disease, information regarding the conditions required for production of both types of inocula and how conidial germination and infection of *P. citricarpa* occurs is of both practical and fundamental value for further biological studies of the pathogen and the development of disease control strategies aimed at restricting the spread of the disease. Therefore, the overall objective of this research was to investigate the conditions essential for inoculum production and the infection process of *P. citricarpa*. The specific objectives were to: (i) characterize the mating-type (*MAT*) loci of *P. citricarpa* and the ubiquitous endophyte, *P. capitalensis* (Chapter 2), (ii) study the interaction between the common twig-colonizing pathogen *Diaporthe citri* and *P. citricarpa* and determine whether they can co-exist to successfully sporulate on dead twigs (Chapter 3), (iii) determine the conditions required for *in vitro* conidial germination of *P. citricarpa* (Chapter 4), and (iv) determine how infection
and colonization of leaves by *P. citricarpa* occurs, potentially leading to the production of
inoculum (Chapter 5).

In Chapter 2, the *MAT* loci of both *Phyllosticta* species were characterized using a draft
 genome approach. In Chapter 3, the relationship between *P. citricarpa* and *D. citri* was
determined by inoculating citrus twigs with a replacement series of conidial inocula of both
species. In addition, a quantitative real-time PCR (qPCR) assay was developed using TaqMan
chemistry for rapid detection of *D. citri*. In Chapter 4, the effects of nutritional and
environmental factors on conidial germination and appressorium formation of *P. citricarpa* were
evaluated in vitro on hydrophobic slides. In Chapter 5, a greenhouse experiment was conducted
to examine the infection process of *P. citricarpa* on citrus leaves using standard paraffin
embedding and sectioning procedures.
Figure 1-1. Fruit symptoms of citrus black spot. A, Hard spot; B, False melanose; C, Freckle spot/Early virulent spot; D, Cracked spot; E, Virulent spot. (Photograph source: IFAS Extension, University of Florida)
Figure 1-2. Leaf symptom of citrus black spot. (Photograph source: IFAS Extension, University of Florida)
Figure 1-3. Life cycle of *Phyllosticta citricarpa*, the citrus black spot pathogen. (Figure source: USDA-APHIS 2010; drawn by Heather Hartzog).
CHAPTER 2
CHARACTERIZATION OF MATING-TYPE (MAT) LOCI OF *Phyllosticta citricarpa*, THE CITRUS BLACK SPOT PATHOGEN AND *P. capitalensis*, THE UBIQUITOUS ENDOPHYTE

Introduction

Even though the role of ascospores in the epidemiology of citrus black spot has been studied (Kotzé 1981; McOnie 1964a; Reis et al. 2006; Spósito et al. 2008), the factors regulating *P. citricarpa* ascospore production are unclear. Sexual reproduction in the Ascomycota is controlled by a single MAT locus with two idiomorphs known as MAT1-1 and MAT1-2 (Debuchy and Turgeon 2006; Lee et al. 2010; Ni et al. 2011). The MAT1-1 idiomorph contains the MAT1-1-1 gene encoding an α-box protein, and the MAT1-2 idiomorph carries the MAT1-2-1 gene encoding a high mobility group (HMG)-domain protein (Lee et al. 2010; Ni et al. 2011). In heterothallic species, sexual reproduction occurs when opposite mating type partners interact (Lee et al. 2010; Ni et al. 2011). In contrast, homothallic fungi contain both MAT1-1-1 and MAT1-2-1 genes either in a fused MAT locus or in proximity which allows the initiation of sexual reproduction without the requirement for an isolate of opposite mating type (Lee et al. 2010; Ni et al. 2011). Although this is the most commonly observed scenario, exceptions to this general MAT locus structure and regulation of mating have been reported (Butler et al. 2009; O’Connell et al. 2012; Vaillancourt et al. 2000). Because *P. citricarpa* is not fertile in single-spore derived cultures, this species is presumed heterothallic but the MAT locus structure and underlying regulation have not been characterized.

In addition to *P. citricarpa*, *P. capitalensis* is commonly isolated from both diseased and healthy citrus fruit (Baayen et al. 2002; Glienke et al., 2011). *P. capitalensis* is a ubiquitous endophyte asymptotically colonizing citrus and many other hosts (Glienke et al., 2011). These two *Phyllosticta* species are morphologically very similar, but *P. capitalensis* can produce fertile
pseudothecia on artificial media (Baayen et al. 2002; Baldassari et al. 2008) whereas *P. citricarpa* cannot (Baayen et al. 2002; Baldassari et al. 2008). From these observations, *P. capitalensis* has been presumed to have a homothallic mating system and *P. citricarpa* a heterothallic system. A single report (Moran Lemir et al. 2000) concerning *P. citricarpa* ascospore production from a single isolate on an artificial medium has not been repeated by other groups (Baldassari et al. 2008) including our own. Whether the conditions for ascospore production by *P. citricarpa* have not been appropriate or whether *P. citricarpa* requires an opposite MAT type isolate to achieve sexual reproduction remains unclear. The objective of this study was to characterize MAT loci of *P. citricarpa* and *P. capitalensis*. The knowledge gained in this study will greatly improve our understanding of the epidemiology of citrus black spot in Florida and facilitate the development of disease control strategies aimed at limiting the spread of the disease.

**Materials and Methods**

**Fungal Isolation and Maintenance**

*P. citricarpa* isolate Gc12 and *P. capitalensis* isolate Gm33 were isolated from symptomatic 'Valencia' fruit collected from Immokalee areas where citrus black spot has been identified (Schubert et al. 2012). The identity of both isolates was verified based on the production of yellow pigment on oatmeal agar (Baayen et al. 2002) and using species-specific primers designed by Peres et al. (2007). Afterward, fungal isolates were single-spored, kept on colonized sterile filter paper in sterile coin envelopes, and stored in plastic containers with desiccants in a −20°C freezer as described by Hincapie et al. (2014). When needed, small pieces of filter paper were transferred onto half-strength PDA (1/2PDA; Difco, BD, Sparks, MD) and maintained at 24°C under a 12-hour photoperiod until use.
DNA Isolation and Genome Sequencing

To isolate genomic DNA (gDNA), the method described in Hu et al. (2014) was adopted. Briefly, fungal mycelia were grown on 1/2PDA with a layer of cellophane at 24°C under a 12-hour photoperiod for 6 days. Following this growth period, 100 mg of fungal mycelia was scraped from the cellophane, transferred into 2.0 ml screw cap tubes with sterile 5 mm stainless steel beads, flash frozen with liquid nitrogen, and homogenized twice for 30 s at 30 Hz using a Qiagen TissueLyser II (Qiagen, Valencia, CA). gDNA was then isolated using the Qiagen Plant Mini kit (Qiagen) based on the manufacturer’s protocol. The quality and quantity of gDNA was determined by measuring the absorbance at 260/280 nm and 260/230 nm using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). All DNA samples were stored at −20°C until use.

To obtain gDNA for genome sequencing, gDNA samples from arbitrarily-selected *P. citricarpa* Gc12 and from *P. capitalensis* Gm33 were further purified by phenol-chloroform extraction and ethanol precipitation. The quality and quantity of gDNA was determined as above and by electrophoresis on a 0.6% agarose gel. More than 5 µg of gDNA each of *P. citricarpa* Gc12 and *P. capitalensis* Gm33 were submitted to the Interdisciplinary Center for Biotechnology Research (ICBR) Genomics Core at University of Florida. Single-end sequencing was performed twice independently for each species on the Ion Torrent Personal Genome Machine (PGM) platform using 200- or 400-bp chemistry on an Ion 318 chip (Life Technologies, Carlsbad, CA).

Identification of *MAT* Loci

Raw sequencing data from Ion Torrent PGM were de novo assembled using the de Bruijn algorithm implemented in the CLC Genomics Workbench version 5.0.1 (CLC bio, Aarhus, Denmark) and the SPAdes genome assembler (version 3.5) (Bankevich et al. 2012). Multiple criteria including N50 value, total contig number, and the average contig length were used to
decide the best assembly. Final assembly parameters in the CLC Genomics Workbench utilized a window size of 20 and 30 nucleotides, a bubble of 175 and 400 nucleotides, and a minimum contig length of 200 nucleotides for sequencing data of *P. citricarpa* Gc12 and *P. capitalensis* Gm33, respectively. Protein-encoding genes were predicted from the assembly using the ab initio algorithm GeneMark-ES (Ter-Hovhannisyan et al. 2008). Homology searches were performed using BLASTn and BLASTp algorithms against the National Center for Biotechnology Information (NCBI) database and displayed using the EPoS 0.9.1 (Griebel et al. 2008) to search for MAT-associated genes including *MAT1-1-1, MAT1-2-1, apn2* (DNA lyase), and *sla2* (cytoskeleton assembly control protein) genes.

During the analysis of genomic data from *P. citricarpa* Gc12, a *P. citricarpa* isolate from China, CGMCC3.14348, was sequenced and deposited in the NCBI database (accession number AOTE00000000). This Chinese *P. citricarpa* genome was annotated and analyzed as above to identify MAT-associated genes.

**Amplification of the Flanking Region and the Coding Sequence of MAT1-2-1 of *P. citricarpa***

To determine the flanking sequence of *MAT1-2-1* of *P. citricarpa*, conventional PCR was performed in a 25 μl reaction volume containing 1 to 10 ng of gDNA, 0.2 μM of each primer, 0.2 mM of each dNTP, 1× PCR reaction buffer, and 1 unit of HotStar Taq Polymerase (Qiagen) using an MJ Research PTC-200 Peltier Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) according to the following conditions: 95°C for 5 min, 40 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min, followed by a final extension of 5 min at 72°C. PCR products were then electrophoresed on a 1% agarose gel with SYBR Safe DNA stain (Invitrogen) in 1× Tris-acetate-EDTA (TAE, 40 mM Tris, 20 mM acetic acid, and 1 mM EDTA, pH 8.4) buffer and visualized under ultraviolet light using a UVP-MultiDoc-It Digital Imaging System (UVP, Upland, CA).
The resulting PCR amplicons were cloned into the pGEM-T Easy vectors (Promega Crop., Madison, WI) and sequenced by Sanger chemistry at the ICBR, followed by BLASTn analysis against the NCBI database.

To determine the coding sequence of MAT1-2-1 of *P. citricarpa*, total RNA was extracted using the Qiagen RNeasy Plant Mini kit (Qiagen) following the instructions of the manufacturer, quantified using a NanoDrop ND-1000, and reverse transcribed into complementary DNA (cDNA) using SuperScript III reverse transcriptase (Invitrogen, Grand Island, NY) and oligo-dT<sub>20</sub> based on the instructions of the manufacturer. PCR reactions using GcitMAT-HMG-1 (5'-TTTCCTCTACTCAAGACCCTTCCC-3') and oligo-dT<sub>20</sub> were performed according to the conditions above, except the annealing temperature was 50°C. Subsequently, a nested PCR was performed as above using primer GcitMAT-HMG-3 (5'-GCTCTCCACACGTTCCAGTCCC-3') and oligo-dT<sub>20</sub> with 100-fold diluted PCR products, and resulting amplicons were cloned and sequenced as above.

**Sequence Alignment and Phylogenetic Analysis**

Dot plot analysis of MAT1-1 and MAT1-2 of *P. citricarpa* was performed using the Nucleic Acid Dot Plot program on the Molecular Toolkit website (http://www.vivo.colostate.edu/molkit/index.html) (Maizel Jr. and Lenk 1981). Synteny analysis between MAT1-1 and MAT1-2 of *P. citricarpa* and between MAT idiomorphs of *P. citricarpa* and *P. capitalensis* was performed using the Artemis Comparison Tool (ACT) (Carver et al. 2005). Sequence identity was analyzed using the Needleman-Wunsch global alignment algorithm available on the NCBI website. Predicted amino acid sequences of the PH-domain proteins of *P. citricarpa* and *P. capitalensis* were aligned using ClustalW2 along with other PH-domain proteins of Dothideomycetes obtained from GenBank and the Joint Genome Institute (JGI) genome databases (Table A-1). The maximum likelihood phylogenetic tree of amino acid
sequences was generated accordingly using MEGA version 6.0.5 (Tamura et al. 2013) with 1000 bootstrap replicates under the Le and Gascuel (LG) + Gamma (G) model.

Results

Identification of MAT Loci

Initial attempts to isolate sequences of the P. citricarpa MAT locus followed a PCR-based strategy using degenerate primers designed from MAT genes from Cochliobolus heterostrophus, Alternaria alternata, and Stagonospora nodorum (Arie et al. 1997; Arie et al. 2000; Bennett et al. 1999). None of the chosen primer combinations amplified a specific MAT locus sequence from P. citricarpa (data not shown). Subsequently, a draft genome sequencing strategy was pursued for P. citricarpa Gc12 and P. capitalensis Gm33 using two runs of semiconductor-based next generation sequencing technology for each species. For P. citricarpa (Gc12), 1.0×10^7 reads totaling 2,360 Mbp (1,889 Mbp of Q20 nucleotides) with a mean read length of 226 bp were obtained. For P. capitalensis (Gm33), 9.3×10^6 reads totaling 2,281 Mbp (1,745 Mbp of Q20 nucleotides) with a mean read length of 245 bp were obtained. These sequences were deposited in GenBank under accession numbers LOEN00000000 and LOEO00000000 for P. citricarpa and P. capitalensis, respectively. The raw sequencing reads from P. citricarpa Gc12 and P. capitalensis Gm33 genomes were assembled into 5,748 contigs with an estimated genome size of 31.1 Mbp (N50 = 21.1 kbp) and 1,341 contigs with an estimated genome size of 32.5 Mbp (N50 = 55.8 kbp), respectively (Table 2-1). Similar G+C contents were observed in P. citricarpa and P. capitalensis genomes (53.1% and 54.6%, respectively). In addition, a total of 9,763 and 10,316 protein-coding genes were predicted from P. citricarpa and P. capitalensis, respectively (Table 2-1).

From the assembled genome and predicted proteins of P. citricarpa isolate Gc12, BLAST queries using C. heterostrophus MAT1-2-1 identified a 1,463 bp-fragment on contig 3787
(contig length = 16,464 bp) with an E-value of 9e−19 (expectation value, a parameter describing the number of hits expected to see when searching a database; the higher E-value is, the less significant the match is.). This fragment encoded a protein with high similarity to MAT1-2-1 from *Sphaeropsis sapinea* (E-value = 3e−30). Contig 3787 also contained a putatively conserved flanking gene of the *MAT* locus, *apn2*. Sequences of genes including *sla2* which have been reported to flank *MAT* loci of other ascomycete fungi were not predicted on this contig. Using BLASTn and BLASTp searches with *C. heterostrophus MAT1-1-1* gene and protein as query sequences, no gene with homology to *MAT1-1-1* was identified in the *P. citricarpa* Gc12 genome. Contig 513 (contig length = 29,282 bp) in the assembled genome of *P. capitalensis* isolate Gm33 was identified by BLAST queries to encode proteins homologous to MAT1-1-1 from *Neofusicoccum parvum* with an E-value of 8e−47 and MAT1-2-1 of *Diplodia seriata* with an E-value of 8e−30. Similar to *P. citricarpa*, *apn2* was the only conserved flanking gene identified in this contig. Following BLAST queries of the Chinese *P. citricarpa* isolate CGMCC3.14348 genome using *C. heterostrophus MAT* genes as above, a gene of 1,941 bp on contig 4071 (9,524 bp) was identified with an E-value of 3e−29. This gene was further verified to encode a homologue of MAT1-1-1 from *N. parvum* (E-value = 5e−51) based on the subsequent BLASTp search of NCBI. No sequences sharing homology with MAT1-2-1 sequences were identified on contig 4071 or elsewhere in the *P. citricarpa* CGMCC3.14348 genome.

The structural organization of the *P. citricarpa* Gc12 *MAT1-2* locus was predicted by comparative sequence analysis with *P. capitalensis* Gm33 contig 513. Subsequently *P. citricarpa* PCR primers GcMAT-HMG-7 (5′-CATCCGCCCAACACATCAAAGCGACCTA-3′) and GcMAT-PH-4 (5′-CGGTGCTCTCCTTTTTCACTGGA-3′) were designed and used to amplify
sequences that joined contigs 3787 and 2477 to provide a complete \textit{MAT1}-2 locus sequence. The AUGUSTUS gene prediction tool was used to generate gene models for this joined contig as well as for contig 513 from Gm33 and contig 4071 from \textit{P. citricarpa} CGMCC3.14348. Multiple sequence alignments with related sequences from GenBank were used to confirm the coding sequence predictions. Reverse transcriptase-PCR was used to confirm the coding sequence of \textit{P. citricarpa} \textit{MAT1}-2-1. The resulting structural prediction of the \textit{P. citricarpa} Gc12 and \textit{P. capitalensis} Gm33 \textit{MAT} loci are shown in Fig. 2-1 and sequences were deposited in GenBank (accession numbers KT708823 and KT708824, respectively).

**Defining Boundaries of the \textit{MAT} Loci**

Synteny mapping between the \textit{P. citricarpa} \textit{MAT} idiomorphs determined the sequence boundaries of the \textit{MAT1}-1 and \textit{MAT1}-2 idiomorphs. The conserved flanking sequences of the \textit{MAT} locus begin upstream at the start of the \textit{oml1} (overlapping \textit{MAT} locus 1) 5' coding sequence and end downstream in the intergenic region of the 40S S9 gene (Fig. 2-1, 2-2A, and 2-2B). While the BLASTp search revealed that OML1 of \textit{P. citricarpa} is homologous to MAT1-1-4 of \textit{D. pinea} (E-value = 2e−06), the \textit{oml1} gene is primarily located in the conserved flanking sequences of both \textit{MAT} idiomorphs of \textit{P. citricarpa} and therefore as a whole is not considered part of the \textit{MAT} locus (Fig. 2-1, 2-2A, and 2-2B). From this analysis, the sequences that comprise the \textit{MAT1}-1 and \textit{MAT1}-2 idiomorphs are 6,005 bp and 6,623 bp, respectively.

Structurally, the \textit{MAT1}-1 idiomorph contained one additional predicted gene downstream of \textit{MAT1}-1-1; this gene encodes a protein with a putative integral membrane domain (PH domain) (Fig. 2-1). The \textit{MAT1}-2 idiomorph also encoded a PH-domain protein downstream of the conserved \textit{MAT1}-2-1 gene but it appears to be only distantly related to the PH-domain gene of the \textit{MAT1}-1 idiomorph as the two sequences share only 56% identity at the nucleotide level. The \textit{MAT1}-2 idiomorph also encoded a unique hypothetical protein located downstream of
MAT1-2-1 and upstream of the PH-domain gene (Fig. 2-1). A BLASTp search of the NCBI database revealed that this hypothetical protein shared low identity to MAT1-2-5 of D. pinea (E-value = 1.9) (Bihon et al. 2014). However, since the designation of MAT1-2-5 has been assigned to the cytochrome c oxidase subunit VIa gene (cox13) in Coccidioides species (Mandel et al. 2007), MAT1-2-9 was instead used to designate this gene in the MAT1-2 idiomorph according to the nomenclature proposed by Turgeon and Yoder (2000).

Gene prediction and BLAST analysis of the P. capitalensis contig 513 revealed that the MAT locus of P. capitalensis contained both MAT1-1-1 and MAT1-2-1 genes in close proximity (Fig. 2-1). This locus also contained the MAT1-2-9 gene, a PH domain-encoding gene and the conserved flanking genes found associated with the P. citricarpa MAT idiomorphs (Fig. 2-1). Synteny mapping between the MAT idiomorphs of P. citricarpa and the MAT locus of P. capitalensis revealed a conservation of gene composition between the species with a fused MAT1-1 and MAT1-2 idiomorph structure in P. capitalensis (Fig. 2-2C). Pairwise comparison between the MAT1-1-1 and MAT1-2-1 genes of P. citricarpa with the P. capitalensis orthologs revealed 68 to 70% nucleotide sequence identity (59 to 65% amino acid identity) (Table 2-2). The oml1 gene of P. capitalensis shared similar nucleotide sequence identity (55 to 57%) with the allelic copies of the P. citricarpa orthologs which were 92% identical to each other (Table 2-2). Consistent with the idea of independent evolution of the PH-domain encoding genes from the two P. citricarpa mating type isolates (56% nucleotide identity), the PH-domain gene from P. capitalensis shared greater nucleotide identity (72%) with the P. citricarpa MAT1-2 PH-domain gene than with the P. citricarpa MAT1-1 PH-domain gene (54%) (Table 2-2).
Comparison of Conserved Regions of MAT and PH-Domain Proteins

The alignment of deduced amino acid sequences of \( \alpha_1 \)-box proteins (MAT1-1-1) from Dothideomycetes showed a conserved motif with conserved intron positions in most fungal species used in this study (Fig. A-1A). The same analysis for deduced amino acid sequences of HMG-domain proteins (MAT1-2-1) also revealed a conserved motif containing an intron position specific to Dothideomycetes (Fig. A-1B). A phylogenetic tree for the concatenated amino acid sequences of the \( \alpha_1 \)-box and the HMG domain generated from maximum likelihood analysis showed well-supported clades for Pleosporales, Botryosphaeriales, and Capnodiales. \textit{P. citricarpa} was observed to group more closely with \textit{P. capitalensis} in Botryosphaeriales than other species in Pleosporales than Capnodiales (Fig. A-2).

To better understand the evolution of PH-domain encoding genes associated with these \textit{Phyllosticta MAT} loci, the chromosomal proximity of homologous sequences relative to MAT loci were examined in other sequenced Dothidiomycete species. The nucleotide distances between the MAT locus and PH-domain gene within genomes of Botryosphaeriales and Capnodiales species ranged from 0.7 to 16.7 kb, whereas the intervening sequences in genomes of Pleosporales were larger than 100 kb (Table A-2). In addition, among the genomes of Dothideomycetes only one copy of the PH-domain gene was found with the exception of \textit{P. citricarpa}, in which each \textit{MAT} idiomorph carries unique copies of a PH-domain gene. Maximum-likelihood phylogenetic analysis using PH-domain amino acid sequences from \textit{P. citricarpa}, \textit{P. capitalensis}, and other Dothideomycetes also showed well-supported clades for Pleosporales, Botryosphaeriales, and Capnodiales (Fig. 2-3). However, while the two PH-domain proteins of \textit{P. citricarpa} clustered most closely with the PH-domain protein from \textit{P. capitalensis},
the separate branching of the MAT1-1 copy of the *P. citricarpa* PH-domain protein reflected its significant divergence (Fig. 2-3).

**Discussion**

In areas of the world where citrus black spot has become established, pseudothecia are produced in the leaf litter and ascospores are thought to be responsible for the majority of new fruit infections and spread within the grove (Kiely 1948; Kotzé 1981; McOnie 1964a; Reis et al. 2006; Spósito et al. 2008). Given this epidemiology, how *P. citricarpa* regulates its sexual reproduction and what factors are critical for this process are of both practical and fundamental value. Deciphering the makeup, and structure of the *MAT* locus and distribution of idiomorphs within the population as reported here is an important step in gaining this understanding. We first discovered that the Floridian isolate of *P. citricarpa* (Gc12) characterized in this study only contained the MAT1-2 idiomorph. The presence of only one idiomorph in this isolate is consistent with the lack of self-fertility in culture (Baayen et al. 2002; Baldassari et al. 2008) and is supported by multiple other lines of evidence. Firstly, from the draft genome sequence established here, the assembled 28.9 kb contig containing the MAT1-2-1 coding sequence contained an additional 13.1 kb of upstream and 14.3 kb of downstream sequence with no sequence homology to known MAT1-1 genes. Further, given the significant sequencing coverage (approximately 76X), if MAT1-1-1 genes existed in close proximity with the MAT1-2-1 gene, as found in homothallic *P. capitalensis* Gm33 in this study, it would be expected to be found within this region. Thirdly, MAT1-1-1 sequences, but no evidence of MAT1-2-1 sequences, were identified in the *P. citricarpa* CGMCC3.14348 genome. Thus, the evidence presented here indicates that *P. citricarpa* is heterothallic.

The boundaries of fungal MAT loci can generally be determined based on the conserved flanking genes and genetic content. However, these criteria were not completely applicable to *P.
citricarpa. Studies have reported conserved genes that commonly flank the MAT loci of ascomycetes and zygomycetes (Debuchy and Turgeon 2006; Gryganskyi et al. 2010; Lee et al. 2010). Of them, apn2 and sla2 are most commonly found adjacent to MAT loci in ascomycetes (Debuchy and Turgeon 2006; Lee et al. 2010). But in Dothideomycetes, only apn2 is commonly found in the flanking region of the MAT locus (Debuchy and Turgeon 2006) and another nonconserved gene, such as the 40S S9 gene that we observed in P. citricarpa and the gap1 gene (GTPase activating protein) in Leptosphaeria maculans (Cozijnsen and Howlett 2003), rather than the sla2 gene commonly defines the other flank. This limited conservation of the downstream flank explains our failure to amplify the MAT locus in initial cloning attempts. Syntenic analysis between the MAT idiomorphs of P. citricarpa revealed that the conserved flanking sequences of the MAT locus begin upstream in the 5' end of the oml1 gene rather than in an intergenic region. This structure is rare but found in the MAT loci of Magnaporthe grisea, Cordyceps takaomontana, Aspergillus fumigatus, and Pyrenopeziza brassicae, where one of their MAT genes or lineage-specific genes extends from the idiomorphic region into the flanking region (Debuchy and Turgeon 2006; Paoletti et al. 2005; Singh and Ashby 1998; Yokoyama et al. 2003). The encoded OML1 protein of P. citricarpa shared homology with MAT1-1-4 of D. pinea that is located in its MAT1-1 idiomorph (Bihon et al. 2014), implying a possibility that the incorporation of the oml1 gene into the MAT locus of P. citricarpa is ongoing. The expansion of MAT loci has been observed in Coccidioides species, Mycosphaerella species, and the most striking case, Cryptococcus neoformans, in which extensive rearrangements of genes were found between the two idiomorphs (over 100 kb) (Arzanlou et al. 2010; Lengeler et al. 2002; Mandel et al. 2007). These findings are consistent with the evolution of MAT loci toward sex chromosomes (Lengeler et al. 2002).
In contrast to *P. citricarpa*, the MAT locus of *P. capitalensis* harbors both MAT1-1-1 and MAT1-2-1 genes as well as the MAT1-2-9 ortholog and a single PH domain-encoding gene. This organization was also present in the MAT locus of another presumably homothallic *Botryosphaeriales* fungus, *B. dothidea*. The structurally homothallic MAT locus in *P. capitalensis* is consistent with the previous observation that *P. capitalensis* can produce pseudothecia and ascospores in single spore-derived cultures (Baayen et al. 2002; Baldassari et al. 2008). The presence of a single PH-domain encoding gene in the homothallic locus as well as in each of the heterothallic idiomorphs presents an additional instance in which the distinction between conserved flanking sequence and idiomorphic sequences becomes blurred. Surprisingly, in the *P. citricarpa* idiomorphs the two PH-domain genes shared only 56% nucleotide identity, whereas the *P. citricarpa* MAT1-2 PH-domain gene shared 72% nucleotide identity with the *P. capitalensis* PH-domain gene. Based on this intra-species divergence, the PH-domain genes are considered part of the *P. citricarpa* idiomorph. Our analysis of other available genome sequences determined that, with the exception of *P. citricarpa*, the PH-domain gene is often found adjacent to the MAT idiomorphs in Dothideomycetes rather than a member of the idiomorph. This evidence combined with our findings of the divergent 5' sequences of *oml1* indicates that *P. citricarpa* has expanded its idiomorphic regions by the incorporation of flanking sequences, including the PH-domain gene that is frequently found adjacent to MAT idiomorphs.

From these results, the following evolutionary model for the MAT loci of *Phyllosticta* species is proposed (Fig. 2-4). The hypothetical *Phyllosticta* ancestor was homothallic and contained MAT1-1-1, MAT1-2-1, MAT1-2-9, and a single copy of the PH-domain gene. In the homothallic lineage, represented by *P. capitalensis*, the organization of the MAT locus remains similar to that of the *Phyllosticta* ancestor. However, in the heterothallic lineage, represented by
P. citricarpa, the ancestor was subjected to gene loss, resulting in strains containing the MAT1-1 idiomorph (containing MAT1-1-1 only) and the MAT1-2 idiomorph (containing MAT1-2-1 and MAT1-2-9). Subsequently, each idiomorph underwent independent sequence divergence and consequently acquired the flanking sequences including the PH-domain gene and 5´ portion of the omll gene. The PH-domain gene copy in the MAT1-1 idiomorph has diverged as evident from the higher interspecies rather than intraspecies sequence identity and closer phylogenetic association of the MAT1-2 PH-domain protein with the P. capitalensis PH-domain gene. This divergence, if accompanied by neofunctionalization, suggests a redundant function for the PH-domain gene in the P. citricarpa genome or a mating-sexual development function that requires only one copy.

In conclusion, this study successfully identified the MAT loci of P. citricarpa and P. capitalensis using draft genome sequencing. Our future work will firstly focus on designing mating type-specific primers to determine the mating type ratio in the Floridian population of P. citricarpa. In addition, with the genome sequences in hand, single sequence repeat (SSR) markers can be identified to provide better resolution for phylogenetic analysis of P. citricarpa, P. capitalensis, and other Phyllosticta species.
Table 2-1. Genome assembly statistics for *Phyllosticta citricarpa* and *P. capitalensis*.

<table>
<thead>
<tr>
<th>Statistic</th>
<th><em>P. citricarpa</em></th>
<th><em>P. capitalensis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Total sequences</td>
<td>5,748</td>
<td>1,341</td>
</tr>
<tr>
<td>Total bases (bp)</td>
<td>31,127,227</td>
<td>32,454,403</td>
</tr>
<tr>
<td>Min sequence length (bp)</td>
<td>200</td>
<td>203</td>
</tr>
<tr>
<td>Max sequence length (bp)</td>
<td>117,156</td>
<td>237,106</td>
</tr>
<tr>
<td>Average sequence length (bp)</td>
<td>5,415</td>
<td>24,201</td>
</tr>
<tr>
<td>Median sequence length (bp)</td>
<td>795</td>
<td>11,958</td>
</tr>
<tr>
<td>N25 length (bp)</td>
<td>37,150</td>
<td>87,493</td>
</tr>
<tr>
<td>N50 length (bp)</td>
<td>21,052</td>
<td>51,729</td>
</tr>
<tr>
<td>N75 length (bp)</td>
<td>9,435</td>
<td>28,479</td>
</tr>
<tr>
<td>N90 length (bp)</td>
<td>3,445</td>
<td>13,658</td>
</tr>
<tr>
<td>N95 length (bp)</td>
<td>983</td>
<td>8,411</td>
</tr>
<tr>
<td>(A + T)s</td>
<td>46.95%</td>
<td>45.44%</td>
</tr>
<tr>
<td>(G + C)s</td>
<td>53.05%</td>
<td>54.56%</td>
</tr>
</tbody>
</table>
Table 2-2. Percent nucleotide and amino acid identity of the mating-type (*MAT*) loci between *Phyllosticta citricarpa* and *P. capitalensis*.

<table>
<thead>
<tr>
<th>P. capitalensis</th>
<th>P. citricarpa</th>
<th>Sequence identity (%)^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Idiomorph</td>
<td>Gene</td>
<td></td>
</tr>
<tr>
<td><em>oml1</em></td>
<td><em>MAT1-1</em></td>
<td>55 (40)</td>
</tr>
<tr>
<td></td>
<td><em>oml1</em></td>
<td>57 (24)</td>
</tr>
<tr>
<td><em>MAT1-1-1</em></td>
<td><em>MAT1-1</em></td>
<td>68 (59)</td>
</tr>
<tr>
<td><em>MAT1-2-1</em></td>
<td><em>MAT1-2-1</em></td>
<td>70 (65)</td>
</tr>
<tr>
<td><em>MAT1-2-9</em></td>
<td><em>MAT1-2-9</em></td>
<td>69 (48)</td>
</tr>
<tr>
<td>PH domain-encoding gene</td>
<td><em>MAT1-1</em></td>
<td>PH domain-encoding gene</td>
</tr>
<tr>
<td></td>
<td><em>MAT1-2</em></td>
<td>PH domain-encoding gene</td>
</tr>
</tbody>
</table>

^a Nucleotide and amino acid sequences were predicted using the AUGUSTUS web interface with *Botrytis cinerea* as the model organism.

^b Sequence identity was analyzed using the Needleman-Wunsch global alignment algorithm available on the website of National Center for Biotechnology Information (NCBI). Nucleotide identities are presented with amino acid identities in parentheses.
Figure 2-1. Structural organization of the mating-type (MAT) loci of *Phyllosticta citricarpa* and *P. capitalensis*. Gene structures of MAT loci were predicted using GeneMark-ES and AUGUSTUS with *Botrytis cinerea* as the model organism and verified by BLASTn and BLASTp against the NCBI database. Arrowed boxes with gene names above indicate the position and orientation of open reading frames. Similar gene sequences are marked by the same color. Black lines within the arrowed boxes indicate introns. The light grey boxes represent the idiomorphic region of the MAT locus of *P. citricarpa*. Black lines underneath MAT genes of *P. citricarpa* indicate amplified regions using MAT-specific primers. *apn2*, DNA lyase; 40*S* S9, ribosomal protein 40S S9; *MAT1-1-1*, α box-containing protein; *MAT1-2-1*, HMG domain-containing protein; PH-domain, putative PH domain-containing protein (integral membrane protein); *oml1*, hypothetical protein (overlapping MAT locus 1); *MAT1-2-9*, hypothetical protein. The figure is to scale.
Figure 2-2. Synteny between MAT1-1 and MAT1-2 idiomorphs of *Phyllosticta citricarpa* and between the two idiomorphs of *P. citricarpa* and the fused idiomorph of *P. capitalensis*. Sequence comparison between the two idiomorphs of *P. citricarpa* was performed using the Dot Plot program (A) and the Artemis Comparison Tool (ACT) (B and C). The straight black diagonal line generated by the dot plot analysis indicates highly similar regions between idiomorphs. Red and blue lines generated by ACT analysis indicate highly similar regions in the forward and reverse directions, respectively, between idiomorphs. These analyses show that the *MAT* loci of *P. citricarpa* and *P. capitalensis* are flanked by *apn2* and 40S S9 genes and the two species share a conserved genetic structure between the *oml1* gene and the 40S S9 gene although *P. citricarpa* keeps two copies of PH-domain genes.
Figure 2-3. Maximum likelihood tree constructed using the amino acid sequences of PH-domain proteins from *Phyllosticta citricarpa* idiomorphs, *P. capitalensis*, and other Dothideomycetes. The tree was constructed using MEGA version 6.0 and rooted with *Saccharomyces cerevisiae*. Levels of branch support obtained with 1000 bootstrap replicates are indicated at nodes when they exceeded 70%. Fungal families are indicated with vertical bars. The scale bar represents 0.5 substitutions per site. The analysis clearly indicates that *P. citricarpa MAT1-2* and *P. capitalensis* are claded together.
Figure 2-4. A model describing the evolution of the mating type (MAT) loci in *Phyllosticta* species. First, a common ancestral homothallic *Phyllosticta* species contained *MAT1-1-1*, *MAT1-2-1*, and other *MAT*-related genes in a linked *MAT* locus. Whereas species in the homothallic lineage such as *P. capitalensis* keep a similar genetic organization as that of the homothallic ancestor during evolution, species in heterothallic lineage such as *P. citricarpa* are subjected to gene loss, resulting in fungal strains containing either *MAT1-1-1* or *MAT1-2-1* and *MAT1-2-9* (the idiomorphic region). Afterwards, the MAT locus of different mating type strains evolves and diverges independently, leading to the acquisition of flanking sequences including the partial 5' region of *oml1* and the PH-domain gene and consequently the expansion of the MAT locus. Arrowed boxes indicate the position and orientation of open reading frames. Similar gene sequences are marked by the same color. The light grey boxes represent the idiomorphic region of the *MAT* locus.
CHAPTER 3
INTERACTION BETWEEN THE CITRUS BLACK SPOT PATHOGEN, *Phyllosticta citricarpa* AND THE COMMON TWIG-COLONIZING PATHOGEN *Diaporthe citri* ON CITRUS TWIGS

**Introduction**

Melanose, caused by *Diaporthe citri* F. A. Wolf (anamorph: *Phomopsis citri* H. Fawc.), is a common fungal disease in most citrus-producing countries of the world and can produce brick red lesions on fruit, leaves, and twigs varying from individual spots to tearstain or mudcake patterns on fruit (Whiteside 1976). While all citrus varieties, especially grapefruit and lemon, are susceptible to melanose, this disease is usually not responsible for large yield reduction but mainly for the reduced value of fresh fruit in the market due to blemished fruit (Whiteside 1976).

The primary source of *D. citri* inoculum is conidia produced in pycnidia on dead twigs, from which conidia are disseminated to leaves, twigs, and fruit by rain-splash within the tree canopy (Whiteside 1976). Ascospores produced in perithecia on dead twigs do not greatly contribute to the total inoculum supply but can play a significant role in long distance dispersal of the disease due to the airborne nature of the ascospores (Whiteside 1976). *D. citri* can produce pycnidia and perithecia on dead twigs in citrus groves, whereas it has been reported that this pathogen does not survive well or sporulate on any living host tissues and can only be isolated from the fresh pustules few days after they appear (Davis and Wilhite 1983; Whiteside 1976). Given that dead twigs are the primary niche where *D. citri* survives and reproduces, the disease severity in citrus groves will increase along with aging of trees and accumulation of dead twigs in tree canopies (Davis and Wilhite 1983; Whiteside 1976).

In Brazil, *P. citricarpa* was shown to infect citrus twigs where conidia are produced for repeated infection cycles during the season (Spósito et al., 2011; Spósito et al., 2007). Considering that both *P. citricarpa* and *D. citri* occupy the same niche on twigs for inoculum
production, a potential interaction between these two fungi might occur, which has not been investigated so far. Therefore, the objective of the research was to investigate the interaction between \textit{P. citricarpa} and \textit{D. citri} and determine whether they can co-exist to successfully sporulate on dead twigs and cause new infections.

**Materials and Methods**

**Fungal Isolates**

\textit{P. citricarpa} isolate Gc12 was isolated from symptomatic 'Valencia' fruit with typical freckle spot lesions from the Immokalee area in Florida in 2010 (Schubert et al. 2012). \textit{D. citri} isolate PC-STL 5-5 was isolated from melanose-affected grapefruit trees in Florida in 2002. These isolates were single-spored and maintained on 1/2PDA in a 24°C incubator under a 12:12 h light:dark regime. For the long-term storage of fungal isolates, sterile filter paper colonized by fungal hyphae was air dried in a laminar flow hood overnight and kept in sterile coin envelopes in plastic containers with desiccants at −20°C as described in Hincapie et al.’s study (2014).

**Inoculum Preparation**

The collection of \textit{P. citricarpa} conidia was according to the method described by Hincapie et al. (2014). Briefly, conidia were collected from 2 to 3 week-old cultures flooded with a 0.02% Tween-20 (v/v) solution and suspended in 1.5 ml Eppendorf tubes. After centrifugation at 2,500 \( \times \) g for 5 min, the supernatant was discarded and the resulting pellet was resuspended in 1 ml sterile deionized water. The washing step was repeated a total of three times. The conidial concentration was measured using a hemocytometer (Bright Line, Hausser Scientific, Horsham, PA) under an Olympus BX41 microscope (Olympus, Center Valley, PA) at 100× magnification and adjusted to \( 10^5 \) conidia/ml for subsequent twig inoculation assays. To collect conidia of \textit{D. citri}, cultures were grown for 6 to 8 weeks until pycnidia formed on the surface of the medium began to exude conidia. Conidia were then collected directly from conidial masses using pipette
tips and suspended in 1.5 ml Eppendorf tubes with 1 ml of sterile deionized water. The conidial suspension was washed three times with sterile deionized water, and the conidial concentration was measured and adjusted to $10^5$ conidia/ml as above.

**Twig Inoculation**

To determine the potential competition between *P. citricarpa* and *D. citri* for saprophytic twigs, similar experiments for twig inoculation as described in the study by Mondal et al. (2007) were performed. Year-old 'Valencia' twigs between 2 to 4 mm in diameter were collected from the field, washed in distilled water, cut into approximately 1.5 cm long pieces, and then autoclaved at 121°C and 104 kPa (15 psi) for 20 min or irradiated in an AECL Gammacell-1000 device with the maximum dose of 250 Gy. Afterward, these twigs were inoculated with either sterile deionized water as negative controls or a series of $10^5$ conidia/ml suspensions constituting various proportions of *P. citricarpa* and *D. citri* conidia in 50 ml Falcon conical tubes (Thermo Fisher Scientific). The input ratios of *P. citricarpa* and *D. citri* conidia were 0.00:1.00, 0.25:0.75, 0.50:0.50, 0.75:0.25, and 1.00:0.00 (Adee et al. 1990). Following the inoculation period of 24 h in a Biometra hybridization oven (OV3; Biometra, Göttingen, Germany) at 3 rpm at 24°C, twigs were air dried for 3 days at room temperature, transferred into mesh bags (1 mm mesh), and incubated in a 24°C incubator with a 12 h photoperiod. To stimulate pycnidial formation of both species, inoculated twigs were wetted in distilled water for 3 to 4 h and air dried in the incubator three times a week for 8 weeks. During the incubation, twigs were examined under a dissecting microscope at 4× magnification for pycnidial formation once a week and a subset of five twigs from each input ratio was removed from mesh bags and processed once every 2 weeks to record pycnidial formation. At the end of the incubation period of 8 weeks, an additional subset of five twigs were placed in 1.5 ml Eppendorf tubes in a moist chamber at room temperature for 2 weeks to stimulate sporulation. To collect conidia produced on inoculated twigs, twig samples
were immersed in 500 µl of sterile deionized water for 1 h at room temperature and then shaken on a vortex mixer for 5 s. The concentration of the conidial suspensions were measured using a hemocytometer as above. If the interaction between *P. citricarpa* and *D. citri* was equal, the relative number of pycnidia or conidia of each species (the number from the mixture of inocula divided by the number from the pure inoculum) produced on twigs would be directly proportional to the starting input ratio.

**DNA Isolation**

Fungal genomic DNA (gDNA) isolation was adapted from the method described in Hu et al. (2014). Briefly, mycelia were grown on a layer of cellophane overlaid on 1/2PDA at 24°C under a 12 h light cycle. After 5 days, 100 to 150 mg of fungal mycelia was transferred from the cellophane into 2.0 ml screw cap tubes with sterile stainless steel beads (5 mm in diameter; Qiagen) using a flame-sterilized microspatula. Samples were then flash frozen in liquid nitrogen, homogenized twice at 30 Hz for 30 s using a Qiagen TissueLyser II (Qiagen), and subjected to gDNA isolation using the Qiagen DNeasy Plant Mini kit (Qiagen) based on the manufacturer’s instructions. The quality and quantity of gDNA was determined based on the ratio of absorbance at 260/280 and 260/230 nm using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). All gDNA samples were stored at −20°C until use.

According to the study by Hu et al. (2014), the MoBio PowerSoil DNA Isolation kit (MoBio Laboratories, Carlsbad, CA) was found to reliably extract good quality of gDNA from partially decomposed leaf litter. Since inoculated detached twigs were in decay process, similar to that of decomposed leaf litter, the MoBio PowerSoil DNA Isolation kit was used throughout the study to extract twig gDNA. Briefly, twig samples were first cut into small pieces and transferred into 2.0 ml screw cap tubes with sterile stainless steel beads (5 mm in diameter).
Twig samples were then subjected to the same procedures as above for sample homogenization, followed by DNA extraction using the MoBio PowerSoil DNA Isolation kit (MoBio Laboratories) according to the manufacturer’s instructions. The quality and quantity of twig gDNA was determined as above and gDNA samples were stored at −20°C until use.

**Primer and Probe Design for *D. citri* qPCR Assays and Validation**

A species-specific primer/probe set for quantification of *D. citri* was designed according to the criteria in the study by Hu et al. (2014). Briefly, the internal transcribed spacer 1 (ITS) region of ribosomal DNA, translational elongation factor 1α (*tef*), β-tubulin (*tub*), and calmodulin (*cal*) genes of *Diaporthe* species were retrieved from the NCBI nucleotide database (Table 3-1) and aligned for identification of regions with high polymorphism between species. Candidate primers and probes were designed using the PrimerQuest Tool available on the website of Integrated DNA Technologies, Inc. (IDT, Coralville, IA; http://www.idtdna.com/Primerquest/Home/Index) and then examined for the potential formation of hairpins, self-, and hetero-dimers using OligoAnalyzer version 3.1 in IDT (http://www.idtdna.com/calc/analyzer). Proposed primer/probe sets were discarded if low ΔG values were identified. In addition, selectivity and specificity of proposed primer/probe sets were verified with BLASTn analysis on NCBI.

To effectively and economically test the selected primer/probe sets without synthesizing the costly fluorophore- and quencher-labeled probes, oligonucleotides modified by 3’ phosphorylation to inhibit DNA polymerization were used to simulate the function of a probe in quantitative PCR (qPCR). These assays were performed in triplicate using SYBR Green chemistry in a 25 µl volume containing 1 to 10 ng of gDNA of *D. citri*, 1× SsoFast EvaGreen supermix (Bio-Rad Laboratories), 400 nM of each primer, and 200 nM of the 3’ phosphorylated
qPCR was conducted with a CFX96 Real-Time PCR detection system (Bio-Rad Laboratories) under the following conditions: 95°C for 30 s, 50 cycles of 94°C for 15 s and 59°C for 1 min. At the end of 50 cycles, melting curve analysis was performed from 65 to 95°C in 0.5°C increments with a dwell time of 5 s at each temperature to confirm that only the correct amplicon but no primer dimers or non-specific amplicons were produced. Sterile deionized water was used as a no-template control (NTC), and the whole experiment was repeated twice. Afterward, the selected *D. citri* probe was labeled with a 5’ 6-FAM fluorophore (6FAM) and a 3’ Black Hole Quencher 1 (BHQ1). All primers and probes were synthesized by IDT.

**Specificity**

The specificity of the DCCAL primer/probe set for *D. citri* was evaluated in triplicate as above with 10 ng of gDNA from citrus-associated fungi and oomycetes commonly found in Florida (Table 3-2). Sterile deionized water served as an NTC and gDNA from healthy sterilized citrus twigs was also included in qPCR assays. The whole experiment was repeated two times. The primer/probe set was considered specific when the fluorescence signal did not exceed the threshold automatically calculated by Bio-Rad CFX Manager software (version 1.6; Bio-Rad Laboratories) after 50 cycles.

**Standard Curves of gDNA from *D. citri***

Standard curves for detection of *D. citri* were constructed based on the study of Hu et al. (2014). Briefly, standard curves for DCCAL assays were prepared in a 10-fold serial dilution of *D. citri* gDNA, ranging from 2.5 ng/μl to 2.5 fg/μl. All qPCR reactions were performed in triplicate in a final volume of 25 μl containing 400 nM of each primer, 200 nM of the probe, 400 μM of each dNTP, 1× PCR buffer, 1.25 units of HotStar Taq Plus DNA polymerase (Qiagen), and 4 μl of gDNA samples or sterile deionized water as an NTC using a CFX96 Real-Time PCR
detection system (Bio-Rad Laboratories). The thermal cycling conditions were as follows: an initial activation step of 95°C for 10 min, followed by 50 cycles of 94°C for 15s and 59°C for 1min. To ensure the comparability across all qPCR assays, data were accepted if PCR amplification efficiencies were confirmed to be between 90 and 110% with $r^2 > 0.99$, as calculated by the software of the CFX96 Real-Time PCR detection system (Bio-Rad Laboratories) according to the formula $E = 100 \times [(10^{-1/slope}) – 1]$. The whole experiment was repeated three times.

Effect of gDNA of Healthy Citrus Twigs on qPCR Assays and Multiplex qPCR

To evaluate the presence of inhibitors in citrus twig samples, healthy 'Valencia' citrus twigs were prepared and autoclaved, and twig gDNA was extracted using the Qiagen DNeasy Plant Mini kit (Qiagen) as above. Afterward, 10 ng of twig gDNA was spiked into each qPCR mixture, and qPCR for DCCAL assays was performed as above.

For the potential to do multiplex qPCR for simultaneously quantifying $P. citricarpa$ and $D. citri$, qPCR assays combined with the standard curve method were performed as above, except for the use of different combinations of primer/probe sets and gDNA as follows: (i) GCITS primer/probe set (Hu et al. 2014) + $P. citricarpa$ gDNA, (ii) DCCAL primer/probe set + $D. citri$ gDNA, (iii) GCITS and DCCAL primer/probe sets + $P. citricarpa$ gDNA, (iv) GCITS and DCCAL primer/probe sets + $D. citri$ gDNA, and (v) GCITS and DCCAL primer/probe sets + $P. citricarpa$ and $D. citri$ gDNA. Equal amount of primers/probes and gDNA were mixed for all combinations. PCR efficiencies were calculated as above to determine whether the interference between the primer/probe sets occurred. Two technical replicates were performed for each qPCR reaction, and the whole experiment was repeated twice.
**Determination of Nuclei Number in *D. citri* Conidia**

The method to determine the nuclei number of *D. citri* conidia was adopted from Hu et al.’s study (2014). Briefly, a conidial suspension of *D. citri* was collected from 6 to 8 week old cultures as above, pipetted onto slides, and dried at approximately 70°C on a hot plate for 1 min. Afterward, conidia were stained with 4’,6-diamidino-2-phenylindole (DAPI, Invitrogen; 1 μg/ml in sterile deionized water) for 1 min and examined under an Olympus BX61 fluorescence microscope (Olympus) at 400× magnification at an excitation wavelength of 350 nm and an emission wavelength of 425 nm. Images of nuclear fluorescence were obtained using an OMAX digital camera (A35140U; OMAX, Kent, WA).

**Effect of Relative Humidity on Twig Colonization by *P. citricarpa***

To determine the effect of relative humidity (RH) on colonization of twigs by *P. citricarpa*, year-old 'Valencia' twigs and conidial suspensions of *P. citricarpa* were prepared for inoculation as above. Sterilized twigs were inoculated with either sterile deionized water as negative controls or $10^5$ conidia/ml of *P. citricarpa* and incubated in a Biometra hybridization oven (Biometra) at 3 rpm at 24°C for 24 h. Following the inoculation period, inoculated twigs were air dried for 3 days at room temperature, transferred into mesh bags (1 mm mesh), and incubated in glass desiccators with controlled humidity environments in a 24°C incubator with a 12 h photoperiod. The controlled humidity environments were achieved using saturated salt solutions of potassium dihydrogen phosphate (KH$_2$PO$_4$, 100% RH), potassium chloride (KCl, 82%), and sodium chloride (NaCl, 72% RH) (Winston 1960). The RH of 43% inside the incubator was used as a control. During the incubation, twig samples were examined under a dissecting microscope for *P. citricarpa* pycnidial formation once a week. Beginning from 3 weeks post-inoculation (wpi), subsets of five twigs from each RH environment were processed once every 2 weeks to record pycnidial formation, conidial production, and gDNA yield. *P.*
*citricarpa* pycnidia were directly counted under a dissecting microscope at 4× magnification, and conidial production was measured using a hemocytometer as above following the immersion of twig samples in 500 µl of sterile deionized water in 1.5 ml Eppendorf tubes for 1 h. Quantification of *P. citricarpa* gDNA on twig samples was performed using qPCR as described by Hu et al. (2014).

**Data Analysis**

A t-test using the procedure PROC TTEST in SAS (version 9.4; SAS Institute Inc.) was used to test whether there was a significant difference among the slopes of the standard curves generated with or without citrus twig DNA. To determine whether there was interference between the GCITS and DCCAL primer/probe sets during multiplex qPCR assays, a one-way analysis of variance (ANOVA) using SAS PROC GLM (version 9.4; SAS Institute Inc.) was used to analyze the difference in slopes of standard curves generated by the different combinations of primer/probe sets and fungal DNA.

Pycnidial and conidial production data were standardized to be the proportion of the input ratios of *P. citricarpa* and *D. citri* conidia. The observed and expected counts were compared by a χ² test using PROC FREQ in SAS (version 9.4; SAS Institute Inc.). If the competition was equal between *P. citricarpa* and *D. citri*, the observed ratio of pycnidial or conidial production between two species would be the same as the inoculum input ratio and a low chi-square value (high *P*-value) would be revealed from the analysis. Fisher’s protected least significant difference (LSD) test was performed to determine the effects of RH on pycnidial formation, conidial production, and gDNA yield at the 5% significance level using SAS PROC GLM (version 9.4; SAS Institute Inc.).
Results

Twig Colonization by \textit{P. citricarpa} and \textit{D. citri}

\textit{D. citri} began to produce pycnidia on both autoclaved and irradiated twigs at 2 wpi regardless of the inoculum ratios (Fig. 3-1). The number of \textit{D. citri} pycnidia produced on inoculated twigs also increased over time (Fig. 3-1). However, \textit{P. citricarpa} only began to produce pycnidia at 4 wpi on autoclaved but not irradiated twigs that were inoculated with only \textit{P. citricarpa} conidia (Pc:Dc = 1:0; Fig. 3-1). Fig. 3-2 shows representative autoclaved twig samples inoculated with various inoculum ratios at 4 wpi. At 8 wpi, the number of \textit{D. citri} pycnidia produced on autoclaved and irradiated twigs seemed to be proportional to the inoculum ratio (Fig. 3-3), whereas \textit{P. citricarpa} pycnidia were still observed only on autoclaved twigs that were inoculated with \textit{P. citricarpa} conidia solely (Fig. 3-3). No fungal structures were observed on sterile deionized water-inoculated twigs throughout the experiments (data not shown).

After incubation of twigs at 8 wpi in a moist chamber at room temperature for additional 2 weeks, approximately $10^6$ to $10^7$ conidia/ml of \textit{D. citri} and $10^4$ to $10^5$ conidia/ml of \textit{P. citricarpa} were collected from those twigs with pycnidia (Fig. 3-4), no conidia were collected from those twigs without fungal fruiting bodies (Fig. 3-4) and from sterile deionized water-inoculated twigs (data not shown). Surprisingly, following the stimulation period, the production of \textit{P. citricarpa} pycnidia and conidia ($10^4$ to $10^5$ conidia/ml) were observed on the irradiated twigs inoculated with sole \textit{P. citricarpa} conidia (Fig. 3-4).

Primer and Probe Design and Specificity

The primer/probe set DCCAL for detection of \textit{D. citri} (Table 3-3) was designed based on the sequence alignment of the partial \textit{cal} genes of multiple \textit{Diaporthe} species (Table 3-1), in which the greatest differentiation among \textit{Diaporthe} species was found. The expected amplicon size of 110 bp for \textit{D. citri} using this primer/probe set was further confirmed by sequencing (data
note shown). No major secondary structures (primer hairpin, self-, and hetero-dimers) or primer-probe interactions were observed for the DCCAL primer/probe set by the in silico analysis through the IDT website and by the melt-curve analysis with or without the modified probe oligonucleotide, showing the presence of a single specific PCR product (data not shown).

The DCCAL primer/probe set was highly specific to *D. citri* as no PCR products were amplified by this primer/probe set from gDNA of common citrus-associated fungi and oomycetes (Table 3-2). In addition, PCR amplification was observed neither from gDNA of healthy citrus twigs nor the NTCs (Table 3-2).

**Standard Curves for qPCR Quantification and Inhibitive Effect of Healthy Citrus Twig on qPCR Assays**

The correlation coefficients from the standard curves for *D. citri* gDNA were greater than 0.99, indicating that the cycle threshold (Ct) values were linear across the range of gDNA tested (Fig. 3-5). The amplification efficiencies of DCCAL qPCR assays were 98.8% (Fig. 3-5), and the detection limit of the qPCR assays was 100 fg of *D. citri* gDNA (Fig. 3-5). The DCCAL qPCR assays spiked with 10 ng of healthy twig gDNA did not show a significant difference in the amplification efficiency from the assays without healthy twig gDNA (*P* < 0.791) (Fig. 3-5). No amplification was observed in the NTCs (data not shown).

**Multiplex Interference**

The equal concentration of the DCCAL primer/probe set did not interfere with the GCITS qPCR assays for *P. citricarpa*, and vice versa (Fig. 3-6). However, when both GCITS and DCCAL primer/probes sets were used for simultaneous detection of *P. citricarpa* and *D. citri* in the presence of gDNA of both species, interference in *D. citri* detection was observed due to the higher amplification efficiency (133.9%) (Fig. 3-6). Under the same conditions, this
interference was not found in *P. citricarpa* detection (Fig. 3-6). No amplification was detected in the NTCs (data not shown).

**Nuclei Number of *D. citri* Conidia**

The single-celled *D. citri* conidia were observed to have a single nucleus through DAPI staining (Fig. 3-7).

**Quantification of gDNA from Inoculated Twig Samples**

Since an additional 2-week incubation in a moist chamber at room temperature stimulated pycnidial and conidial production of *P. citricarpa* on the irradiated twigs (at 8 wpi), we hypothesized that *P. citricarpa* may actually survive on those twigs where no *P. citricarpa* pycnidia were observed. To test this hypothesis, a subset of autoclaved and irradiated twig samples at 8 wpi were subjected to DNA extraction using the MoBio PowerSoil DNA Isolation kit (MoBio Laboratories) and qPCR analysis using GCITS and DCCAL primer/probe sets as above. The results are presented in Fig. 3-8.

Compared with irradiated ones, more gDNA was obtained from autoclaved twigs (Fig. 3-8). Similar amounts of *D. citri* gDNA were detected on twig samples inoculated with just sole *D. citri* conidia or the mixture of *P. citricarpa* and *D. citri* conidia (*P. citricarpa*: *D. citri* = 0.5:0.5) (Fig. 3-8), whereas much less gDNA of *P. citricarpa* was obtained from twig samples inoculated with the conidial mixture of *P. citricarpa* and *D. citri* (Fig. 3-8). Surprisingly, although no *P. citricarpa* pycnidia were observed on twig samples inoculated with the conidial mixture of both species, approximately 10 pg/twig of *P. citricarpa* gDNA was detected, indicating the presence of *P. citricarpa* in those twig samples (Fig. 3-8).

**Effect of RH on Twig Colonization by *P. citricarpa***

Pycnidial formation of *P. citricarpa* began at 3 wpi on twigs incubated at 100% and 82% RH but not at 72% and 43% RH (Fig. 3-9). Even at 7 wpi, no pycnidia were observed on twigs
incubated at 72% and 43% RH (Fig. 3-9). In addition, more *P. citricarpa* pycnidia were found on twigs incubated at 100% RH than at 82% RH over the incubation period (Fig. 3-9). Fig. 3-10 shows representative inoculated twig samples incubated at various RH at 7 wpi. *P. citricarpa* conidia were collected from those twigs with pycnidia (Fig. 3-11), and approximately two to fivefold more conidia were collected from twigs incubated at 100% RH than at 82% RH (Fig. 3-11). Similar to the results of pycnidial production, more gDNA of *P. citricarpa* was obtained from twigs incubated at 100% and 82% RH than at 72 and 43% RH (Fig. 3-12).

To further confirm whether *P. citricarpa* remained alive under the low humidity environments, inoculated twig samples previously incubated at 72% and 43% RH were transferred and incubated at 100% RH to stimulate its pycnidial and conidial production. As seen in Fig. 3-9, following stimulation for 2 weeks, *P. citricarpa* pycnidia were observed on twig samples previously incubated at 72% RH but not at 43% RH. However, those twigs did not produce conidia even after stimulation for 4 weeks (Fig. 3-11). With the production of pycnidia on twig samples previously incubated at 72% RH, more gDNA of *P. citricarpa* was also obtained (Fig. 3-12). The yield of *P. citricarpa* gDNA from twig samples previously incubated at 43% RH only slightly increased after stimulation (Fig. 3-12).

**Discussion**

In the past, most of the epidemiological studies in citrus black spot have been focused mainly on ascospores produced in leaf litter with less attention to conidia produced on the lesions of symptomatic fruit, dead twigs, and in leaf litter (Kiely 1948; Kotzé 1981; Reis et al. 2006). This biased attention to ascospores started to change with the findings of especially Spósito et al. (2011) that indicated *P. citricarpa* was capable of infecting citrus twigs where conidia were produced for recurrent infections during the season. Furthermore, our preliminary results on the screening of mating types in the Floridian population of *P. citricarpa* indicates the presence of a
single mating type in Florida, implying that the existence of ascosporic inoculum of *P. citricarpa* is unlikely and, as a consequence, the disease spread would solely rely on the conidia. *D. citri* is a common twig-colonizing fungus in Florida and produces the primary inoculum (conidia) also on dead twigs (Mondal et al 2004; Mondal et al 2007). Given that both *P. citricarpa* and *D. citri* occupy the same niche on twigs for inoculum production, understanding how these two species interact with each other on dead twigs for inoculum production is of practical value. In this study, we first inoculated autoclaved or irradiated twigs with a replacement series of conidial inocula of both species to determine the interaction between *P. citricarpa* and *D. citri*. Our results showed that pycnidial and conidial production of *P. citricarpa* on autoclaved and irradiated twigs were not observed in the presence of *D. citri* even at an inoculum ratio of 0.75:0.25 conidia (*P. citricarpa*: *D. citri*). One possible explanation with respect to their interaction is an antagonistic relationship between these two pathogens. In the plate assays with *P. citricarpa* and *D. citri* side-by-side on 1/2PDA, no antagonism between two species was noticed and *D. citri* mycelia grew over the colonies of *P. citricarpa* and formed pycnidia normally on 1/2PDA (data not shown). Another possible explanation is that *P. citricarpa* and *D. citri* competed for nutritional resources needed in inoculum production. Considering *D. citri* is a relatively fast-growing fungus compared to *P. citricarpa*, *D. citri* might be capable of promptly colonizing the dead twigs and inhabiting the niche before *P. citricarpa* can, resulting in limited-nutrients and space for *P. citricarpa* development. In future studies, it would be interesting to determine the inoculum ratio that provides equal opportunities for both fungi to compete for nutritional resources.  

*D. citri* colonized and produced pycnidia on both autoclaved and irradiated twigs, whereas *P. citricarpa* only produced pycnidia on autoclaved twigs. These findings imply that
sterilization methods may create different nutritional environments on twigs that, in turn, differentially favor *P. citricarpa* and *D. citri*. Sterilization by autoclaving uses pressurized moist steam to heat and hydrolyze cells and as a result nutritional resources from autoclaved twigs would be accessible immediately. In contrast, sterilization by irradiation only profoundly damages DNA of cells and thus retains cell vitality (Munarin et al. 2013), rendering twig samples into a more natural state, simulating a slow senescent state for fungal development. Therefore, nutritional resources from the irradiated twigs would be released gradually during the decay process. The observation of pycnidial production of *D. citri* on autoclaved and irradiated twigs is in agreement with the fact that *D. citri* can infect both live and dead twigs and does not undergo latent infections (Mondal et al. 2004). However, the limited resource environment on irradiated twigs may somehow affect *P. citricarpa* development and may force *P. citricarpa* to form latent infections that would take much longer to form structures and produce inoculum. It is also possible due to the nature of *P. citricarpa* whose development is not favored within green non-senescent host tissue.

A qPCR detection system for *D. citri* was developed in aid of understanding the proportion of *P. citricarpa* gDNA to *D. citri* gDNA on twigs inoculated with a series inoculum ratio of both species and determining whether *P. citricarpa* survived on those twigs. Among primer/probe sets designed from the available ITS, *tef*, *tub*, and *cal* sequences in the GenBank databases, those sets designed from the *cal* sequences showing the least homolog and deleterious interactions exhibited the least potential for formation of hairpins, self-, and hetero-dimers and therefore were selected for further tests. Rather than ordering costly probes containing fluorophores and quenchers directly for qPCR assays, nucleotides modified with 3’ phosphorylation were used to simulate the probe’s function in qPCR reactions. Since the 3’ OH
group of the nucleotides was modified by phosphorylation, these nucleotides did not have the ability of extension. Therefore, with the 3’ modification of nucleotides and SYBR green chemistry, we greatly reduced the cost to test multiple primer/probe sets prior to ordering a real TaqMan probe.

In hopes of efficiently quantifying *P. citricarpa* and *D. citri* simultaneously, our initial testing was performed in multiplex reactions with GCITS and DCCAL primer/probe sets. With the use of the equal amount of gDNA of both species in multiplex qPCR reactions, the detection of *P. citricarpa* remained consistent, although the *D. citri* detection was greatly affected. This inhibition was similar to that observed in Hu et al.’s study (2014), in which a DNA concentration-dependent inhibition in multiplex qPCR reactions was noticed when the concentration difference was greater than 10^3 copy numbers/reaction. Although the equal amount of gDNA of both species was used, *P. citricarpa* was predicted to have approximately 100 fold more target gene copies than *D. citri* based on the C_t values. Therefore, rather than multiplex reactions, GCITS and DCCAL primer/probe sets were used separately in single reactions throughout the study.

The qPCR assays detected approximately 1 ng/twig of *P. citricarpa* gDNA on irradiated twigs inoculated with just *P. citricarpa*, which was approximately 100-fold lower than that on autoclaved counterparts. These results indicate that *P. citricarpa* indeed survived on those twigs and waited for favorable conditions to break latency. In leaves, *P. citricarpa* penetrates through the cuticle layer and forms a small knot (vegetative stroma) between the cuticle layer and the first layer of epidermal cells. Afterward, in most cases *P. citricarpa* appears to remain in a long period of latency (McOnie 1967). *P. citricarpa* rarely produces leaf symptoms, but when it does, some hyphae were observed in the center and around the edge of leaf lesions (McOnie 1967).
However, as a whole, *P. citricarpa* does not grow and increase the overall fungal biomass in living leaves until leaf abscission. It is not known whether *P. citricarpa* has a similar pattern of a long latent period on twigs, but we would expect that *P. citricarpa* has a long-term latency on twigs until twigs die.

The dominance of *D. citri* in the interaction with *P. citricarpa* and the observation of very few *P. citricarpa* conidia produced on inoculated twigs made us wonder whether the conditions for *P. citricarpa* development on twigs were optimal. Therefore, the effect of RH on pycnidial and conidial production of *P. citricarpa* was tested. *P. citricarpa* formed pycnidia and conidia on twigs incubated at 100 and 82% RH but not at 72 and 43% RH. These findings indicate a possible threshold between 82 and 72% RH for pycnidial production of *P. citricarpa*. It may be worth trying to narrow down the RH range using other saturated salt solutions that can provide in-between RH such as sodium dihydrogen phosphate (NaH$_2$PO$_4$, 81% RH) (Winston 1960) or an incubator equipped with a relative humidity controller.

Following the stimulation via incubation in 100% RH, *P. citricarpa* began to form pycnidia and conidia on twigs previously incubated at 72% RH but not at 43% RH. In agreement with these results, the qPCR assays further revealed a consistent level of *P. citricarpa* gDNA on twigs previously incubated at 72% RH prior to stimulation and a great increase in gDNA after stimulation, providing more evidence showing the growth and development of *P. citricarpa* on those twigs. In contrast, *P. citricarpa* gDNA from twigs incubated previously at 43% RH decreased drastically by 7 wpi. Although the gDNA level increased after stimulation, the increase was not as large as those on twigs previously at 72% RH. These findings imply that a long-term incubation of *P. citricarpa* at a consistent low RH environment such as 43% RH may be highly detrimental to *P. citricarpa*. 

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During the twig inoculation assays under controlled humidity environments, we noticed RH changes, particularly in the container with the saturated KH$_2$PO$_4$ solution. The saturated KH$_2$PO$_4$ solution is capable of providing an approximately 96% RH environment in closed space at 25°C (Winston 1960). Indeed, 96% RH was measured at the beginning of the experiment, but the RH increased from 96% toward 100% soon after the collection of inoculated twig samples from the glass desiccator. The RH drift toward 100% from the theoretical value is a common but not widely reported issue, particularly in high humidity environments. RH drift is caused by the process of saturated solutions absorbing water from the air, resulting in a diluted solution at the surface area. If the diluted solution at the surface area is not mixed well with the bulk of the saturated solution, it would become less capable of holding water in the solution and as a result provide a higher than theoretical RH in a closed space. This situation can be improved by mechanical mixing of the solution every time it is exposed to fresh air. In addition, when designing the experiment, the ratio of the surface area of a solution to the total air volume within a chamber should be taken into account. As suggested by Martin (1962), the ratio should be less than 10 to ensure that a saturated solution is capable of maintaining its given humidity level.

In this study, pycnidia production of *P. citricarpa* and *D. citri* on twigs varied drastically even within the subsamples of the same treatment, which may result from the variation of the twig size. It has been noted that there is a relationship between wetting duration and twig thickness with respect to pycnidial production of *D. citri* on twigs (Davis and Wilhite 1983; Mondal et al. 2004). In general, *D. citri* pycnidia formed better on small twigs (Davis and Wilhite 1983; Mondal et al. 2004). Mondal et al. (2004) found that the number of pycnidia on twigs greater than 6 mm in diameter was low under the wetting regime of 3 to 4 h per time, three times a week, which could be improved when twigs were soaked in water longer. However, a
prolonged wetting period could be detrimental to pycnidial production of *D. citri* on small twigs as it limits the time for the production due to rapid decay of twigs (Mondal et al. 2004). For this reason, not only the length but also the thickness of twigs should be taken into account when collecting twigs from the field for twig inoculation assays in order to minimize the variance.

As *P. citricarpa* can produce inoculum on dead twigs, the removal of dead twigs from the tree canopy might be expected to be a practical disease control measure. However, it is still unclear how long *P. citricarpa* can continuously produce inoculum on dead twigs. In the case of *D. citri*, Mondal et al (2004) reported that *D. citri* appears to not undergo latent infections on twigs and required 45 to 60 days at warmer conditions (28°C) and 90 to 120 days at cooler conditions (20°C) for inoculum production on twigs that died in the last 6 months. However, removing dead twigs from the tree canopy in a timely manner as a useful melanose control measure is difficult to apply due to the cost (Mondal et al. 2004). Given different life styles between *P. citricarpa* and *D. citri*, we will need to conduct experiments to acquire information regarding the time frame for *P. citricarpa* inoculum production on twigs under different temperatures before recommending this practice as a useful control measure for citrus black spot.

In conclusion, preliminary results of the interaction between *P. citricarpa* and *D. citri* on twigs suggest that *D. citri* is the dominant fungus in the presence of equal amounts or similar ratios of inocula of both species on twigs. However, due to the fact that pycnidial production of *D. citri* on detached twigs in the laboratory ceased approximately after 6 months (Mondal et al. 2004), we wonder whether *P. citricarpa* could begin to develop after *D. citri* no longer actively grew on those twigs. In addition, a qPCR detection method for *D. citri* was developed in this study, which, combined with other traditional means, will be a very useful tool to study fungal interactions. The preliminary results of the RH effect on *P. citricarpa* development on twigs
indicate that there is a possible humidity threshold between 82 and 72% RH for fungal
development and the latency of *P. citricarpa* on twigs can be broken when favorable conditions
are met. Therefore, future studies will continuously focus on the interaction between *P.
citricarpa* and *D. citri* and the RH effect on *P. citricarpa* development on twigs, which, in turn,
will provide information as a basis for disease forecasting models and help improve the
management of citrus black spot in Florida.
Table 3-1. GenBank accession numbers of internal transcribed spacer 1 (ITS) region, translation elongation factor 1α (tef), β-tubulin (tub), and calmodulin (cal) genes of *Diaporthe* species used in the development of quantitative polymerase chain reaction (qPCR).

<table>
<thead>
<tr>
<th>Species</th>
<th>Accession number&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ITS</td>
</tr>
<tr>
<td><em>Diaporthe citri</em></td>
<td>KC843320</td>
</tr>
<tr>
<td><em>D. amygdali</em></td>
<td>KC343020</td>
</tr>
<tr>
<td><em>D. arecae</em></td>
<td>KC343032</td>
</tr>
<tr>
<td><em>D. hickoriae</em></td>
<td>KC343118</td>
</tr>
<tr>
<td><em>D. vaccinii</em></td>
<td>KC343223</td>
</tr>
<tr>
<td><em>D. crotalariae</em></td>
<td>KC343056</td>
</tr>
<tr>
<td><em>D. ganjae</em></td>
<td>KC343112</td>
</tr>
<tr>
<td><em>D. neoarctii</em></td>
<td>KC343145</td>
</tr>
<tr>
<td><em>D. batatas</em></td>
<td>KC343040</td>
</tr>
<tr>
<td><em>D. phaseolorum</em></td>
<td>KC343175</td>
</tr>
<tr>
<td><em>D. melonis</em></td>
<td>KC343142</td>
</tr>
<tr>
<td><em>D. sojae</em></td>
<td>KC343197</td>
</tr>
<tr>
<td><em>D. vexans</em></td>
<td>KC343229</td>
</tr>
</tbody>
</table>

<sup>a</sup> –, species not used for the design of qPCR primer/probe sets.
Table 3-2. Specificity test of the *Diaporthe citri*-specific primer/probe set.

<table>
<thead>
<tr>
<th>Sample</th>
<th>DCCAL</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Diaporthe citri</em></td>
<td>21.57</td>
</tr>
<tr>
<td><em>Alternaria alternata</em></td>
<td>NA</td>
</tr>
<tr>
<td><em>Elsinoë fawcettii</em></td>
<td>NA</td>
</tr>
<tr>
<td><em>Mycosphaerella citri</em></td>
<td>NA</td>
</tr>
<tr>
<td><em>Phyllosticta capitalensis</em></td>
<td>NA</td>
</tr>
<tr>
<td><em>P. citricarpa</em></td>
<td>NA</td>
</tr>
<tr>
<td><em>Phytophthora nicotianae</em></td>
<td>NA</td>
</tr>
<tr>
<td><em>P. palmivora</em></td>
<td>NA</td>
</tr>
<tr>
<td>Healthy citrus twigs</td>
<td>NA</td>
</tr>
<tr>
<td>Sterile deionized water (non-template control)</td>
<td>NA</td>
</tr>
</tbody>
</table>

*a* 10 ng of genomic DNA (gDNA) from each fungal species and healthy citrus twigs were used for the specificity test.

*b* DCCAL, $C_t$ values of the primer/probe set for the calmodulin gene of *D. citri*; NA, no detectable amplification after 50 cycles.
Table 3-3. Species-specific TaqMan primer and probe sequences used for quantitative polymerase chain reaction (qPCR).

<table>
<thead>
<tr>
<th>Designation</th>
<th>Type</th>
<th>Sequences (5’ to 3’) and labeling&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diaporthe citri</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DCCALF1</td>
<td>Forward primer</td>
<td>CCTTCTCCCTTCTTTGTAAGTCA</td>
<td>110</td>
</tr>
<tr>
<td>DCCALR1</td>
<td>Reverse primer</td>
<td>ATCCTTATCCTACAAAGCCGA</td>
<td></td>
</tr>
<tr>
<td>DCCALP1</td>
<td>TaqMan probe</td>
<td>6FAM-CCC GCC CCT CTTACT CCTT GC-BHQ1</td>
<td></td>
</tr>
<tr>
<td><strong>Phyllosticta citricarpa</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GCITSF1</td>
<td>Forward primer</td>
<td>CCTGAAAGGTGATGGAAGGG</td>
<td>84</td>
</tr>
<tr>
<td>GCITSR1</td>
<td>Reverse primer</td>
<td>CGCAAAGCAACATGTTGATA</td>
<td></td>
</tr>
<tr>
<td>GCITSP1</td>
<td>TaqMan probe</td>
<td>Cy5-AGCCGCCC GCCTACCTTCA-Iowa black RQ</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Probe labels are highlighted in bold.

<sup>b</sup> The GCITS primer/probe set for detection of *P. citricarpa* is from Hu et al. (2014).
Figure 3-1. Pycnidial production of *Phyllosticta citricarpa* or *Diaporthe citri* on (A) autoclaved or (B) irradiated twigs over time. Twig samples were either autoclaved or irradiated and then inoculated with a series of $10^5$ conidia/ml suspensions consisting of various proportions of *P. citricarpa* and *D. citri* conidia, including input ratios of 0.00:1.00, 0.25:0.75, 0.50:0.50, 0.75:0.25, and 1.00:0.00. Abbreviated fungal names in parentheses indicate either *P. citricarpa* (Pc) or *D. citri* (Dc) pycnidia observed on twig samples.
Figure 3-2. Colonization of autoclaved citrus twigs by *Phyllosticta citricarpa* and *Diaporthe citri* after treatment with wetting and drying cycles for 4 weeks. Twig samples were autoclaved and then inoculated with a series of $10^5$ conidia/ml suspensions consisting of various proportions of *P. citricarpa* and *D. citri* conidia, including input ratios of 0.00:1.00, 0.25:0.75, 0.50:0.50, 0.75:0.25, and 1.00:0.00.
Figure 3-3. Pycnidial production of *Phyllosticta citricarpa* and *Diaporthe citri* on (A) autoclaved or (B) irradiated twigs at 8 weeks post-inoculation (wpi). Twig samples were either autoclaved or irradiated and then inoculated with a series of $10^5$ conidia/ml suspensions consisting of various proportions of *P. citricarpa* and *D. citri* conidia, including input ratios of 0.00:1.00, 0.25:0.75, 0.50:0.50, 0.75:0.25, and 1.00:0.00. The dotted lines represent the expected number of pycnidia produced on inoculated twigs for equal competition between two species and the solid lines represent the observed number of pycnidia produced on inoculated twigs.
Figure 3-4. Conidial production of *Phyllosticta citricarpa* or *Diaporthe citri* on (A) autoclaved or (B) irradiated twigs at 10 weeks post-inoculation (wpi). Twig samples were either autoclaved or irradiated and then inoculated with a series of $10^5$ conidia/ml suspensions consisting of various proportions of *P. citricarpa* and *D. citri* conidia, including input ratios of 0.00:1.00, 0.25:0.75, 0.50:0.50, 0.75:0.25, and 1.00:0.00. Twigs collected at 8 weeks post-inoculation (wpi) were incubated in a moist chamber at room temperature for additional 2 weeks to stimulate pycnidial and conidial production. The dotted lines represent the expected number of conidia produced on stimulated twigs for equal competition between two species and the solid lines represent the observed number of conidia produced on stimulated twigs.
Figure 3-5. Standard curves of genomic DNA of *Diaporthe citri* alone (blue solid diamond) or spiked with 10 ng of citrus twig DNA (black solid circle).
Figure 3-6. Interference between GCITS and DCCAL primer/probe sets in multiplex quantitative polymerase chain reaction (qPCR) for the simultaneous detection of (A) Phyllosticta citricarpa and (B) Diaporthe citri. In (A), blue solid diamond, GCITS primer/probe set with P. citricarpa gDNA; black solid circle, GCITS primer/probe set and DCCAL primer/probe set with P. citricarpa gDNA; red solid square, GCITS primer/probe set and DCCAL primer/probe set with P. citricarpa gDNA and D. citri gDNA. In (B), blue solid diamond, DCCAL primer/probe set with D. citri gDNA; black solid circle, GCITS primer/probe set and DCCAL primer/probe set with D. citri gDNA; red solid square, GCITS primer/probe set and DCCAL primer/probe set with P. citricarpa gDNA and D. citri gDNA.
Figure 3-7. 4', 6-diamidino-2-phenylindole (DAPI) staining of *Diaporthe citri* conidia.
Figure 3-8. Quantification of genomic DNA from *Phyllosticta citricarpa* and/or *Diaporthe citri*-inoculated twig samples at 8 weeks post-inoculation (wpi) using quantitative polymerase chain reaction (qPCR). Twig samples were either autoclaved or irradiated and then inoculated with a series of $10^5$ conidia/ml suspensions consisting of various proportions of *P. citricarpa* and *D. citri* conidia, including input ratios of 0.00:1.00, 0.50:0.50, and 1.00:0.00.
Figure 3-9. Effect of relative humidity (RH) on pycnidial production of *Phyllosticta citricarpa* on inoculated citrus twigs over time. The arrow indicates the timing when inoculated twigs were transferred and incubated at 100% RH.
Figure 3-10. Colonization of autoclaved citrus twigs by *Phyllosticta citricarpa* after incubation at various relative humidities at 24°C for 7 weeks.
Figure 3-11. Effect of relative humidity (RH) on conidial production of *Phyllosticta citricarpa* on inoculated citrus twigs over time. The arrow indicates the timing when inoculated twigs were transferred and incubated at 100% RH.
Figure 3-12. Effect of relative humidity (RH) on genomic DNA yield obtained from *Phyllosticta citricarpa*-inoculated citrus twigs using quantitative polymerase chain reaction (qPCR). The arrow indicates the timing when inoculated twigs were transferred and incubated at 100% RH.
CHAPTER 4
THE EFFECTS OF NUTRITIONAL AND ENVIRONMENTAL FACTORS ON CONIDIAL GERMINATION AND APPRESSORIUM FORMATION OF *PHYLLOSTICTA CITRICARPA*, THE CAUSAL AGENT OF CITRUS BLACK SPOT

**Introduction**

The first step of successful infection and colonization of host tissue by fungal pathogens is the attachment of spores to host tissue, followed by germination, appressorium formation and penetration via an infection peg (Gareth Jones 1994). These processes are affected by many factors such as surface wettability, nutrients, and environmental conditions (incubation period, temperature, pH, etc.) (Blank and Murray 1998; Emmett and Parbery 1975; Kuo and Hoch 1996; Liu and Xiao 2005). However, conditions required for these processes have been poorly understood for *P. citricarpa*, which limits further biological studies. Previously, Shaw et al. (2006) reported that conidia of *Phyllosticta* spp. germinated at significantly higher rates on hydrophobic surfaces, indicating a common phenomenon of the contact/attachment among *Phyllosticta* spp. prior to germination. However, conidial germination of *P. citricarpa* was found to occur neither on artificial media such as water agar and PDA nor in sterile water on a hydrophobic surface (Darnell-Smith 1918; Kiely 1948; Korf 1998), implying the requirement of additional stimuli for conidial germination. Darnell-Smith (1918) first reported that orange peel extract had the capacity to stimulate conidial germination of freshly discharged *P. citricarpa* conidia in 12 h. Kiely (1948) further tested the capabilities of extracts of various fruit tissue at different ages for germination and proposed that some organic acids might play an important role in stimulating conidial germination. Afterward, he tested some organic acids (citric acid, tartaric acid, lactic acid, and oxalic acid) for stimulating germination and found that 0.1 to 0.5% citric acid greatly improved conidial germination to over 80%. However, such a high germination rate of *P. citricarpa* conidia in citric acid solutions has not been repeated by other laboratories (Korf...
1998), including our own. Furthermore, Korf (1998) reported that the addition of 2% Valencia juice into the conidial suspension of *P. citricarpa* drastically increased the germination rate to approximately 60% after 48 h incubation at 22°C. However, Valencia juice is a complex solution and the critical components in Valencia juice required for *P. citricarpa* germination have not been determined. Moreover, the quality of Valencia juice depends on the maturity of the fruit, which may greatly influence the subsequent assays of conidial germination and hamper the reproducibility of the experiments.

Therefore, in the present study, our objectives were to investigate (i) the effects of citrus juice sources, concentrations, and pH on conidial germination and appressorium formation; (ii) the effects of incubation period and temperature on conidial germination and appressorium formation; (iii) the effects of carbon and nitrogen sources on conidial germination and appressorium formation; and (iv) the effects of synthetic citrus juice on conidial germination and appressorium formation. Our results contribute to a further understanding of nutritional and environmental factors required for the germination processes of *P. citricarpa* and to the development of a robust and convenient system for the evaluation of fungicide efficacy.

**Materials and Methods**

**Culture, Growth Conditions, and Preparation of Conidial Suspensions**

A single spore isolate of *P. citricarpa* Gc3 isolated from a symptomatic fruit of 'Valencia' sweet orange from Immokalee, Florida was used throughout the study after confirmation of the yellow pigment production on oatmeal agar (Baayen et al. 2002) and the specific amplification using species-specific primers (Peres et al. 2007). *P. citricarpa* Gc3 was maintained on half-strength PDA (1/2PDA; Difco, Sparks, MD) at 24°C under a 12 h light-12 h dark cycle. To harvest conidia of *P. citricarpa*, 2 to 3 week-old cultures were flooded with a 0.02% Tween 20 solution (v/v) (Hincapie et al. 2014). The resulting conidial suspension was then dispensed into
1.5 ml Eppendorf tubes and centrifuged at 2,500 × g for 5 min. After discarding the supernatant, the pellet was resuspended in 1 ml sterilized water. This washing step was repeated two more times. The final conidial suspension was counted with a hemocytometer (Bright Line, Hausser Scientific, Horsham, PA) under an Olympus BX41 microscope (Olympus, Center Valley, PA) at 200× magnification and adjusted to a concentration of at least 10^6 conidia/ml for the subsequent assays of conidial germination and appressorium formation.

**Evaluation and Observation of Conidial Germination**

Unless otherwise specified, the conidial suspensions in this study were mixed with various solutions at a 1:3 ratio of conidial suspensions to treatment solutions and pipetted onto 12-well Teflon-coated hydrophobic slides (5 μl/well) (Thermo Fisher Scientific, Waltham, MA). These slides were then placed onto moist P5 filter paper (Thermo Fisher Scientific) in petri dishes sealed with Parafilm to maintain the moisture. Petri dishes were transferred into a plastic moist chamber and incubated at 24°C for 24 h. One hundred conidia were counted in each of 5 replicates for each experiment using an Olympus BX41 microscope (Olympus) at 400× magnification. All experiments were performed three times. The sterile deionized water and 2% (v/v) Valencia juice were used as negative and positive controls, respectively. The definition of a germinated conidium was that the conidium formed an appressorium or the length of the germ tube was equal to or greater than the width of the conidium. Images of the observations were acquired using a Leica DMR-HC microscope (Leica, Wetzlar, Germany) equipped with differential interference contrast (DIC) optics and a SPOT digital camera (version 3.2; Diagnostic Instruments Inc., Sterling Heights, MI) at 30 min intervals until the processes of conidial germination were complete. To be the consistent across the study, germination data
were considered when the percent conidial germination in 2% Valencia juice (positive controls) achieved at least 80%.

Effects of Citrus Juice Sources, Concentration, and pH

To determine conidial germination and appressorium formation of *P. citricarpa* in response to different nutritional environments, fresh fruit juice was squeezed from seven citrus cultivars in June 2010, including 'Valencia' sweet orange [*Citrus sinensis* (L.) Osbeck], 'Hamlin' sweet orange [*C. sinensis* (L.) Osbeck], 'Navel' sweet orange [*C. sinensis* (L.) Osbeck], 'Dancy' mandarin (*C. tangerine* Tanaka), 'Marsh' grapefruit (*C. x paradisi* MacFadyen), 'Eureka' lemon [*C. limon* (L.) Burm. f. 'Eureka'), and 'Key' lime [*C. aurantiifolia* (Christm.) Swingle], followed by filtering with P5 filter paper (Thermo Fisher Scientific) and stored at −20°C as stocks. Each juice stock was then diluted to 2% (v/v) and autoclave-sterilized before use in assays. The pH of each diluted juice was also measured using an Accumet pH Meter AB15 (Thermo Fisher Scientific). To determine the optimal juice concentration for germination and appressorium formation of *P. citricarpa*, a series of 'Valencia' juice solutions with concentrations of 0.5, 1.0, 2.0, 4.0, 8.0, and 16.0% (v/v) were prepared and used as above, resulting in the final concentrations of 0.375, 0.75, 1.5, 3.0, 6.0, and 12.0% (v/v), respectively. To evaluate the relationship between the pH of the juice solution and germination processes, pH of 2% 'Valencia' juice was adjusted from 2 to 9 at 1-pH intervals with 1 N HCl or 1 N NaOH using an Accumet pH Meter AB15 (Thermo Fisher Scientific). Afterward, 'Valencia' juice solutions with various pH levels were filter-sterilized through 0.45-µm membrane filters (Thermo Fisher Scientific) and applied as above.

To further determine the optimal pH of 'Valencia' juice for germination and appressorium formation, the pH of 2% 'Valencia' juice was adjusted from 3 to 5 at 0.2-pH intervals with 1 N
HCl or 1 N NaOH, followed by filter-sterilization using 0.45-μm membrane filters (Thermo Fisher Scientific) and application to the germination tests as above.

**Juice Quality**

To analyze juice quality, total soluble solids (°Brix), total titratable acid, and oil levels were determined according to Procedures for Analysis of Citrus Products published by FMC FoodTech (Cheng 2002). Briefly, measurements of °Brix values of various juice samples were performed in triplicate using a digital Leica Mark II Plus Abbe refractometer (Leica). Acidity was determined by titrating 25 ml of juice with 0.3123N NaOH to the end point of phenolphthalein. The amount of oil in each juice sample was determined based on the Scott oil analysis (Cheng 2002).

**Effect of Temperature and Incubation Time**

To determine the optimal temperature required for conidial germination and appressorium formation of *P. citricarpa*, conidial suspensions were prepared as above, mixed with 2% Valencia juice at a 1:3 ratio of conidial suspensions to solutions, and applied to hydrophobic slides (Thermo Fisher Scientific). Petri dishes with slides were placed in plastic moist chambers and incubated at 4°C intervals from 4 to 32°C for 24 h. In addition, the optimal incubation time was determined in the time-course experiments at 24°C, in which conidial germination and appressorium formation were recorded through observation with an Olympus BX41 microscope (Olympus) at 400× magnification every 2 h up to 12 h and then at 6 h intervals from 12 to 36 h.

**Effect of Carbon and Nitrogen Sources**

All chemicals were purchased from Sigma (Sigma-Aldrich, St. Louis, MO) unless otherwise stated. *P. citricarpa* conidia were harvested as above and individually mixed with various autoclave-sterilized nutrients at a 1:3 ratio of conidial suspensions to solutions to give
final concentrations indicated as follows: 75 mM of carbon sources (D-glucose, D-fructose, D-galactose and D-mannitol, sucrose, and maltose), 75 mM of nitrogen sources (ammonium acetate, ammonium chloride, ammonium sulfate, ammonium nitrate, sodium nitrate, potassium nitrate, calcium nitrate, and urea), three-fourth strength potato dextrose broth (PDB), 0.75% (w/v) of yeast extract, and 0.75% (w/v) of tryptone.

Effect of Synthetic Citrus Juice

Synthetic citrus juice was prepared according to the recipe published in Cánovas et al. (1997) to determine the critical components of citrus juice for conidial germination and appressorium formation of *P. citricarpa*. Briefly, five different basal solutions were prepared and filter-sterilized using 0.45-μm membrane filters (Thermo Fisher Scientific) as follows (w/v): sugars (Su; 23 g/l of glucose, 23 g/l of fructose, and 46 g/l of sucrose), salts (Sa; 0.5 g/l of K₂HPO₄, 0.5 g/l of KH₂PO₄, 0.2 g/l of NH₄Cl, and 0.001 g/l of FeCl₃), 10 g/l of citric acid (C), 1 mg/l of thiamine (T), and 45 mg/l of limonin (L). Various formulations of synthetic citrus juice were then prepared using these basal solutions, diluted to 2% with deionized water, adjusted to pH 3.4 with 1 N HCl or 1 N NaOH, and filter-sterilized using 0.45-μm membrane filters (Thermo Fisher Scientific) for the subsequent assays of conidial germination and appressorium formation.

Data Analysis

Prior to evaluating effects of citrus juice sources, carbon and nitrogen sources, and synthetic citrus juice on conidial germination and appressorium formation, residual plots and statistics of the data sets generated using SAS PROC UNIVARIATE (version 9.4; SAS Institute Inc., Cary, NC) were examined to validate the model assumptions. If the model assumptions were violated, the data set would be further subjected to Box-Cox transformation (Box and Cox
1964). Afterward, Fisher’s protected least significant difference (LSD) test was performed using SAS PROC GLIMMIX (SAS Institute Inc.) to separate the treatment means at the 5% significance level. The correlation between the juice quality (juice pH, Brix/Acid ratios, and juice oil content) and conidial germination or appressorium formation was analyzed using Pearson’s correlation coefficient ($r$) (SAS PROC CORR; SAS Institute Inc.). To describe the relationship between the effect (juice concentration, juice pH, temperature, or incubation time) and conidial germination and appressorium formation, nonlinear regression analysis was carried out using SAS PROC NLIN with the Marquardt option (SAS Institute Inc.). The coefficient of determination ($r^2$) and the root mean square error (RMSE) calculated by linear regression analysis between the predicted values obtained from the model and the measured values of the data set were used to evaluate the performance of models.

**Results**

**Conidial Germination and Appressorium Formation**

Conidia of *P. citricarpa* are surrounded with a layer of a barely visible mucoid sheath extended into an apical appendage and are filled with lipid (Fig. 4-1A). Conidial germination began approximately 2 h after incubation in 1.5% Valencia juice at 24°C with the emergence of a germ tube (Fig. 4-1B). After 3 h incubation, the germ tube began to swell to initiate appressorium formation (Fig. 4-1C). During the process of appressorium formation, the lipid-like bodies migrated into the developing appressorium (Fig. 4-1D to 4-1E). Once the migration of the lipid-like bodies was complete, melanization of the appressorium began, and a septum was formed to separate the conidium from the appressorium (Fig. 4-1F to 4-1G). At 7 h post-incubation, the melanized appressorium became apparent (Fig. 4-1H).
Citrus Juice Sources

The presence of nutritional sources significantly enhanced conidial germination and appressorium formation of *P. citricarpa* (Fig. 4-2). The rates of conidial germination and appressorium formation were significantly higher in citrus juice sources after 24 h of incubation at 24°C than in sterile deionized water where less than 1% of germination and appressorium formation were observed (Fig. 4-2). Among citrus juice sources tested, 'Valencia' and 'Navel' juice yielded significantly greater percent conidial germination and appressorium formation (> 80%) compared to 'Marsh', 'Hamlin', 'Dancy', 'Key lime', and 'Eureka' juice (Fig. 4-2). The pH of citrus juice sources ranged from 4.32 ('Hamlin') to 2.87 ('Key lime') (Table 4-1). However, the pH of citrus juice sources was not correlated with conidial germination or appressorium formation with *P*-values of 0.084 and 0.073, respectively, according to Pearson’s correlation test (Table 4-1). The analysis of the overall oil content in juice sources showed that the oil content ranged from 0.086% in 'Valencia' juice to 0.003% in 'Dancy' juice and no correlation between the oil content and germination and appressorium formation was observed with *P*-values of 0.465 and 0.466, respectively (Table 4-1). The analysis of Brix/Acid ratios of citrus juice sources revealed that 'Navel' juice had the highest Brix/Acid ratio of 23.2, and 'Eureka' lemon and 'Key' lime juices had the lowest Brix/Acid ratio of 1.4 (Table 4-1). Surprisingly, Pearson’s correlation test revealed a moderate correlation between the Brix/Acid ratio and germination or appressorium formation with \( r = 0.783 \) \((P < 0.05)\) and \( 0.800 \) \((P < 0.05)\), respectively (Table 4-1).

Juice Concentration and pH

The juice concentration and pH significantly affected the germination and appressorium formation of *P. citricarpa* (Fig. 4-3 and 4-4). After incubation for 24 h at 24°C, the percent conidial germination and appressorium formation increased alongside the increase of 'Valencia' juice concentration and reached the peak at 1.5% 'Valencia' juice (89.07 and 84.73%).

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respectively) (Fig. 4-3). As the 'Valencia' juice concentration increased from 1.5% to 12%, the percentages of conidial germination and appressorium formation decreased to 69.53 and 36.73%, respectively (Fig. 4-3). Conidial germination occurred in 1.5% 'Valencia' juice with pH values from 3 to 6, and the peak germination rate was observed in the original 1.5% 'Valencia' juice with the pH of 3.45 (Fig. 4-4A). No conidial germination was observed in 1.5% 'Valencia' juice with pH below 2 or above 7. A similar trend for appressorium formation was also observed (Fig. 4-4A). Furthermore, the subsequent examination of the optimal pH of 'Valencia' juice showed that conidial germination and appressorium formation occurred in the pH range of 3 to 5 with the optimal pH of 3.4 (Fig. 4-4B), which matched our previous observation that the optimal pH of 'Valencia' juice for germination and appressorium formation was approximately 3.45.

**Temperature and Incubation Time**

After 24 h of incubation in 1.5% Valencia juice, the germination rate increased as the temperature increased from 12 to 24°C with the peak rate at 24°C (85.33%) and then decreased as the temperatures increased from 24 to 32°C (Fig. 4-5). While a similar trend for appressorium formation was observed, the rates of appressorium formation at 12 and 32°C were much lower than those of conidial germination (Fig. 4-5). No conidial germination or appressorium formation was observed at the temperatures below 8°C or over 36°C (Fig. 4-5). Conidial germination initiated at 2 h after incubation in 1.5% Valencia juice at 24°C, whereas appressorium formation was observed beginning from 6 h after incubation (Fig. 4-6). The percent germination and appressorium formation reached a maximum after 18 h of incubation and remained constant afterward.

**Carbon and Nitrogen Sources**

Compared with 1.5% Valencia juice, simple carbon and nitrogen solutions did not significantly increase conidial germination and appressorium formation over a 24 h period of
incubation at 24°C, while ammonium nitrate significantly increased the germination over the water control (Table 4-2). In contrast, a complex carbon and nitrogen source, PDB, enhanced conidial germination (71.27%) with no significant difference from 1.5% Valencia juice (84.27%) (Table 4-2). However, appressorium formation in PDB (30%) was significantly reduced compared to that of 1.5% Valencia juice (84.27%). Another complex carbon and nitrogen source, yeast extract, only supported a low level of conidial germination and appressorium formation (Table 4-2).

**Synthetic Citrus Juice**

Over 80% *P. citricarpa* conidia germinated and formed appressoria in 1.5% complete synthetic citrus juice (Su+Sa+C+T+L) without a significant difference from those in 1.5% 'Valencia' juice (Fig. 4-7). The elimination of limonin from the synthetic citrus juice (Su+Sa+C+T) did not have a significant effect on conidial germination and appressorium formation (Fig. 4-7). Within 3-component solutions (Su+Sa+C, Su+Sa+T, Su+C+T, and Sa+C+T) tested, conidial germination was significantly reduced in the absence of sugars (Sa+C+T) or citric acid (Su+Sa+T). While the percent germination in the solution without salts (Su+C+T) or thiamine (Su+Sa+C) were not significantly different from those observed in 1.5% Valencia juice, the appressorium formation was reduced by approximately 20% in the absence of thiamine and reduced sharply by approximately 40% in the absence of salts (Fig. 4-7). The use of 2-component solutions (Su+Sa, Su+C, and Sa+C) for germination assays resulted in 30 to 40% reduction in germination and appressorium formation (Fig. 4-7). In addition, single component solutions (Su, Sa, and C) were found to only stimulate a basal level of germination and appressorium formation of approximately 20%.
Discussion

A successful parasitic relationship between fungal pathogens and host cells is established upon the satisfaction of many prerequisites involved in infection processes, including surface wettability of a substratum, exogenous nutrients, and environmental conditions (light, moisture, and temperature) (Blank and Murray 1998; Emmett and Parbery 1975; Kuo and Hoch 1996; Liu and Xiao 2005). Given the emergence of *P. citricarpa* in Florida (Schubert et al. 2012), elucidating what factors influence its conidial germination is not only of fundamental value in understanding fungal biology but also of practical interest for better disease control strategies. Previously, the universal prerequisite for conidial germination in *Phyllosticta* spp. was reported to be the attachment of conidia to a hydrophobic surface (Shaw et al. 2006). However, the attachment to a hydrophobic surface was not solely supportive of conidial germination of *P. citricarpa*. Its conidia rarely germinated in sterile water even on a hydrophobic surface (Kiely 1948; Korf 1998), implying additional requirements for germination. In the present study, we evaluated the effects of seven different citrus juices on conidial germination and appressorium formation of *P. citricarpa* and demonstrated that all citrus juices significantly favored conidial germination and appressorium formation with the maximum rate of over 80% observed in 1.5% Valencia juice. The requirement for additional nutrients to promote germination and the full development of appressoria has also been observed in other fungi such as *Potebniamyces pyri*, *Penicillium digitatum*, *Botrytis cinerea*, and *Colletotrichum graminicola* (Liu and Xiao 2005; Nassr and Barakat 2013; Pelser and Eckert 1977; Skoropad 1967). However, in contrast to our results, Korf (1998) reported that only approximately 60% germination of *P. citricarpa* was recorded in 1.5% 'Valencia' juice. The discrepancy in germination levels between these two studies is likely due to the different pH values of 'Valencia' juices used as the 'Valencia' juice with a higher pH value of 4.34 was applied to Korf's germination assays compared to ours with a
pH value of 3.45. Indeed, analysis of the effect of 'Valencia' juice pH on germination and appressorium formation clearly showed that the highest germination rate occurred in 1.5% Valencia juice with a pH value of approximately 3.4. Moreover, Korf’s observation of approximately 60% germination at pH 4.34 matched our predicted value according to the mathematical equation (described in Fig. 4-4B), further confirming the tendency of the pH-dependent conidial germination in *P. citricarpa*. The germination of *P. citricarpa* conidia in response to pH also appears to be source-dependent as the optimal pH of 0.5% citric acid and 0.2% glucose solutions for germination was approximately 4.0 and 6.0, respectively (Korf 1998; Lu et al. 2012).

A moderate relationship between the Brix/Acid ratio and conidial germination (*r* = 0.783) was revealed from the juice quality analysis test, but no relationship between juice pH or the oil content and germination was found with the same test. In general, a Brix/Acid ratio, the amount of soluble solids divided by the acid concentration, is a term commonly used in the juice processing industry to express the relationship between sweetness and acidity (Kilburn 1958). This ratio is greatly affected by citrus cultivar and fruit maturity as the acid concentration in juice decreases during the fruit ripening process while the Brix value remains constant or increases slightly (Kilburn 1958). Therefore, with such a small sample size of cultivars (*n* = 7) and only one maturity stage of fruit used, the observation of the moderate correlation between the Brix/Acid ratio and germination may not be meaningful, as was observed in this study where 'Valencia', 'Navel', and 'Marsh' juices all favored the conidial germination of *P. citricarpa* without a significant difference while the variation of the Brix/Acid ratios among them was quite large, ranging from 9.4 to 23.2. To further elucidate the possible role of juice quality in
germination, a set of citrus juices collected from fruit of a specific cultivar at various ripening stages should be included in a set of germination assays.

Conidial germination and appressorium formation of *P. citricarpa* were not substantially improved by most simple carbon or nitrogen sources alone, except for ammonium nitrate that was able to support a germination rate of approximately 20%. These results are likely due to the toxicity of the higher concentration (75 mM) used in germination assays. Nassr and Barakat (2013) reported that the conidial germination of *B. cinerea* declined sharply in the presence of more than 10 mM of cations (Ca$^{2+}$, Mg$^{2+}$, K$^+$, and Fe$^{2+}$). Since ammonium nitrate is the only tested compound containing nitrogen sources (NH$_4^+$ and NO$_3^-$) as the sole ions, the lack of those extra cations (Ca$^{2+}$, Na$^+$, and K$^+$) or anions (Cl$^-$, CH$_3$COO$^-$, and SO$_4^{2-}$) in ammonium nitrate may be the reason why *P. citricarpa* conidia can germinate even at the relatively high concentration.

Given that natural citrus juice is a complex solution and its quality is largely influenced by cultivar and fruit maturity, that components in citrus juice responsible for conidial germination are not known hampers the reproducibility of germination assays between laboratories. Therefore, in this study synthetic citrus juice prepared based on the recipe of Cánovas et al. (1997) was tested and shown to be as effective as the natural 'Valencia' juice in promoting conidial germination and appressorium formation. The elimination of limonin did not significantly result in reduction of conidial germination and appressorium formation, suggesting that limonin as the triterpenoid bitter principle is not essential for the germination processes of *P. citricarpa*. As to salts and thiamine, it was shown that the omission of salts or thiamine did not significantly affect the germination. However, the appressorium formation was reduced by approximately 20% in the absence of thiamine and reduced sharply by approximately 40% without the presence of salts, suggesting that both salts and thiamine play a certain role in the
processes of appressorium formation of *P. citricarpa*. These findings were similar to those reported in Pelser and Eckert’s study (1977) where the mixture of macro-, micro-elements, and vitamins were not required for germination of conidia but essential for vigorous growth of germ tubes of *P. digitatum*.

The sugar solution was found to be essential to stimulate conidial germination to the level observed in Valencia juice and the absence of sugars in the solution resulted in significant germination reduction. While we did not further test the effectiveness of each sugar (glucose, fructose, and sucrose) in supporting germination, Pelser and Eckert (1977) reported that glucose was superior to fructose and sucrose in stimulating conidial germination of *P. digitatum*, which is likely due to more efficient delivery of glucose into cells and/or easier access to glucose by constitutive enzymes in cells, and therefore suggested to replace the original sugar mixture with a double concentration of glucose without reduction of conidial germination (Pelser and Eckert 1977). In contrast, fructose was found to more efficiently stimulate germination of *B. cinerea* conidia than glucose or sucrose (Blakeman 1975; Nassr and Barakat 2013). These findings indicate that the sugar preference for stimulation of conidial germination is fungal species-dependent and needs to be determined in *P. citricarpa* to further simplify the preparation of the synthetic citrus juice for germination assays.

Citric acid was shown to support over 80% conidial germination of *P. citricarpa* (Kiely 1948), however the effectiveness of citric acid in stimulating germination has not been repeated by others (Korf 1998). In this study, while citric acid alone only stimulated a basal level of germination, citric acid combined with other components in the synthetic juice was found to be essential to support the high percent germination of *P. citricarpa*. This finding along with the fact that *P. citricarpa* prefers approximately pH 3.4 for conidial germination indicates that citric
acid may function in buffering the medium to ensure the optimal pH for germination. This observation is also consistent with that reported in *P. digitatum* by Pelser and Eckert (1977). Kiely’s findings (1948) showing that citric acid alone was capable of stimulating germination of *P. citricarpa* may have resulted from the effectiveness of other compounds being washed off from fruit wounds or surface during the preparation of conidial suspensions.

It was found that *P. citricarpa* conidia germinated at temperatures ranging from 12 to 32°C with the optimal temperature at 24°C. Korf (1998) also reported a similar temperature range for germination with the optimal temperature at 22°C. As to the mycelial growth, *P. citricarpa* can grow across a wide range of temperatures from 5 to 38°C with the optimal temperature at 26°C (Er et al. 2014). This observation is consistent with that obtained by Brodrick and Rabie (1970), showing that 27°C is the optimal temperature for mycelial growth and symptom development. Despite the difference in the optimal temperature for conidial germination versus mycelial growth, these findings suggest that the response of conidial germination of *P. citricarpa* to temperature is in general agreement with the temperature response of mycelial growth of the fungus.

The role of melanin accumulation during appressorium development has been well characterized in *Magnaporthe grisea* where it plays an important role in supporting the generation of high turgor pressure that contributes to the force required for penetration (Howard and Ferrari 1989; Howard et al. 1991). In addition, appressoria, while not as long-term survival structures (Emmett and Parbery 1975), may have an auxiliary capacity to endure the adverse environmental conditions such as UV light and desiccation to increase the chance of successful infection. These findings suggest that appressorium formation plays an important role in establishing successful fungal-plant interactions. *P. citricarpa*, similar to many other fungal
pathogens such as *M. grisea* and *P. ampelicida* (Bourett and Howard 1990; Shaw et al. 1998), develops melanized appressoria as an essential prerequisite for successful penetration of the host cuticle. However, excess nutrients during appressorium formation of *P. citricarpa* appear to delay or inhibit its formation as approximately 60% of germinating conidia only developed long germ tubes without the formation of appressoria in PDB and 12% Valencia juice. The similar response of fungal conidia to excess nutrients has also been observed in *Colletotrichum* species. In these studies, conidia often continued germ tube elongation and branching rather than developing appressoria in the presence of high nutrient levels (Emmett and Parbery 1975). These findings imply that starvation may favor the initiation of appressorium formation. In the case of *P. citricarpa*, conidial germination requires the stimulus of additional nutrients. Therefore, under this circumstance balancing the needs between exogenous nutrients for germination and exhaustion of cellular energy reserves for appressorium formation is critical toward the establishment of a successful fungal-plant interaction.

During our germination assays, unexpected low germination rates occasionally occurred. This observation was also reported by Kiely (1948), in which inconsistent results with low germination rates were obtained even when fresh 0.1 to 0.5% citric acid solutions were used. These findings indicate that other factors such as the source of conidia may contribute to inconsistent results. The first possible clue comes from studies of conidia produced by pure fungal cultures on artificial medium. Kiely (1948) mentioned that these type of conidia particularly tended to generate poor results. But even with the use of conidia freshly harvested from pycnidia on lesions of symptomatic mature Valencia fruit, inconsistent results remained, indicating that the sources (artificial medium or lesions on symptomatic fruit) where conidia were produced were probably not the cause for the inconsistent results. Therefore, other possible
factors were proposed, including the environmental conditions during conidial production and the age of conidia. Kiely (1948) mentioned that the moisture condition during conidial production was critical. In this work, conidia produced from pre-soaked pycnidia on lesions of symptomatic fruit were more capable of germinating during the assays. Afterward, he tested the longevity of conidia by conducting germination assays over a long period of time with the same conidial source and found that the germination rate drastically decreased from approximately 80% to 20% after 5 days, indicating a quick decline of conidial germinate ability and its short-lived nature (Kiely 1948). A similar age effect on conidial germination was also observed in B. cinerea in Nassr and Barakat’s study (2013) where a significantly lower germination rate (67%) was recorded in 14-day-old conidia on the hydrophobic surface compared with 91% in 5-day-old conidia. However, the age effect on conidial germination of B. cinerea can be masked by additional nutrients as older conidia germinated as well as younger conidia in Gamborg B5 basal salt solution amended with fructose (Nassr and Barakat 2013). Given that our fungal cultures were incubated in Parafilm-sealed plates, the moisture condition during pycnidial and conidial production should not be an issue. On the other hand, since 2-week old fungal cultures were chosen for the experiments, the possibility of mixed conidial ages may be linked to the inconsistent results. This suggests that the harvest timing for P. citricarpa conidia produced on artificial medium should be further fine-tuned to minimize inconsistency.

P. citricarpa conidia have been confirmed to be the sole inoculum in the citrus field in Florida (Wang et al. 2016, submitted). Given that citrus black spot management mainly relies on regular fungicide sprays, chances are that a fungicide-resistant population may arise from the frequent use of a single fungicide. Therefore, alternative fungicides with different modes of actions would be needed to reduce the risk of resistance development. This can be achieved
using a robust and convenient technique for screening the efficacy of fungicides *in vitro*. Indeed, this system has been successfully applied to the determination of baseline sensitivity of the Floridian population of *P. citricarpa* to quinone outside inhibitor (QoI) fungicides (Hincapie et al. 2014), which will subsequently serve as the foundation for monitoring fungicide resistance in the future.

In conclusion, our study has shown that conidial germination and appressorium formation of *P. citricarpa* is nutrition and environment-dependent and can reach the maximum rate when conidia are incubated on a hydrophobic surface in 1.5% Valencia juice at pH 3.4 for at least 18 h at 24°C. Sugars, salts, citric acid, and thiamine were determined to be critical for the germination processes of *P. citricarpa*, suggesting that the effectiveness of the natural or synthetic juice in stimulating germination is attributed to complicated interactions of many nutritional and environmental factors instead of one or few unique components. Our findings in the current study will not only contribute to increasing the reproducibility of germination assays of *P. citricarpa* between different laboratories via simplifying the preparation of the nutritional solution, but also provide a further understanding of fungal biology that may be useful for developing better disease control measures.
Table 4-1. The relationship between juice pH and conidial germination or appressorium formation of *Phylllosticta citricarpa*.

<table>
<thead>
<tr>
<th>Juice source</th>
<th>Germination (%)</th>
<th>Appressorium (%)</th>
<th>pH</th>
<th>Brix/Acid ratio&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Oil (%)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Valencia sweet orange</td>
<td>87.13</td>
<td>85.80</td>
<td>3.45</td>
<td>13.4</td>
<td>0.086</td>
</tr>
<tr>
<td>Hamlin sweet orange</td>
<td>75.87</td>
<td>74.53</td>
<td>4.32</td>
<td>20.5</td>
<td>0.014</td>
</tr>
<tr>
<td>Navel orange</td>
<td>82.47</td>
<td>82.00</td>
<td>4.04</td>
<td>23.2</td>
<td>0.052</td>
</tr>
<tr>
<td>Marsh grapefruit</td>
<td>82.07</td>
<td>79.60</td>
<td>3.42</td>
<td>9.4</td>
<td>0.004</td>
</tr>
<tr>
<td>Dancy tangerine</td>
<td>74.60</td>
<td>72.47</td>
<td>3.74</td>
<td>14.6</td>
<td>0.003</td>
</tr>
<tr>
<td>Eureka lemon</td>
<td>38.53</td>
<td>30.53</td>
<td>2.93</td>
<td>1.4</td>
<td>0.062</td>
</tr>
<tr>
<td>Key lime</td>
<td>49.93</td>
<td>45.00</td>
<td>2.87</td>
<td>1.4</td>
<td>0.080</td>
</tr>
</tbody>
</table>

Correlation coefficient (r) for pH<sup>a</sup> 0.694 (P = 0.084) 0.712 (P = 0.073)
Correlation coefficient (r) for ratio<sup>a</sup> 0.783 (P = 0.038) 0.800 (P = 0.031)
Correlation coefficient (r) for oil<sup>a</sup> −0.334 (P = 0.465) −0.333 (P = 0.466)

<sup>a</sup> The correlation between juice quality (juice pH, Brix/Acid ratios, and juice oil content) and conidial germination or appressorium formation was evaluated using Pearson’s correlation coefficient (r).

<sup>b</sup> The juice quality was evaluated based on the methods described in Procedures for Analysis of Citrus Products (Cheng 2002).
Table 4-2. The effects of various carbon or nitrogen sources on conidial germination and appressorium formation of *Phyllosticta citricarpa*.

<table>
<thead>
<tr>
<th>Solution(^a)</th>
<th>pH</th>
<th>Germination (%)(^b)</th>
<th>Appressorium (%)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Carbon</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Fructose</td>
<td>4.80</td>
<td>3.13±1.23 d</td>
<td>2.60±1.10 cd</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>4.91</td>
<td>0.87±0.29 efg</td>
<td>0.73±0.24 efg</td>
</tr>
<tr>
<td>D-Galactose</td>
<td>4.68</td>
<td>0.67±0.13 fghi</td>
<td>0.60±0.12 efg</td>
</tr>
<tr>
<td>D-Mannitol</td>
<td>4.74</td>
<td>0.53±0.18 ghi</td>
<td>0.47±0.18 fg</td>
</tr>
<tr>
<td>Sucrose</td>
<td>4.74</td>
<td>1.60±0.23 def</td>
<td>1.47±0.29 cedf</td>
</tr>
<tr>
<td>Maltose</td>
<td>4.58</td>
<td>0.40±0.12 hij</td>
<td>0.33±0.07 gh</td>
</tr>
<tr>
<td><strong>Nitrogen</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ammonium acetate</td>
<td>6.67</td>
<td>0.13±0.07 jk</td>
<td>0 i</td>
</tr>
<tr>
<td>Ammonium chloride</td>
<td>4.72</td>
<td>1.80±0.00 de</td>
<td>1.73±0.07 cde</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>4.90</td>
<td>1.20±0.20 efg</td>
<td>0.07±0.07 hi</td>
</tr>
<tr>
<td>Ammonium nitrate</td>
<td>4.73</td>
<td>23.73±4.91 b</td>
<td>22.87±4.10 b</td>
</tr>
<tr>
<td>Potassium nitrate</td>
<td>5.08</td>
<td>0.07±0.07 k</td>
<td>0.07±0.07 hi</td>
</tr>
<tr>
<td>Sodium nitrate</td>
<td>5.18</td>
<td>0.07±0.07 k</td>
<td>0 i</td>
</tr>
<tr>
<td>Calcium nitrate</td>
<td>4.96</td>
<td>1.13±0.29 efg</td>
<td>0.87±0.29 defg</td>
</tr>
<tr>
<td>Urea</td>
<td>8.84</td>
<td>0.53±0.07 ghi</td>
<td>0.27±0.07 gh</td>
</tr>
<tr>
<td><strong>Complex carbon/nitrogen</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDB</td>
<td>5.01</td>
<td>71.27±1.58 a</td>
<td>30.00±3.82 b</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5.75</td>
<td>9.67±1.25 c</td>
<td>4.27±2.56 c</td>
</tr>
<tr>
<td>Tryptone</td>
<td>6.98</td>
<td>0.40±0.00 ghij</td>
<td>0.33±0.07 gh</td>
</tr>
<tr>
<td>Valencia juice</td>
<td>3.45</td>
<td>84.27±4.28 a</td>
<td>84.00±4.20 a</td>
</tr>
<tr>
<td>Sterile deionized water</td>
<td>6.59</td>
<td>0.33±0.07 ij</td>
<td>0.27±0.07 gh</td>
</tr>
</tbody>
</table>

\(^a\) The final concentrations of solutions were as follows: 75 mM carbon and nitrogen sources, 0.75% yeast extract and tryptone, three-fourth strength PDB, and 1.5% Valencia juice.

\(^b\) Data are means of three independent experiments with five replicates of each experiment ± standard errors. Means followed by the same letter are not significantly different from each other according to Fisher’s protected least significant difference (LSD) test (\(P = 0.05\)).
Figure 4-1. Conidial germination and appressorium formation of *Phyllosticta citricarpa*. A, Conidia of *P. citricarpa* are surrounded with a layer of barely visible mucoid sheath (arrow) and an apical appendage (arrowhead). They contain abundant lipid that obscure other cytoplasmic organelles. Dumbbell-shaped spermatia are also observed in conidial suspensions. B, At 2 h after incubation in 1.5% Valencia juice at 24°C, a germ tube emerged from the spore and remained free of lipid-like bodies. C, At 3 h after incubation, the germ tube started to swell as a sign of initiation of appressorium development. D, At 3.5 h after incubation, the germ tube continuously swelled, and the lipid-like bodies migrated into the swollen tube. E, Within approximately 4 h after incubation, most of lipid-like bodies had migrated into the immature appressorium. F, At 5 h after incubation, the appressorium developed almost completely and began to melanize. The arrow indicates the barely visible sheath surrounding the empty spore. G, At 5.5 h after incubation, a septum (arrow) that separates the conidium from the appressorium became discernible. H, After 7 h incubation, the melanized appressorium became clearly visible. Scale bar = 10 µm.
Figure 4-2. The effect of various juice sources on conidial germination (A) and appressorium formation (B) of *Phylllosticta citricarpa* at 24°C after incubation for 24 h. Each point represents the mean of three independent experiments with five replicates of each experiment. Error bars show standard errors of the mean. Columns labeled with the same letter are not significantly different from each other according to Fisher’s protected least significant difference (LSD) test (*P* = 0.05).
Figure 4-3. The effect of juice concentration on conidial germination and appressorium formation of *Phylllosticta citricarpa* at 24°C after incubation for 24 h. Each point represents the mean of three independent experiments with five replicates of each experiment. Error bars show standard errors of the mean.
Figure 4-4. The effect of pH on conidial germination and appressorium formation of *Phyllosticta citricarpa* at 24°C after incubation for 24 h. The juice pH was adjusted from 2 to 9 at 1-pH intervals (A) and from 3 to 5 at 0.2-pH intervals (B) with 1 N HCl or 1 N NaOH. Each point represents the mean of three independent experiments with five replicates of each experiment. Error bars show standard errors of the mean.
Figure 4-5. The effect of temperature on conidial germination and appressorium formation of *Phylosticta citricarpa* after incubation for 24 h. Each point represents the mean of three independent experiments with five replicates of each experiment. Error bars show standard errors of the mean.
Figure 4-6. The effect of incubation time on conidial germination and appressorium formation of *Phyllosticta citricarpa* at 24°C. Each point represents the mean of three independent experiments with five replicates of each experiment. Error bars show standard errors of the mean.
Figure 4-7. The effects of synthetic citrus juice solutions on conidial germination and appressorium formation of *Phyllosticta citricarpa*. The synthetic citrus juice was prepared according to Cánovas et al. (1997). The final concentration of all solutions was 1.5% after being mixed with conidial suspensions at a 1:3 ratio. Each point represents the mean of three independent experiments with five replicates of each experiment. Error bars show standard errors of the mean. Columns labeled with the same letter (uppercase and lowercase letters for germination and appressorium formation, respectively) are not significantly different from each other according to Fisher’s protected least significant difference (LSD) test ($P = 0.05$). Su, sugars (glucose, fructose, and sucrose); Sa, salts (K$_2$HPO$_4$, KH$_2$PO$_4$, NH$_4$Cl, and FeCl$_3$); C, citric acid; T, thiamine; L, limonin.
CHAPTER 5
HISTOLOGICAL OBSERVATION OF THE LEAF INFECTION PROCESS BY *Phyllosticta citricarpa*, THE CITRUS BLACK SPOT PATHOGEN

Introduction

Leaf infection by foliar fungal pathogens is in large part considered the first phase of fungal-plant interactions (Fernandez et al. 2014), but the process of leaf infection by *P. citricarpa* remains poorly understood. Following successful leaf infection, *P. citricarpa* remains latent for up to 36 months before leaf abscission (Kiely, 1948; McOnie, 1967). McOnie (1967) observed appressorium formation on leaf surfaces after infection by ascospores as well as the formation of the penetration peg and a ‘small knot’ of fungal tissue (vegetative stroma) that stayed between the cuticle and the first epidermal layer. Up to 96 h post-infection, the small knot did not penetrate further into the plant tissue, and cells underneath the fungal tissue seemed to deform possibly due to certain interactions between the pathogen and the host. Noronha (2002) also observed appressorium formation from conidia on the leaf surface following a wetness duration of 24 to 36 h at 25 to 30°C. Truter et al. (2007) reported that conidia of *P. citricarpa* from pure cultures or symptomatic fruit cannot infect and colonize the detached 'Eureka' lemon leaves under laboratory or field conditions. Furthermore, Truter (2010) found that the susceptible periods of green 'Eureka' lemon and 'Valencia' leaves to *P. citricarpa* were 10 and 8 months, respectively, which were determined by the recovery of *P. citricarpa* from the leaf tissue. However, once leaf infection by *P. citricarpa* occurred, the pathogen could only be reisolated for up to 5 months post-infection (Truter, 2010), implying that *P. citricarpa* may become quiescent and wait for favorable conditions for further development. Thus, the objective of the study was to determine how infection and colonization of leaves by *P. citricarpa* occurred, potentially leading to the production of inoculum.
Materials and Methods

**Fungal Isolate, Bacterial Strain, and Plasmid**

Single-spored *P. citricarpa* isolate Gc12 was maintained on 1/2PDA at 24°C under a 12h photoperiod and subcultured once a week. For the long-term storage, ground mycelia of *P. citricarpa* Gc12 were evenly dispensed on 1/2PDA with a layer of sterile P5 filter paper (Thermo Fisher Scientific) and incubated at 24°C under a 12h photoperiod for 6 days. After the incubation period, filter paper colonized by fungal mycelia were transferred into sterile coin envelopes and air dried in a laminar flow hood overnight. Dried filter paper and envelopes were then stored in plastic containers with desiccants in a −20°C freezer until use.

The *Escherichia coli* (*E. coli*) strain DH5α with plasmid pCT74 was maintained on ampicillin-amended LB agar (Lennox; Difco) (100 μg/ml) or stored in 15% glycerol solution at −80°C until use (Lorang et al. 2001).

Plasmid pCT74 harboring the synthetic green fluorescent protein gene (*sgfp*) under the control of the promoter from the *Pyrenophora tritici-repentis* host-selective toxin gene *toxA* and the hygromycin resistance gene (Fig. B-1) was used for protoplast transformation (Lorang et al. 2001). The plasmid was isolated from the *E. coli* strain using the Qiagen QIAprep Spin Miniprep kit (Qiagen) according to the instructions of the manufacturer and quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). The purified plasmids were stored at −80°C until use.

**Protoplast Preparation**

Protoplasts of the wild type *P. citricarpa* Gc12 strain were prepared based on the procedures described by Rollins (2003). Two to three-week old mycelia of *P. citricarpa* grown on 1/2PDA were transferred into 1.5 ml Eppendorf tubes with 300 μl of sterile deionized water
and ground into small pieces using small plastic pestles. Afterwards, 125 ml flasks containing 50 ml of potato dextrose broth (PDB; Difco) were inoculated with ground mycelia and incubated on a shaker at 100 rpm at room temperature for 5 to 6 days. Following the incubation, the mycelial mass was washed once each with sterile deionized water and protoplast buffer (0.8 M MgSO₄·7H₂O, 0.2 M sodium citrate dihydrate, pH 5.5) through a sterile powder funnel with four layers of sterile cheesecloth, chopped coarsely into small pieces with a sterile spatula in another 125 ml flask, and incubated on a shaker at 100 rpm at 28°C for 4 h in the dark in the protoplasting solution containing 1% lysing enzymes from Trichoderma harzianum (L1412; Sigma-Aldrich) dissolved in a solution containing Novozyme buffer (1 M sorbitol, 50 mM sodium citrate dihydrate, pH 5.8) and protoplast buffer at a ratio of 3:17. Protoplasts were then separated from the residual hyphae by filtration through four layers of sterile Miracloth (Thermo Fisher Scientific) and pelleted by centrifugation at 3,000 × g at 4°C for 10 min. The resulting pellet was washed twice with STC buffer (1 M sorbitol, 50 mM Tris-HCl, pH 8, 50 mM CaCl₂·2H₂O) and centrifuged as above. Protoplasts (equal to 67.5% of the final volume) were resuspended at a concentration of 10⁸ protoplasts/ml in storage buffer (equal to 32.5% of the final volume) containing dimethyl sulfoxide, 0.5% heparin (in STC buffer), and 40% polyethylene glycol (PEG) solution [two parts of 60% PEG 4000 in water and one part of KTC buffer (1.8 M KCl, 150 mM Tris-HCl, pH 8, 150 mM CaCl₂·2H₂O)] at a ratio of 1:5:20. These protoplast stocks were stored at −80°C until use (Fig. B-2).

**Protoplast Transformation and Regeneration**

For protoplast transformation, 5 µg of plasmid DNA was mixed with 2 µl of 50 mM spermidine and 5 µl of 0.25% heparin (in STC buffer), kept on ice for 20 min, and added to 100 µl of the protoplast stocks. Following incubation on ice for 30 min, the protoplast-DNA
suspension was gently mixed with 1 ml of 40% PEG solution and incubated at room temperature for 20 min. The suspension was then mixed with 3 ml of liquid RM medium (0.7 M sucrose, 0.05% yeast extract) and incubated on a shaker (< 100 rpm) at room temperature for 4 h. Afterwards, the suspension was gently mixed with 16 ml of warm melting RM medium (0.7 M sucrose, 0.05% yeast extract, 1.5% agar) and 20 µl of hygromycin B (100 mg/ml in water) to reach the final hygromycin concentration of 100 µg/ml. The entire solution was evenly dispensed into two plates and incubated at room temperature for 5 to 7 days. Subsequently, hygromycin-resistant transformants were transferred onto hygromycin-amended 1/2PDA (10 µg/ml) and purified a minimum of three times with a hyphal tip method. Those transformants were also observed under a fluorescence microscope (Leica DMR-HC microscope; Leica) at 400× magnification to visualize GFP expression, and images were acquired using a SPOT digital camera (version 3.2; Diagnostic Instruments Inc.) equipped on the microscope.

**PCR Confirmation of GFP expression in *P. citricarpa***

To further confirm the presence of the plasmid in the GFP-expressing strains of *P. citricarpa*, a PCR-based method was performed using M13 forward (−20) (5’-GTAAAGCAGGCTATGACCAG-3’) and M13 reverse (5’-GGAAACAGCTATGACCAG-3’) primers as follows. Briefly, gDNA of GFP-expressing stains was isolated as described by Hu et al. (2014). PCR reactions were performed in a total volume of 25 µl containing 1 to 10 ng of gDNA, 0.2 µM of each primer, 0.2 mM of each dNTP, 1× PCR reaction buffer, and 1 unit of HotStar Taq Plus Polymerase (Qiagen) using an MJ Research PTC-200 Peltier Thermal Cycler (Bio-Rad Laboratories). The thermal cycling conditions were as follows: 95°C for 5 min, 40 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 2 min, with a final extension of 5 min at 72°C. The resulting PCR products were analyzed by electrophoresis on 1% agarose gels stained...
with SYBR Safe DNA stain (Invitrogen) in 1× TAE buffer at 100 V for 40 min. The gels were imaged using a UVP-MultiDoc-It Digital Imaging System (UVP).

**Greenhouse Experiment**

To understand the early infection process by *P. citricarpa*, conidial suspensions at a concentration of $10^6$ conidia/ml were harvested as described by Hincapie et al. (2014). Mature 'Valencia' leaves were collected from the greenhouse, washed in tap water, and dried on paper towels. Multiple 25 mm$^2$ areas on collected leaves were then marked and inoculated with 10 µl of a $10^6$ conidia/ml suspension of *P. citricarpa* before incubation in a moist chamber at ambient conditions. Four leaves were collected every 12 h up to 96 h and processed using conventional paraffin embedding and sectioning procedures as described below.

To determine how infection and colonization of leaves by *P. citricarpa* potentially leads to inoculum production, attached leaf assays were conducted in the quarantine greenhouse in the Division of Plant Industry (DPI) in Gainesville, FL as follows. Briefly, conidial suspensions at a concentration of $10^6$ conidia/ml were harvested as described by Hincapie et al. (2014), except that the conidial suspensions were resuspended in 0.1% agar solution. One to two month-old leaves of 'Valencia' orange were inoculated by pipetting 5 µl of the conidial suspension or sterile deionized water as negative controls onto the marked areas (25 mm$^2$) of labeled leaves. Those inoculated trees were immediately wrapped in plastic bags for 24 h to maintain the humidity required for conidial germination and appressorium formation. Three leaves were then collected every day for the first week, weekly thereafter up to 10 weeks, biweekly for the next 8 weeks, and then once every 4 weeks up to 26 weeks. The collected leaf samples were subjected to paraffin embedding and sectioning as described below to observe the leaf infection process by *P. citricarpa*. After inoculation for 15 months, the rest of inoculated leaves were collected from the
quarantine greenhouse, incubated in a 24°C incubator with a 12 h photoperiod, and subjected to wetting and drying cycles (30 min wetting and then drying) at room temperature three times a week for up to 5 months to stimulate the formation of fungal fruiting structures.

**Paraffin Embedding and Sectioning**

Collected leaf samples were fixed and embedded as described by Li and Rollins (2009) with some modifications. Briefly, 25 mm$^2$ of leaf tissue were cut from inoculated leaf areas and fixed in Trump’s fixative solution (0.1 M Sorensen’s phosphate buffer, pH 7.2, 4% paraformaldehyde, 1% glutaraldehyde, 100 mM sucrose, and 0.1 mM CaCl$_2$·2H$_2$O) at 4°C overnight or until use. Fixed leaf samples were then washed three times with rinse buffer (0.1 M Sorensen’s phosphate buffer, pH 7.2, 200 mM Sucrose, 0.1 mM CaCl$_2$·2H$_2$O) for 30 min each at room temperature. Subsequently, leaf samples were dehydrated by successive 1 h incubations in each of 30, 50, 70, 80, 95, and two times in 100% ethanol at room temperature, followed by successive 1 h incubations in ethanol:tert-butyl alcohol (TBA, Thermo-Fisher Scientific) at a 1:1 ratio, ethanol:TBA at a 1:3 ratio, and 100% TBA at room temperature. Dehydrated leaf samples were incubated successively in TBA:paraffin (Paraplast Plus tissue embedding medium; Thermo Fisher Scientific) at a 1:1 ratio and three times in 100% paraffin at 60°C overnight. Leaf samples were then cast in paraffin molds, sectioned at 5 to 10 μm in a Leica RM2155 rotatory microtome (Leica Biosystems Inc., Buffalo Grove, IL), and mounted on glass slides. Following the drying period on a 37°C hot plate overnight, sections were dewaxed by incubation twice in HistoClear solution (National Diagnostics, Atlanta, GA) and rehydrated by successive 5 min incubations in each of 100, 95, 80, and 70% ethanol at room temperature before the final wash in distilled deionized water for 3 min. Rehydrated sections were stained with 1% safranin O (in water) and 5% cotton blue (in lactophenol) at room temperature for 30 and 2 min, respectively.
Subsequently, those slides were dehydrated in a graded series of 30, 70, and 100% ethanol for 1 min each at room temperature, cleared by xylene twice for 1 min, mounted using Cytoseal mounting medium (Thermo-Fisher Scientific, and then examined under an Olympus BX41 microscope (Olympus) at 400× magnification for observation of early infection events including germination, appressorium formation, penetration, and colonization between the cuticle and the first epidermal layer. Images were recorded using a Motic digital camera driven by Motic Image Plus software (version 3.2; Motic, British Columbia, Canada).

Results

Expression of GFP in *P. citricarpa* Transformants

Following PEG transformation, four independent transformants were selected. Hyphae of all transformants exhibited green fluorescence after light activation, whereas, as expected, the wild type strain of *P. citricarpa* did not show any fluorescence under the same condition (Fig. 5-1). In addition, green fluorescence in these transformants was observed to diffuse in the cytoplasm but sometimes became attenuated probably due to the formation of dark pigments (melanin), particularly in older fungal tissue (data not shown). Furthermore, conventional PCR was to verify the integration of the *sgfp* gene into genomes of the transformants (Fig. 5-2). As seen in Fig. 5-2, an expected size band of approximately 3 kb was amplified from gDNA of these transformants using M13 forward (−20) and M13 reverse primers (Fig. 5-2, lanes 1 to 4), while it was not found using gDNA of the untransformed and wild type strains of *P. citricarpa* (Fig. 5-2, lanes 5 and 6, respectively).

Surprisingly, conidia of *P. citricarpa* transformants did not show any GFP expression although hyphae of the mother transformants exhibited bright green fluorescence (data not shown). We further tried to obtain regenerated colonies from germinated conidia of the mother transformants on 1/2PDA amended with 10 µg/ml hygromycin B. However, those regenerated
strains did not show any fluorescence and tested negative for the presence of the \textit{sgfp} gene using conventional PCR (Fig. 5-2).

\textbf{Leaf Infection Process by \textit{P. citricarpa}}

Conidia of the wild type strain \textit{P. citricarpa} Gc12 germinated and formed appressoria on the surface of inoculated leaves at 24 h post-inoculation (Fig. 5-3). On the surface of water-inoculated leaves, no conidia of \textit{P. citricarpa} or other fungi were observed after 24 h (Fig. 5-3). At 36 h post-inoculation, a peg-like structure (approximately 2 to 3 \(\mu\text{m}\) in diameter) penetrating the cuticle layer of the leaf was observed on sections of inoculated detached leaves (Fig. 5-4A). A similar peg-like structure was also observed on sections of inoculated attached leaves at 7 days post-inoculation (Fig. 5-4B), whereas the further progress of penetration and colonization by \textit{P. citricarpa} on inoculated leaf samples was not found from this point.

After the rest of inoculated leaves were collected and subjected to wetting and drying treatments, those leaf samples gradually turned brown and started decomposing as a sign of leaf senescence (Fig. 5-5). However, following the treatments for 5 months, no \textit{Phyllosticta}-like fruiting bodies were observed on the leaf surface under a dissecting microscope and on mounted slides under a compound microscope (data not shown), indicating that wetting and drying treatments did not stimulate the formation of \textit{Phyllosticta}-like fruiting bodies on those leaf samples.

\textbf{Discussion}

The long latent period after successful infection has been a major characteristic of \textit{P. citricarpa}-host interactions (Kiely, 1948; McOnie, 1967). It has been reported that \textit{P. citricarpa} can remain latent for up to 36 months prior to leaf abscission (Kiely, 1948; McOnie, 1967). However, how \textit{P. citricarpa} breaks the latency and what factors contribute to this turning point leading to the later inoculum production are not clear. Previously, McOnie (1967) not only
observed ascosporal germination and appressorium formation on the leaf surface but also found a peg-like structure penetrating the leaf cuticle layer. Beneath the peg-like structure, a ‘small knot’ was observed and considered to be the result of latent infection, although no further penetration from this knot at 96 h post-inoculation was recorded (McOnie 1967). In this study, our preliminary results showed that as early as 36 h post-inoculation, a peg-like structure underneath the appressorium of *P. citricarpa* was found to penetrate the leaf cuticle layer. With the attached leaf assays, a similar peg-like structure was also observed at 7 days post-inoculation. Although McOnie (1967) observed a small knot between the cuticle and the first epidermal layer, this structure was not found in our sectioned samples, which may be due to the display of sections resulting from the cutting angle during section preparation or the fact that ascospores and conidia of *P. citricarpa* may develop different structures during the latent period. As this study remains ongoing, we would definitely need more samples to confirm our findings.

Our GFP-expressing strains of *P. citricarpa* tested positive for the presence of the *sgfp* gene by conventional PCR and exhibited bright green fluorescence under a fluorescence microscope even after subculture for a year, indicating that GFP is functionally expressed under the control the *toxA* promoter in *P. citricarpa*. However, such fluorescence was not observed in their conidia (<1%). The differential expression of GFP between mycelia and conidia could be due to the fact that the GFP expression in *P. citricarpa* transformants is constitutive but insufficient or completely unfunctional in conidia, as was reported in *Gibberella pulicaris* by Salch and Beremand (1993). If this explanation was true, the discrepancy in GFP expression would possibly result from a poor ability of *P. citricarpa* to recognize the *toxA* promoter; the use of other promoter sequences such as that from the gpd (glyceraldehyde-3-phosphate dehydrogenase) gene of *A. nidulans* might circumvent this issue. However, even though we
recovered isolates from germinated conidia of the mother *P. citricarpa* transformants, those isolates did not show fluorescence and tested negative for the presence of the *sgfp* gene, implying that the *sgfp* gene was completely removed from the genome and therefore other factors may be involved in the differential GFP expression between mycelia and conidia, such as mitotic instability during sporulation/sexual reproduction. Mitotic instability has been found to be associated with the integration pattern and position on host chromosomes (Salch and Beremand 1993; Soares et al. 2005) and also been reported in other filamentous fungi such as *C. heterostrophus* (Keller et al. 1991), *F. graminearum* (Dickman and Partridge 1989), *Glomerella cingulata* f. sp *phaseoli* (Rodriguez and Yoder 1987), and *T. reesei* (Penttila et al. 1987). Salch and Beremand (1993) observed three different integration patterns in *G. pulicaris* transformants: a single copy, a partial copy, or multiple tandem copies of the vector at one or multiple positions per genome. *G. pulicaris* transformants with a single or partial copy of the vector were genotypically and phenotypically stable during sexual and asexual reproductions. In contrast, those transformants with multiple tandem copies of the integrated DNA in the genome showed a high propensity for mitotic instability likely through a progressive loss of multiple copies of the insert (Salch and Beremand 1993). To further test this hypothesis, our GFP-expressing strains could be successively cultured on non-selective 1/2PDA for several generations and then on 1/2PDA amended with hygromycin B at the previously-determined concentration. Mitotically stable strains would be able to grow on amended medium and maintain the GFP expression. Moreover, Southern blot analysis could be performed to determine the copy number of the integrated DNA in their genomes as well as the integration pattern to identify whether *P. citricarpa* transformants have multiple tandem copies of the vector.
Besides the current study, two other groups in Brazil thus far have successfully obtained GFP-expressing *P. citricarpa* strains using *Agrobacterium tumefaciens*-mediated transformation (Figueiredo et al. 2010; Rodrigues et al. 2013). The *P. citricarpa* transformants created by Figueiredo et al. (2010) showed GFP expression in mycelia and conidia, but whether germinating conidia and mycelia from them thereafter retained fluorescence is unknown. In addition, information with respect to the percentage of conidia actually expressing GFP is lacking. In another study, Rodrigues et al. (2013) only reported the presence of green fluorescence in mycelia of the GFP-expressing strains. Therefore, at this stage, we are not clear whether *P. citricarpa* conidia not capable of expressing GFP is a common result, and more transformants obtained using different vectors would be needed to solve this mystery.

Our preliminary results showed that a peg-like structure was observed from an appressorium of *P. citricarpa* at 7 days post-inoculation, but the further development such as penetration and colonization beyond the first epidermal cells was not evident at this stage. Our original experimental design indeed included leaf samples up to 26 weeks post-inoculation in hopes of observing the breaking of the latency and the further development. In addition, the conidial suspension of *P. citricarpa* was made in 0.1% agar solution to increase the stickiness of the solution to ensure the suspension would remain in marked areas on the leaf surface. Many leaf samples were damaged during paraffin embedding due to the exposure to the high temperature of 60°C, resulting in tearing of the thin agar layer containing germinated conidia from leaf samples. To circumvent this problem, low melting-temperature paraffin wax could be a good substitute for the high melting-temperature counterpart.

Alternate wetting and drying cycles have been found to be essential for stimulating inoculum production of *P. citricarpa* in decomposing leaf litter (Kotzé 1981; Reis et al. 2006;
However, no *Phylllosticta*-like fruiting bodies were observed on leaf samples after being subjected to alternate wetting and drying cycles for 5 months. These findings imply that *P. citricarpa* may remain quiescent and await favorable conditions for further development, similar to that reported by Truter (2010). It is also possible that a long-term low-humidity environment in the air-conditioned quarantine greenhouse where inoculated trees were maintained may adversely affect the survival of *P. citricarpa* on leaf. Although not direct evidence, this adverse effect of low humidity on fungal survival was also observed in twig inoculation assays in Chapter 3, in which relatively low amount of *P. citricarpa* gDNA was detected from inoculated twigs incubated in low-humidity environments, particularly at 43% RH. Thus, when repeating this experiment in the future, it may be worth increasing the humidity in the quarantine greenhouse to be more favorable for *P. citricarpa* development.

In conclusion, we successfully obtained the GFP-expressing strains of *P. citricarpa* using PEG transformation; however GFP expression was not present in conidia of those strains, which, as a result, hinders its application in leaf inoculation assays. Furthermore, we demonstrated the paraffin embedding and sectioning protocol as a useful tool to investigate the leaf infection process by *P. citricarpa*. Although thus far we have only successfully processed few samples, information acquired in this study such as the risk of sample damage from the exposure to the high temperature and the potential adverse effect on *P. citricarpa* survival on leaf due to the long-term low humidity environment could serve as a basis for improving the design of future studies.
Figure 5-1. A representative GFP-expressing strain of *Phyllosticta citricarpa* cultured in half-strength PDA (1/2PDA) containing 10 µg/ml of hygromycin B.
Figure 5-2. PCR confirmation of the integration of pCT74 into genomes of GFP-expressing strains of *Phyllosticta citricarpa* using M13 forward (−20) and M13 reverse primers. Lane M, GeneRuler 1kb plus DNA ladder; lane 1, Gc12GFP-1; lane 2, Gc12GFP-2; lane 3, Gc12GFP-3; lane 4, Gc12GFP-4; lane 5, Gc12GFP-5 (untransformed strain); lane 6, wild type.
Figure 5-3. Conidial germination and appressorium formation of *Phyllosticta citricarpa* on the surface of citrus leaves at 24 h post-inoculation. A, B, and C, representative *P. citricarpa*-inoculated leaves; D, water-inoculated leaf as a negative control.
Figure 5-4. Penetration of *Phyllosticta citricarpa* conidia into (A) detached leaves at 36 h post-inoculation and (B) attached leaves at 7 days post-inoculation.
Figure 5-5. Stimulation of the development of *Phyllosticta citricarpa* pycnidia on inoculated leaves after 15 months with alternative wetting and drying cycles. The regime of wetting and drying treatments was 3 to 4 h per treatment, three treatments per week for 5 months.
CHAPTER 6
FINAL CONCLUSIONS AND SUMMARY

In areas of the world where citrus black spot has become established, pseudothecia are produced in the leaf litter and ascospores are thought to be responsible for the majority of new fruit infections and spread within the grove. Given this epidemiology, how \textit{P. citricarpa} regulates its sexual reproduction and what factors are critical for this process are of both practical and fundamental value. Deciphering the makeup and structure of the \textit{MAT} locus is an important step in gaining this understanding. We first discovered that the Floridian isolate of \textit{P. citricarpa} (Gc12) characterized in this study only contained the \textit{MAT1-2} idiomorph. The presence of only one idiomorph in this isolate is consistent with the lack of self-fertility in culture and is supported by multiple other lines of evidence, including the extended sequences upstream and downstream of the \textit{MAT} locus, the significant sequence coverage, and the presence of either \textit{MAT1-1-1} or \textit{MAT1-2-1} genes in \textit{P. citricarpa} genomes. We also found that \textit{P. capitalensis} Gm33 contained a single \textit{MAT} locus consistent with a homothallic mating system and the previous observation that \textit{P. capitalensis} can produce pseudothecia and ascospores in single spore-derived cultures.

Our preliminary results on the screening of mating types in the Floridian population of \textit{P. citricarpa} indicates the presence of a single mating type in Florida, implying that the existence of ascosporic inoculum of \textit{P. citricarpa} is unlikely and, as a consequence, the disease spread would solely rely on the conidia.

\textit{D. citri} is a common twig-colonizing fungus in Florida and produces the primary inoculum (conidia) also on dead twigs. Given that both \textit{P. citricarpa} and \textit{D. citri} occupy the same niche on twigs for inoculum production, their interaction on twigs was studied to determine whether both species can co-exist to successfully sporulate on dead twigs and cause new infections. Our results showed that pycnidial and conidial production of \textit{P. citricarpa} on
autoclaved and irradiated twigs were not observed in the presence of *D. citri* even at an inoculum ratio of 0.75:0.25 (*P. citricarpa:* *D. citri*), indicating that *D. citri* is the dominant fungus in the presence of equal amounts or similar ratios of inocula of both species on twigs. Following the stimulation of conidial production on inoculated twigs by incubation in a moist chamber, *P. citricarpa* pycnidia and conidia were observed on only *P. citricarpa*-inoculated irradiated twigs previously containing no *P. citricarpa* pycnidia. These results suggest that *P. citricarpa* may undergo latency and await favorable conditions for further development on irradiated twigs.

The effect of RH on *P. citricarpa* development on twigs was studied. Results showed that *P. citricarpa* formed pycnidia and conidia on twigs incubated at 100 and 82% RH but not at 72 and 43% RH, indicating a possible threshold between 82 and 72% RH for pycnidial production of *P. citricarpa*. Following the stimulation via incubation in 100% RH, *P. citricarpa* began to form pycnidia and conidia on twigs previously incubated at 72% RH but not at 43% RH. The qPCR assays further revealed a consistent level of *P. citricarpa* gDNA on twigs previously incubated at 72% RH prior to stimulation and a great increase in gDNA after stimulation, providing more evidence showing the growth and development of *P. citricarpa* on those twigs. In contrast, *P. citricarpa* gDNA from twigs incubated previously at 43% RH decreased drastically at 7 wpi. Although the gDNA level increased after stimulation, the increase was not as high as those on twigs previously at 72% RH. These findings imply that a long-term incubation of *P. citricarpa* at a consistent low RH environment such as 43% RH may be highly detrimental to *P. citricarpa*.

The effects of citrus juices, concentration, pH, various carbon and nitrogen sources, and environmental conditions on conidial germination and appressorium formation of *P. citricarpa* were evaluated in vitro. All tested juices, especially 'Valencia' (> 85%, *P < 0.05*), favored
conidial germination and appressorium formation, whereas sterile water rarely stimulated germination (< 1%). Juice quality analysis using Pearson’s correlation test revealed a moderate relationship between the Brix/Acid ratio and germination ($r = 0.783$, $P < 0.05$). The 'Valencia' juice effect was concentration- and pH-dependent, and the maximum rate was reached in 1.5% juice with pH of 3.4. Most carbon, nitrogen, or complex sources did not favor germination or appressorium formation with exception of PDB, ammonium nitrate, and yeast extract. An incubation period of 18 to 24 h at 24°C was required for peak germination and appressorium formation. Further analysis of critical juice components using synthetic juice revealed sugars, salts, citric acid, and thiamine combined were most important for germination and AF (> 80%, $P > 0.05$).

Our preliminary results showed that a peg-like structure was observed from an appressorium of *P. citricarpa* at 36 h and 7 days post-inoculation, but the further development such as penetration and colonization beyond the first epidermal cells was not evident at this stage. No *Phyllosticta*-like fruiting bodies were observed on leaf samples after being subjected to alternate wetting and drying cycles for 5 months. These findings imply that *P. citricarpa* may remain quiescent and await favorable conditions for further development.

In conclusion, identification of the MAT loci of *P. citricarpa* and *P. capitalensis* provides a molecular basis for further studies such as designing mating type-specific primers to determine the mating type ratio in the Floridian population of *P. citricarpa*. Although *D. citri* seems to be dominant in the interaction with *P. citricarpa* on twigs, more data will be needed before drawing a conclusion. In addition, results of the germination assays of *P. citricarpa* renders a robust and convenient system for further applications such as screening for efficacious fungicides. Overall, findings from this research will provide useful information on conditions required for inoculum
production and the infection process by *P. citricarpa*, which, in turn, will help improve disease control programs.
Table A-1. Mating type (MAT) and PH-domain protein sequences of Dothideomycetes and outgroup species from this study, GenBank, or the Joint Genome Institute (JGI) for BLAST search, sequence alignment, and phylogenetic analysis.

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Table A-2. Sequence distances between the mating-type (MAT) locus and the PH-domain gene in genomes of selected Dothideomycetes.

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a Genomes of selected fungal species in Dothideomycetes are available in the Joint Genome Institute (JGI) databases, and MAT locus sequences of *P. citricarpa MAT1-1* idiomorph, *P. citricarpa MAT1-2* idiomorph, and *P. capitalensis* are available in the National Center for Biotechnology Information (NCBI) database (GenBank accession numbers AOTE01003969, KT708823, and KT708824, respectively).
b ?, the sequence distance cannot be determined because the MAT gene and the PH-domain gene are located in different assembled contigs.
Figure A-1. Alignment of the conserved amino acid regions of MAT1-1-1 (A) and MAT1-2-1 (B) from *Phyllosticta citricarpa*, *P. capitulata*, and other Dothideomycetes collected from GenBank and the Joint Genome Institute (JGI). The alignments were performed using ClustalW2 and colored based on the Clustal X color scheme using Jalview. The consensus sequence is listed below the alignment, and fungal families are indicated with vertical bars. Arrows indicate conserved intron positions. Arrowheads indicate intron positions specific to certain species. Fungal species used for alignment are listed in Table A-1.
Figure A-2. Maximum likelihood tree constructed using the concatenated conserved regions of MAT1-1-1 and MAT1-2-1 of *Phyllosticta citricarpa, P. capitalensis*, and other Dothideomycetes. The tree was constructed using MEGA version 6.0 with 1000 bootstrap replicates under the Jones-Thornton-Taylor (JTT) + Gamma (G) model and rooted with *Candida albicans*. Levels of branch support obtained with 1000 bootstrap replicates are indicated at nodes when they exceeded 70%. Fungal families are indicated with vertical bars. The scale bar represents 0.5 substitutions per site. The phylogenetic analysis reveals that *P. citricarpa* and *P. capitalensis* are grouped together with *Botryosphaeria dothidea* in the family Botryosphaerales.
Figure B-1. Map of GFP-expression vector, pCT74. M13 forward (−20) and M13 reverse primers used for verification of successful integration of the plasmid are labeled. The map is not to scale.
Figure B-2. Protoplasts of *Phyllosticta citricarpa*.
LIST OF REFERENCES


Spósito, M. B. 2004. Temporal and spatial dynamics of citrus black spot (Guignardia citricarpa) and quantification of the damages caused to citrus culture. Ph.D., University of São Paulo, Piracicaba, Brazil. pp. 124.


BIOGRAPHICAL SKETCH

Nan-Yi Wang was born in Tainan City, Taiwan. He attended National Chung-Ching University where he majored in plant pathology and graduated in 2001. His undergraduate research project was to develop a fermentation process to scale up the mass production of the biological control agent, *Streptomyces griseobrunneus* S3. Afterward, he was admitted for the Master of Science program in the Department of Plant Pathology at National Chung-Ching University in 2005. During his master’s program, he was involved in multiple research projects but mainly engaged in understanding the hypersensitive reaction on quinoa using a *Zucchini yellow mosaic virus* (ZYMV) vector with various point mutations in helper component-protease. In 2009, he joined Dr. Kuang-Ren Chung’s lab as a research assistant at the Citrus Research and Education Center (CREC), University of Florida where he worked on elucidating signal transduction pathways in *Alternaria alternata*.

With a plant pathology career in mind, he decided to pursue a Ph.D. degree in plant pathology at the University of Florida in 2011 and was fully funded as a graduate research assistant. Under the supervision of Dr. Megan M. Dewdney, he focused on the epidemiology of citrus black spot that is an emerging fungal disease in Florida caused by *Phyllosticta citricarpa*. His doctoral research includes identifying mating type genes that are the master gene regulators for fungal sexual reproduction using next-generation sequencing technology, determining the pivotal environmental conditions for inoculum production on citrus twigs, deciphering the critical components in citrus juice that are essential for conidial germination and appressorium formation, and investigating the leaf infection process using a paraffin embedding and sectioning technique and microscopy. In the future, he hopes to share his expertise with growers and pursue a career as a research scientist in plant pathology.