EVALUATION OF *ISARIA FUMOSOROSEA* FOR CONTROL OF THE ASIAN CITRUS PSYLLID, *DIAPHORINA CITRI* KUWAYAMA (HEMIPTERA: PSYLLIDAE)

By

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To my husband, Harry James and daughter, Lynn Marie Stauderman

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The Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), is a serious pest of citrus and the vector of citrus greening disease, also called huanglongbing (HLB), in Florida. Alternative low risk pesticides are needed given the risks of over using broad spectrum chemical pesticides and the need to conserve beneficial arthropods in citrus production. This study evaluates insect-specific (entomopathogenic) fungi for the control of *D. citri*. A bioassay protocol was developed to evaluate strains of the entomopathogenic fungus, *Isaria fumosorosea* Wize, against *D. citri*. Up to 100% of adult psyllids were killed at concentrations between 10^6 and 10^7 blastospores/ml after 12 days with derived LC_{50} values (at 7 days) of 1.37×10^5 (ARSEF 3581), 2.03×10^6 (Apopka–97), 1.36×10^5 (FE 9901), 1.47×10^7 blastospores/ml and 1.47×10^7 for a conidial formulation of Apopka–97. Average survival times were dosage dependent for all strains, i.e. between 10.2 days at 10^3 blastospores/ml and 3.1 days at 10^9 blastospores/ml. Rates of symptomatic fungal mycosis observed in dead psyllids were also concentration-dependent, with up to 100% sporulation observed at concentrations of 10^8 blastospores or higher but declined at lower concentrations. Based on the laboratory screening, the Apopka-97 strain (commercially available as a

bioinsecticide called PFR-97) was tested against *D. citri* infestations in citrus plants under greenhouse conditions. Half of the formulations included an emulsifiable vegetable oil at 2.5 percent vol/vol that was hypothesized to improve fungal efficacy, such as through improved deposition or germination on the insect cuticle. Fungal treatments at label rates reduced psyllid populations by approximately 50 percent over four weeks. The combination of PFR-97 and the emulsifiable oil did not increase ACP mortality compared with either agent alone. Subsequent greenhouse tests conducted under humid conditions were hampered by natural dissemination of the fungus to untreated psyllid populations, suggesting that the fungus is spread easily by wind or other factors. The insecticide imidacloprid applied as a drench was highly effective, killing 100% of psyllids within 3 weeks. This study demonstrated the potential of entomopathogenic fungi for environmentally safe control of *D. citri*, although it may not be so effective as chemical insecticides.

CHAPTER 1
INTRODUCTION

Literature Review

A serious disease currently affecting the Florida citrus industry is Huanglongbing or greening disease. A problematic aspect of the disease is that it is transmitted by an insect vector, the Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae). The objective of this chapter is to present an overview of the disease and its cause, of the vector with respect to its biology, host range and pest status, of the control and integrated pest management of *D. citri*, and of how the control and management has changed since HLB was discovered in Florida. Alternative low risk pesticides are needed both because of the risks of over using broad spectrum chemical pesticides and the need to conserve beneficial arthropods (parasitic wasps, predatory bugs, spiders, etc) in the groves.

Citrus Greening Disease (HLB)

Description

Huanglongbing (HLB) in Chinese refers to a ‘yellow shoot disease’ or ‘yellow dragon disease’ (da Graca and Korsten 2004; Zhao 1981). The earliest record of HLB was in Shinchiku, Taiwan in 1907 (Kuwayama 1908) and it has successfully invaded citrus producing areas throughout the world. Citrus fruit from infected trees do not produce color but remain green on the tree. Early symptoms of HLB include blotchy-mottled leaves with a yellowing of a single sector of the tree canopy (Fig. 1-1). By contrast, nutritional deficiencies occur symmetrically along the leaf veins. Later symptoms include twig and limb dieback, sparse, stunted leaves that point upward and premature fruit drop. Infected citrus trees may only live 5–8 years and produce irregular

shaped, bitter, unmarketable fruit (Bové 2006; Halbert and Manjunath 2004). According to the U.S. Department of Agriculture, HLB is a growing threat to citrus production in Texas, California and Florida (Santa Ana 2012; USDA 2011b).

There are at least three recognized forms of phloem-limited gram negative bacteria that are causal agents of HLB worldwide (Bové 2006). *Candidatus Liberibacteria asiaticus* is the most widespread and severest of the three. It thrives under low humidity in both cool and warm temperatures (heat-tolerant up to 35°C). Vectored by *D. citri*, HLB occurs throughout Asia, India and neighboring islands, Saudia Arabian peninsula, Brazil, Louisiana and Florida (Garnier and Bové 1993). The African form, *Candidatus Liberibacter africanus*, is a milder form of the disease that is restricted to south of the Sahara in Africa, is vectored by the African citrus psyllid*, Trioza erytreae* Del Guerico (Magomere *et al.* 2009; Van de Berg 1990), and is considered heat-sensitive (Bové 2006; Le Roux *et al*. 2006). More recently, in 2004, a third form was discovered in Brazil, *Candidatus Liberibacter americanus* (Coletta-Filho *et al*. 2004; Teixeira *et al*. 2004). This form is only found in Brazil where it is vectored by *D. citri*.

Causal Agent

The *Candidatus Liberibacter asiaticus* bacterium is either spherical or rod shaped, gram-negative and is found in phloem cells. These bacteria average 930 nm in length and 410 nm in width. According to Shokrollah *et al*. (2010), the cell walls are irregular with varying thickness. It is believed that the bacteria damages cell walls by penetrating and moving throughout the cells. The bacteria have not been cultured in media and, therefore, there is insufficient information on the movement of the pathogen in citrus plants (Shokrollah *et al*. 2010).

HLB is transferred to other plants primarily by piercing-sucking insects. Psyllids are the main vector of this disease. The disease is also known to be graft transmitted, transferred by humans in movement of host material, transmitted experimentally via dodder (Gottwald 2010), and possibly seedborne (Sullivan and Zink 2007).

Asian Citrus Psyllid

Description and Morphology

D. citri is endemic to Asia and known to have a wide host range within the plant family Rutaceae, specifically citrus and related species including orange jessamine, *Murraya paniculata* L. Jack (Halbert and Manjunath 2004; Swingle and Reece 1967). *D. citri* was first detected in Florida in 1998 (Knapp *et al*. 2006) and has since spread throughout the state (Childers and Rogers 2005; FDACS 2008; Qureshi and Stansly 2007b; Tsai *et al*. 2000). *D. citri* has also been found in Texas (French *et al*. 2001), Louisiana, Alabama, Georgia, Mississippi, South Carolina, California, Puerto Rico and Guam (Hummel and Ferrin, 2010; USDA 2008; USDA 2011b) and all of the islands of Hawaii (Conant *et al*. 2009). How it arrived in Florida and exactly where it came from is not known; however, discount garden centers and retail nurseries may have helped distribute psyllids and plants carrying the HLB pathogen in Florida (Manjunath *et al*. 2008).

Mead (1977) described *D. citri* adults as having mottled brown wings held “roof-like” over the body and as ranging in length from 2–4 mm. Nymphs are yellow in color. *D. citri* can be distinguished from six other similar species based upon the pattern of marks in the forewings (Halbert and Manjunath 2004). Eggs of *D. citri* are oval shaped and about 0.3 mm long; as they mature; they turn dark yellow to orange. The eggs hatch after 3–4 days and the psyllid develops through 5 instars. Nymphs continue to

mature for 12 to 14 days, although this period has been shown to vary with environmental conditions of temperature and humidity (Liu and Tsai 2000; McFarland and Hoy 2001). Some *D. citri* may survive moderate freeze events in citrus groves and adults may become cold acclimated during exposure to cooler temperatures during the winter (Hall *et al*. 2011). Both nymphs and adults produce white waxy secretions containing honeydew that accumulates on the leaf surfaces. Honeydew promotes the growth of sooty mold fungi, which is unsightly in ornamental settings and can reduce effective leaf area for photosynthesis (Browning *et al*. 2009).

Feeding and Reproduction

The two factors most important for *D. citri* feeding and reproduction are the presence of new citrus flush and warm temperatures. Ideal temperatures in the range from 20–30°C correspond directly with the abundance of psyllids in the field (Tsai *et al*. 2002). These environmental conditions are also correlated with shoot flush cycles in citrus. Extended temperatures above 32°C will decrease the female psyllid lifespan and egg production. Tsai *et al*. (2002) reported that Florida weather conditions decreased psyllid populations during mid-summer months compared to late spring and early fall. Additionally, they noted that orange jessamine, *Murraya paniculata* L. (an ornamental shrub), serves as an alternate host to the Asian citrus psyllid. The flushing pattern of the citrus relative is continuous in southern Florida and this enables psyllid densities to peak in May, August, and October through December (Tsai *et al*. 2002).

When young, tender unexpanded leaves are present, adult psyllid commonly aggregate on this new flush where they feed and mate. With piercing-sucking mouthparts, their feeding results in deformed, twisted leaves and reduced shoot lengths and possible transmission of HLB (Michaud 2004). Psyllid also siphon large amounts of

plant phloem and excrete the excess as honeydew and wax at the feeding site (Aubert 1987; Triplehorn and Johnson 2005). After mating, the female psyllid must feed on young flush in order to produce mature eggs. Gravid females eventually develop a yellowish to orange abdomen, but recent studies show that both sexes can reach reproductive maturity by 2–3 d post-eclosion (hatching) before this color change occurs (Wenninger and Hall 2007; Wenninger *et al*. 2009). Eggs are inserted into the leaf tissue inside the folds of the new flush of leaves. The life span ranges from 30–50 days depending upon humidity, temperatures and host plant (Liu and Tsai 2000; McFarland and Hoy 2001). Under optimum conditions more than 10 generations of psyllids can be produced per year (McFarland and Hoy 2001).

Although *D. citri* damages plants directly through its feeding activities, the most serious concern of *D. citri* in Florida, and world-wide, is its ability to vector the phloem-limited bacterium *Candidatus Liberibacter asiaticus* that causes huanglongbing (HLB), also known as citrus greening disease (Hung *et al*. 2004; Manjunath *et al*. 2008). HLB is transmitted by the probing action of psyllids during feeding. As little as 30 minutes of feeding has been known to transmit the bacterial pathogen (Bové 2006). Once the psyllid has acquired the bacteria, there is a latent period that varies from 7–25 days before it can transmit the pathogen, which is thought to occur through salivary secretions. Adult psyllid can live for 1–2 months and once they have acquired the HLB bacterium they carry it for life, transmitting it to additional citrus during feeding (Bové 2006).

Economic Impact

When *D. citri* first arrived in Florida, it was not considered by some to be a serious pest per se. Healthy mature citrus trees could withstand significant damage to young

growing shoots, although young plants may succumb during high populations of the psyllid (Michaud 2004). Additionally, *D. citri* was a pest that was easily controlled by the routine insecticidal sprays used by most growers. However, when HLB was detected in south Florida in 2005 on two homeowner trees (FDACS 2005) and spread to numerous locations across the state's citrus growing regions, the problem became more serious.

HLB has seriously impacted Florida's citrus economy (Hodges and Spreen 2012). Citrus acreage in the state has declined to its lowest level in years. Citrus acreage for the 2010–2011 season decreased 6% from the previous season to 541,000 acres, which is considerable reduction from the peak of 940,000 acres that existed during the 1990s (USDA 2011c). Elimination and removal of trees because of residential and industrial development, poor quality soils, nematodes, infections of citrus greening or citrus canker, and abandoned groves were some of the many factors that helped contribute to the gross loss of 19,918 acres (Mossler 2011; USDA 2011a).

According to the Citrus Research & Development Foundation Inc. (CRDF 2011), most recently funded projects mainly fall within three areas—reducing the bacteria inoculum within the tree, providing vector control strategies, and developing new rootstocks and scions for Florida citrus (CRDF 2011; Grosser *et al*. 2011; USDA 2011a).

Management of *D. citri* in Citrus Groves

Chemical Control

In an IPM program, a priority should be placed on natural mortality of the ACP wherever possible. Currently, an infected tree with HLB cannot be cured. Consequently controlling psyllid populations with petroleum oil and foliar and systemic insecticides is currently recommended to reduce the risk of HLB infection (Childers and Rogers 2005;

Rae *et al*. 1997; Rogers and Timmer 2007). In other areas around the world, the use of insecticides to control the psyllid vector has been the major emphasis of greening management strategies. For example, chemical control using pesticides was important in the battle against this pest in its native range in China where 10–13 sprays of pesticide yearly (Tolley 1990) were required to rehabilitate citrus production in an area affected with HLB.

According to the 2011 Florida Citrus Pest Management guide (Brlansky *et al.* 2011), the use of certified disease-free trees is essential to minimize further spread of the disease. Soil-applied systemic insecticides provide the best protection. Currently aldicarb and imidacloprid are two active ingredients that are available to effectively control psyllids on young non-bearing trees (Qureshi and Stansly 2008; Rogers *et al.* 2011; Sétamou *et al.* 2010). In addition, several other broad spectrum foliar insecticide applications are used in winter on bearing citrus trees. Recently, general guidelines were established for growers using air-blast type sprayers (Hoffman *et al.* 2010) in the control of ACP in citrus groves. The method of low-volume application is commonly used by growers in grove applications, which allows them to target the pest quickly, effectively and using minimal product. They were able to maximize efficiency in droplet size in order to penetrate the tree canopy. By applying pesticides very early in the spring, growers can help prevent the need for further sprays during bloom when pollinators are present (Childers and Rogers 2005; Rogers and Timmer 2007). Trivedi *et al.* have successfully isolated and characterized multiple beneficial bacterial strains from citrus roots by selecting bacteria antagonistic to *Candidatus Liberibacter asiaticus*. These strains have the potential to enhance plant growth and suppress HLB.

Alternatively, cultural methods, such as removing diseased trees and planting disease-free nursery stock, are also recommended as management strategies to limit the spread of HLB (Brlansky *et al*. 2007; Rogers and Timmer 2007). By applying foliar nutritional applications to infected trees soon after infection, HLB symptoms may be reduced and the tree may tolerate the effects of the disease, thereby increasing tree survival and yield. Nutrient supplementation may also induce plant resistance mechanisms that protect against the negative symptoms of infection. However, there is little evidence that these plant resistance mechanisms actually prevent diseases like HLB from occurring (Spann *et al*. 2010).

Unfortunately, several problems with excessive reliance on pesticide programs have recently been documented. Recent studies have shown that *D. citri* populations have developed resistance to several insecticides (Tiwari *et al*. 2011). Also, insecticides may be toxic to parasitoids released in citrus groves to help control *D. citri* (Hall and Nguyen 2010). It is known that naturally occurring predators and parasitoids play a vital role in regulating populations of *D. citri* and that their elimination through reckless pesticide use could increase pest pressure and enhance the spread of HLB disease (Qureshi *et al.* 2009). Therefore, integrated control programs based on conservation of natural enemies of *D. citri* through judicious use of insecticides and releases of new parasitoids are needed for sustainable management of pest and disease.

Integrated Crop Management

The classic formula for the onset of a disease is generally characterized as a triangle. The factors include a conducive environment, the presence of the pathogen and a susceptible host. In the case of HLB, however, a vector serves as a fourth element for a disease epidemic. The objective of managing psyllid populations is to

manage their transmission potential in commercial citrus groves. Therefore, the best approach to optimize a sustainable program to reduce the psyllid populations is through the use of an integrated pest management (IPM) approach that relies on a variety of approaches. Reduced conventional pesticide application results in lowering production costs for growers, benefits to the environment, and reduced potential health hazards of pesticide exposure to farm workers and, if no conventional materials are used, the possibility of organic based certification. Alternative strategies will be especially important in organic production practices. Today, soil and foliar applied insecticides currently arrest ACP. This is not necessarily a long-term sustainable strategy for crop management. Other alternative bio-friendly measures should be pursued as part of an overall IPM approach (NRC 2010). For example, biological control using released predators and parasitic wasps is an alternative to pesticides during the year when lower population levels are present (Stansly and Qureshi 2008; Qureshi and Stansly 2007a; 2007b). The use of pesticides that are safe and compatible with natural control agents in the citrus groves is also important. The use of such products can provide resistance management tools for existing pesticides that are used by growers. At this time, there does not appear to be a strategy that can match the insecticide approach. However, if sustainable IPM programs are introduced, they may offer the possibility of long term psyllid management with sustainable benefits (NRC 2010).

Entomopathogenic Fungi for Control of *D. citri*

Insect-killing (entomopathogenic) fungi, natural pathogens of many insects, play an important role in the natural regulation of insect populations (Goettel *et al*. 2005). Because they are safe to people, birds, fish and the majority of non-target arthropods (Goettel *et al*. 1990), entomopathogenic fungi can be used in IPM strategies for some

insect pests (Goettel *et al*. 2005). The ability of their spores or conidia to pierce or puncture the host cuticle directly (Fig. 1-2) makes them an attractive biological control agent for phloem-feeding insects (i.e., Asian citrus psyllid) because the insect does not need to consume the fungus; surface inoculation is sufficient for effective infection (Avery *et al.* 2009, Hajek and St. Leger 1994). Entomopathogenic fungi are especially important natural and artificial control agents for aphids and whiteflies under warm and humid conditions (Cabanillas and Jones 2009; Lacey *et al*. 2008; Latgé and Paperiok 1988; Steinkraus 2007).

There has been relatively little research about managing psyllids using entomopathogenic fungi. Using a detached leaf-bioassay, Puterka *et al.* (1994) found that the nymphs of pear psyllid *Cacopsylla pyricola*, were susceptible to strains of *Beauveria bassiana* (Balsamo) Vuillemin, *Metarhizium anisopliae* (Metschnikoff) Sorokin, *M. flavoviride* (Gams & Rozsypal), *Isaria fumosorosea* (=*Paecilomyces fumosoroseus*) Wize (Hypocreales: Cordycipitaceae) and *Lecanicillium (=Verticillium*) *lecanii* (Zimmerman) Viegas. Spray solutions containing 5.4×10^{13} conidiospores/ha killed up to 37% of pear psyllid nymphs in pear orchards in West Virginia (Puterka 1999; Puterka *et al*. 1994). Recently, another study reported that different strains of fungi were pathogenic against the potato psyllid, *Bactericera cockerelli* (Hemiptera: Triozidae) (Lacey *et al.* 2009). Laboratory studies showed that *I. fumosorosea* (strain Apopka PFR-97) and *M. anisopliae* (strain F 52) killed 95–99% of adults in 2–3 days and 91–99% of nymphs 4 days after application (Lacey *et al*. 2009).

Isaria fumosorosea shows particular promise for use against *D. citri* in citrus groves in Florida. Strains of *I. fumosorosea* are highly active against whiteflies and

have been sold as a biological pesticide for this purpose in Europe (Faria and Wraight 2001; 2007). Also, a strain of *I. fumosorosea* was found naturally infecting an adult Asian citrus psyllid collected from the underside of foliage on orange trees in Polk County, Florida so this pathogen may be considered native (Meyer *et al*. 2008). In addition, recent studies show that *I. fumosorosea* does not normally infect natural biological control agents of psyllids and thus would most likely be suitable in citrus IPM programs (Avery *et al*. 2008; 2009).

Figure 1-1. Symptoms of HLB in citrus foliage (upper panel) and fruit, and the vector *D. citri* (lower right). Photographs courtesy of Tim R. Gottwald and Steve M. Garnsey, USDA, ARS, U.S. Horticultural Research Laboratory, 2120 Camden Road, Orlando, FL 32803. Other members of the Rutaceae can serve as alternate hosts for HLB.

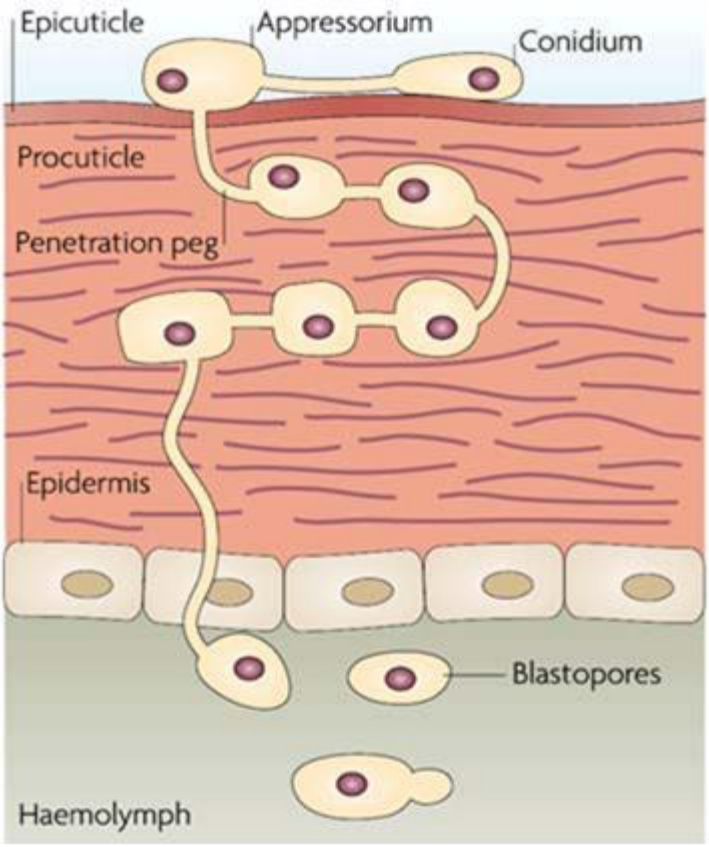

Figure 1-2. Schematic showing how entomopathogenic fungi infect their insect hosts through their cuticles. Source: Nature Reviews Microbiology 5: 377–383 (May 2007).

CHAPTER 2
MATERIALS AND METHODS

All experiments were conducted at the University of Florida Mid-Florida Research and Education Center (MREC) in Apopka. The potential of the entomopathogenic fungus, *Isaria fumosorosea,* as an integrated crop management tool for managing *D. citri* was investigated. In the first phase of the project (Objective 1), laboratory experiments were used to determine the pathogenicity and virulence of *I. fumosorosea* strains applied to *D. citri* under optimum environmental conditions for the fungus. A bioassay system using a mechanized spray tower and detached citrus leaf bioassay was used for this screening procedure. In the second phase (Objective 2), greenhouse tests assessed the psyllid mortality on orange jessamine plants that were sprayed with aqueous suspensions of *I. fumosorosea*. An additional aim of the greenhouse study was to investigate the value of incorporating various spray adjuvants along with fungal suspensions. Most fungal spores, including *I. fumosorosea* (Kim *et al*. 2011), are lipophilic. Formulation with certain types of oils can dramatically improve deposition of the entomopathogenic fungi on the insect cuticle as well as its germination and rain fastness; hence, formulation will significantly improve its effectiveness in the field (Inglis *et al*. 2002).

Two hypotheses were tested: (1) strains of *I. fumosorosea* will have different pathogenicities and virulence to *D. citri* and (2) the use of oils as formulating ingredients will improve the effectiveness of *I. fumosorosea* spores applied as a biological pesticide to control *D. citri* in potted orange jessamine and hybrid Benton citrange (*Citrus sinensis x Poncirus trifoliata*). An insecticidal standard was also included in initial tests for

comparison purposes. The insecticidal standard was confirmed to be highly effective and was dropped in the last test.

Objective 1. Determine the Pathogenicity and Virulence of *I. fumosorosea* Strains to *D. citri*

Fungal Isolates

Five treatments (listed below) were screened: One control plus four commercial strains or formulations of *Isaria fumosorosea* were assayed to test the pathogenicity and virulence of each against *D. citri* under optimum conditions for the fungus; treatment 5 was a control. All of the commercial strains were originally isolated from whiteflies, *Bemisia* and/or mealybugs, *Phenacocus solani*. Each treatment was a dose that consisted of six leaf discs.

1. Control: sterile water + Tween 80® (0.025% vol/vol) (Fisher Scientific, Fairlawn, NJ).
2. *Isaria fumosorosea*: Apopka-97 (PFR-97 20% WDG, Certis, USA, Colombia, MD)—a water-dispersible granule blastospore formulation + Tween 80® (0.025% vol/vol).
3. *I. fumosorosea*: Apopka-97 (Certis USA)—a conidial experimental formulation + Tween 80® (0.025% vol/vol).
4. *I. fumosorosea*: ARSEF 3581, (USDA-ARS, NCAUR, Peoria, IL)—a blastospore experimental formulation + Tween 80® (0.025% vol/vol).
5. *I. fumosorosea*: FE 9901, produced as NoFly® in Europe, (Natural Industries, Inc., Houston, TX)—a blastospore formulation + Tween 80® (0.025% vol/vol).

Viability tests were performed on each strain at the beginning of the study by streaking 0.5 ml of a suspension containing 10^5 spores per ml onto potato dextrose agar (4 replicate Petri-dishes) and counting germinated spores that produced a clear germ tube after 19 hours at 25°C. Viability rates were good for ARSEF 3581 (≥ 99%), Apopka-97 blastospore formulation (79.4%) and FE 9901 (85%), but was low in the conidial formulation of Apopka-97 (24.8%).

An auto-load Potter Precision Spray Tower® (Burkard Scientific Ltd., Uxbridge, Middx, UK) was used to apply controlled dosages of fungal spores to leaf discs. Fungal suspensions were prepared in sterile water containing 0.025% vol/vol Tween 80®. Stock spore suspensions were obtained by concentrating extracts from fungal materials. To achieve the required concentrations, 2.5 g of fungal materials were suspended in 20 ml water, vortexed for 1 minute, sonicated for 2 minutes to help dislodge spores from the substrate (model FS20, Vollrath, Sheboygan, WI), and then filtered through 2 layers of cheese cloth to remove larger inert materials. The remaining suspensions (approximately 6 ml) were vortexed a second time and diluted through serial dilutions to approximately10^6 spores/ml in sterile water containing 0.05% vol/vol Tween 80®. Counting was accomplished using an improved Neubauer hemacytometer (Hausser Scientific, Horsham, PA) and compound microscope at × 100 magnification. Based on hemacytometer counts, test solutions containing 10^3, 10^4, 10^5, 10^6, 10^7, 10^8 and 10^9 spores/ml were created by serial dilutions in sterile water + 0.025% vol/vol Tween 80®. Controls contained sterile water and Tween 80® only. The highest concentration of the 9901 strain was not used because the formulation clogged the spray nozzle.

‘*Marsh*’ grapefruit was chosen to be used in the experiment because the leaves had a large surface area and a large mid-vein to allow psyllid feeding and the leaves were known to be free from pesticide residue. The original certified clean psyllid colony that was supplied by the USDA field station in Ft. Pierce was maintained in an isolated greenhouse on grapefruit. Two-cm-diameter leaf discs were removed with a sterilized corkborer from 1-week-old leaves of young grapefruit trees *cv. Marsh* grown in an isolated greenhouse. Leaf discs were placed on saturated cotton wool (to prevent them from drying) in a 9-cm-diameter Petri dish and placed at the bottom of the spray tower. A five-ml sample of each fungal suspension was shaken and loaded at the top of the spray towers and samples were sprayed at 15 psi starting with lowest concentrations to minimize the risk of contamination. Control discs were sprayed first.

Each sprayed leaf disc was allowed to surface-dry before placing it into a 1-oz plastic cup containing 10 ml of water agar (1.5% w/v, Difco®, Detroit, MI) and capping it with a tightly fitting lid. The water agar prevented the leaf discs from desiccation throughout the test. Five adult psyllids, from a colony maintained in a greenhouse in isolation, were aspirated into a clean individual vial then transferred to one cup. The cups were placed in an incubator set to 26 ± 1°C, 80% relative humidity, and 16L: 8D photoperiod. Psyllid mortality and mycosis, hyphal growth and sporulation were recorded daily for 12 consecutive days.

Statistical Analysis

Six replicate leaf discs per fungal concentration were in each bioassay (hence, 30 psyllids per treatment) and each bioassay was conducted on at least 3 separate occasions for each fungal strain used. Response curves showing the proportions of

healthy versus dead and infected psyllids in relation to dose were subjected to probit regression analysis to determine the lethal concentration to kill 50% of the population. In addition, comparisons of the average survival time (days) of infected and control psyllids using Kaplan-Meier survival estimates were used as a measure of virulence. Differences in mean survival times were tested for significance using a log rank test (SAS 2004) with SPSS statistical software.

Objective 2. Assessment of *D. citri* Mortality Using *I. fumosorosea* with and without Emulsifiable Oils in Greenhouse Tests.

Bioassay Procedure

The original psyllid populations at MREC were established on a 'Marsh' grapefruit tree inside a secure cage that served as the mother colony for the bioassays. Meanwhile, clean *Murraya* plants were produced from seed in the greenhouse and allowed to grow to the size of 60 cm in height and were then transplanted into 3.8 L pots with sterile potting soil mix containing 55% peat, pine bark, perlite and vermiculite (2B, Fafard, Agawam, MA). Plants were fertilized with slow-release citrus fertilizer (12-5-8 Vigoro®, The Scotts Company LLC, Marysville, OH) and maintained in greenhouse cages inside of which air temperatures ranged from 16.1–41.4°C (average 25.2°C) and 15–100% r.h. (average 77.7%). These served as host plants for the 2010 greenhouse trial. Two weeks prior to testing, the plants were pruned to encourage new shoot flushes.

At the start of the first test conducted in the fall of 2010, 36 orange jessamine plants were placed individually in 60 cm square cages (PVC frame with fitted nylon mesh cover) and each plant was infested with 20 adult psyllids aspirated from the colony. Examination of psyllids showed a sex ratio of 23:77 male: female (N=73). The

insects were left for 10 days in the greenhouse allowing them to mate and oviposit on the plants and for nymphs to emerge. There were six treatments:

1. Control: distilled water and Tween 80® (0.025% vol/vol)
2. Imidacloprid (3 ml product/plant) (Merit 2F®, Bayer Crop Science, Research Triangle Park, NC).
3. Emulsifiable vegetable oil (2.5% vol/vol) (Addit®, Koppert Biological Systems, AD Berkel and Rodenrijs, The Netherlands) + 0.025% vol/vol Tween 80®.
4. *Isaria fumosorosea* (2.1 g product/L) (PFR-97 blastospore strain) + 0.025% vol/vol Tween 80®.
5. *Isaria fumosorosea* (2.1 g product /L) (PFR-97 blastospore strain) + 2.5% vol/vol Addit® + 0.025% vol/vol Tween 80®.
6. Highly refined paraffinic oil (2% vol/vol) (SuffOil-X®, BioWorks, Victor, NY) + 0.025% vol/vol Tween 80®.

The PFR-97 was weighed (2.1 g/L) and mixed in approximately 100 ml distilled water for at least 30 minutes prior to use. The final rate applied was equivalent to 8.28 mls product/3.8 L (upper label rate). A standard viability test indicated that spores suspended in water germinated at a rate of 82% when cultured on PDA media for 20 hours at 25°C. However, the impact of emulsifiable oils on spore germination was not specifically assessed in this study. Also, since products were applied at label rates (weight), actual spore concentration per ml was not counted in greenhouse tests (see discussion). Emulsifiable oil treatments were applied with and without *Isaria. '*Addit®*'* was used since it is recommended for use with another entomopathogenic fungus, 'Mycotal,' in Europe for many years and was expected to have good compatibility. SuffOil-X® was applied within label rate (labeled rate = 1–2% vol/vol). SuffOil-X® is labeled for citrus in the U.S. for control of whiteflies, scales, mites and mold. Imidacloprid (Merit 2F®) was used as an insecticide standard. It was applied as a soil

drench (3 ml product per plant diluted in 75 ml water) using a watering can. Distilled water served as the untreated control. Tween 80® was added to all foliar treatments as a surfactant.

Foliage was sprayed to 'run off' for 10 seconds using a 3.7-Liter hand held pressurized sprayer (Flow-master® 1401WMX, Root-Lowell Manufacturing Co., Lowell, MI). The sprayer output was calibrated to 3.88 ml/s. Each plant received 38 ml of inoculate (not all went on the plant). All foliar treatments were reapplied after 7 days. There were 6 replicate plants in each treatment group arranged in a completely randomized design inside a single greenhouse bay. The treatments were applied late afternoon/early evening to take advantage of higher relative humidity at that time of day. An overhead misting system was operated for 20 seconds after all treatments were applied and was shown, using dataloggers inside cages (HOBO U10, Onset Computer Corp., Cape Cod, MA), to maintain > 95% r.h. in the greenhouse for 8 hours. The mister was used to elevate the ambient air humidity and to encourage fungal spores to germinate, but it did not significantly wet the leaves of the plants inside the cages.

In the first experiment, the psyllid populations on the *Murraya* plants were counted initially as a baseline prior to any spray applications and then every seventh day for four consecutive weeks. The total number of psyllids (adults and nymphs) was counted from a minimum of three shoot terminals per plant. In the fourth week, the plants were destructively sampled and a final count was taken. Psyllids showing signs of fungal sporulation were collected, transferred to PDA medium under sterile conditions, and monitored for fungal outgrowths. After 2 weeks incubation, the fungal colony was cultured and inoculated onto new psyllids using a Potter spray tower to confirm Koch's

postulates. Temperatures throughout the 2010 greenhouse tests ranged from 16.1–41.5°C (mean 25.2°C) with a relative humidity between 15–100% r.h. (mean 77.7%).

The greenhouse experiment was repeated in summer and fall of 2011, however, Benton citrange, [*Citrus sinensis* (L.) Osb. x *Poncirus trifoliate* (L.) Raf] plants were used instead of Marsh grapefruit. This change in host plants was due to an inability to acquire Marsh grapefruit plants that had not been treated with imidacloprid. The summer treatments were:

1. Control: distilled water + Tween 80®(0.025% vol/vol)
2. Merit 2F® (3 ml product/plant)
3. Addit ® (2.5% vol/vol) + Tween 80® (0.025% vol/vol)
4. PFR-97 (2.1 g product/L) + Tween 80® (0.025% vol/vol)
5. PFR-97 (2.1 g product/L) + Addit® (2.5% vol/vol) + Tween 80® (0.025% vol/vol)
6. SuffOil-X® (2% vol/vol) + Tween 80® (0.025% vol/vol)

As additional modifications, in the fall 2011 greenhouse test there were 5 replicate plants per treatment and slightly different oil-combinations were tested. Imidacloprid (Merit 2F®) was not included since its high efficacy was clearly shown in the 2010 and summer 2011 greenhouse tests. Orocit® (Oro Agri Inc., Dallas, TX), an adjuvant containing alcohol ethoxylate, was evaluated to determine if it would improve fungal pathogenicity of *Isaria*. Orocit® is currently labeled for citrus as an adjuvant to improve efficacy of miticides, insecticides, fungicides and herbicides. SuffOil-X ® was applied at 1% v/v to determine if it was also effective at the lower label rate. The fall 2011 treatments were:

1. Control: distilled water + Tween 80® (0.025% vol/vol)

2. Orocit® (0.25% vol/vol) + Tween 80® (0.025% vol/vol)
3. SuffOil-X® (1% vol/vol) + Tween 80® (0.025% vol/vol)
4. PFR-97 (2.1 g product/L) + Tween 80® (0.025% vol/vol)
5. PFR-97 (2.1 g product/L) + Orocit® (0.25% vol/vol) + Tween 80® (0.025% vol/vol)
6. PFR-97 (2.1 g product/L) + SuffOil-X® (1% vol/vol) + Tween 80® (0.025% vol/vol)

The germination rate of the fungal spore used in these tests was determined to be 66–77% after 20 hours at 26°C on PDA plates. This germination was assessed prior to mixing with the various oil additives.

Temperatures throughout the summer 2011 greenhouse tests ranged from 22.2–39.7°C (mean 28.8°C) with a relative humidity between 42–100% (mean 83.0%). Temperatures throughout the fall 2011 greenhouse tests were slightly cooler and less humid, and ranged from 18.5–40.9°C (mean 25.0°C) with a relative humidity between 15–100% (mean 69.1%).

Statistical Analysis

The numbers of infested terminals and live psyllid (adults and nymphs) were compared between fungus and control and chemical treatments through analysis of variance (ANOVA) in a randomized design following log (n+1) transformation. Where appropriate, means were further compared with Fishers protected LSD tests at $P < 0.05$.

CHAPTER 3
RESULTS

Stages of Development of *I. fumosorosea* In Vitro and in *D. citri*

Blastospores from *I. fumosorosea* rapidly developed germ tubes on culture media (Fig. 3-1A, B) and subsequently sporulated (Fig. 3-1C and D). Symptoms of inoculated psyllids included twitching of legs and antennae 1–2 days before death. Immediately following death, infected psyllids had fungal hyphae emerging from the tarsi and intersegmental regions of the legs (Fig. 3-1E). Within 24–48 hours post mortem, significant mycelial growth developed on the dead insect (Fig. 3-1F), followed by development of phailides (Fig. 3-1G) and conidiogenesis (Fig. 3-1H).

Survival and Mycosis of *D. citri* in Laboratory Bioassays

Adult psyllids died at different rates over 12 days (Fig. 3-2). By day 12 some of the control psyllids (approximately 30–45%) had also died, probably as a result of the bioassay conditions and natural age-related mortality. However, the bioassay approach was still successful since, when compared with controls, survival was significantly reduced following exposure to all strains of *I. fumosorosea* with a clear concentration-response in all cases. Overall, 100% of psyllids died within 12 days at the higher concentrations, while generally > 80% of psyllids died at the lower concentrations. Average survival times calculated from the data showed differences between the strains, notably the fastest mortality was observed for the ARSEF 3581, followed by FE 9901 and the Apopka-97 blastospore formulation (Table 3-1). Probit analysis conducted on the fungus concentration-response data showed that the lethal dose required to kill 50% of the psyllids after 7 days was in the range of $1–2 \times 10^5$ blastospores/ml for ARSEF 3581 and FE 9901, and significantly higher—2×10^6 blastospores/ml—for

Apopka-97 (Table 3-2). A higher value again (based on non-overlapping confidence intervals) was obtained for the conidial formulation of Apopka-97. A 7-day exposure period was selected since control mortality was still relatively low at this time (< 20%), hence results would reflect the effect of the fungal exposure.

The proportion of psyllids expressing symptomatic response to fungus was also different according to the fungus strain and concentration applied to the leaf discs (Fig. 3-3). In general, up to 100% of psyllids sporulated at concentrations of 10^8 or higher but declined at lower concentrations. This decline was most apparent in the conidial formulation of Apopka-97 strain where < 30% psyllids became symptomatic at concentrations of 10^5 conidia/ml or less.

Evaluation of Foliar Sprays of *I. fumosorosea* and Emulsifiable Oils against *D. citri* Infestations on Citrus under Greenhouse Conditions

In the fall 2010 test, the induced flush ensured oviposition and large numbers of *D. citri* nymphs were present when treatments were first applied, and 2nd generation (F1) adults were present by week 2 (Table 3-3). All treatments significantly reduced these F1 ACP adults compared to the control treatment, by weeks 2 and 3. Merit was the most effective treatment followed by SuffOil-X and PFR-97, Addit and PFR-97+Addit (Table 3-3). The combination of PFR-97 and the emulsifiable oil did not increase ACP mortality compared with either agent alone. Only Merit and SuffOil-X significantly reduced the number of infested terminals (on enclosed plants) from week 2–4. In the destructive count, Merit 2F was the most effective with 99.9% reduction with respect to the untreated control (UTC), followed by SuffOil-X (85.6% reduction), Addit (56% reduction), PFR-97 (52.2% reduction), PFR-97+Addit (49.8% reduction) (Table 3-6). In the Koch's postulate test, slightly increased mortality was observed from adult ACP exposed to

fungal suspensions compared with controls after 7 days (i.e. 26.7% ± 6.7 versus 9.6% ± 5.7), however this difference was not significant ($F_{1,10} = 2.35$, $P = 0.16$). An average of 20% of psyllids that died following exposure to fungal suspensions and incubated in the laboratory produced outgrowth consistent with *I. fumosorosea* symptoms confirming that *I. fumosorosea* was present, although the mortality rate was lower than expected.

In the 2011 tests, contamination by *Cladosporium* sp. was observed in all treatments (Fig. 3-4). In the summer test, while all treatments reduced the number of ACP nymphs by week 1 with Merit being the most effective, relatively few F1 adults were subsequently observed in control cages (Table 3-4). Observations showed widespread growth of *Cladosporium* among all treatments, which compromised the ability to determine treatment effects. A similar observation was found in the fall 2011 test (Table 3-5), where treatments significantly reduced live nymph counts on week 1 and 2, but numbers of F1 adults remained low. Overall, treatments containing oils alone or in combination with PRF were most effective in the fall 2011 test.

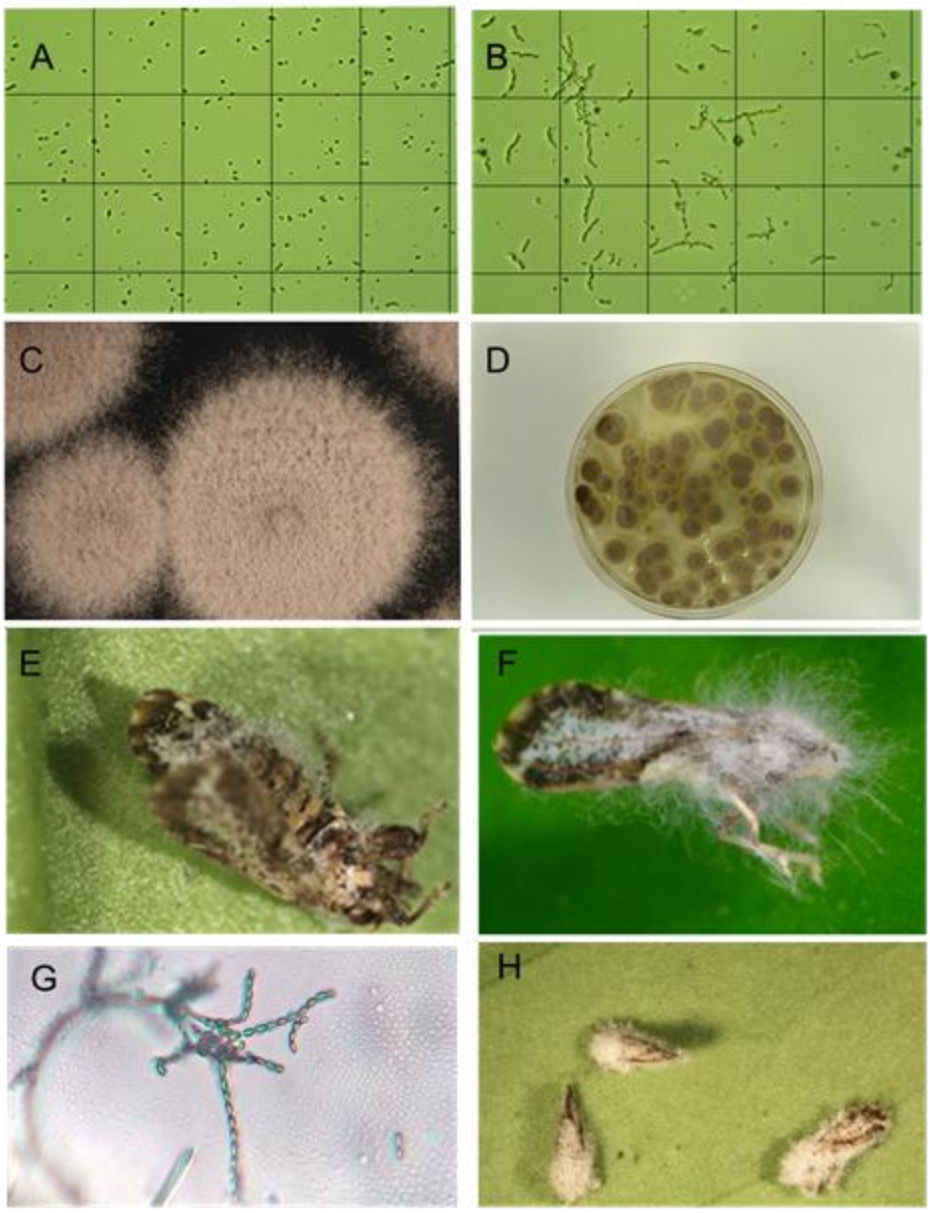

Figure 3-1. Photographs showing stages of development of *I. fumosorosea* in *D. citri*, (a) Non-germinated blastospores × 100 magnification, (b) blastospore germination on PDA media × 100 magnification, (c) and (d) colony forming units on PDA, (e) initial and (f) late mycelial growth protruding from dead insect × 40 magnification, (g) chains of conidiophores developed on phailides, note whorled branching of hyphae with conidia × 400 magnification, (h) sporulating insect cadavers × 40 magnification. Photo credits (F and G) Pasco Avery.

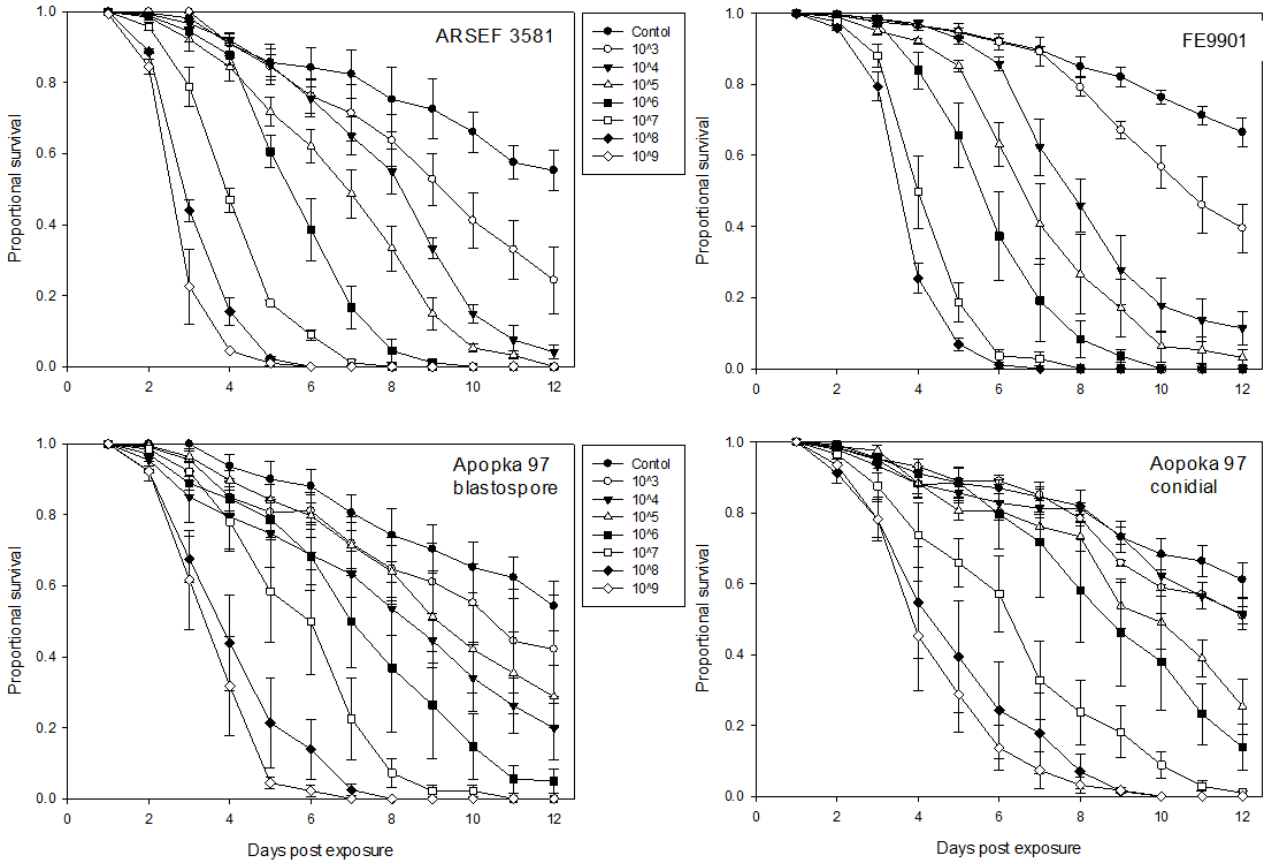

Figure 3-2. Proportional survival of adult *D. citri* following exposure to citrus leaves treated with different formulations of *Isaria fumosorosea* at concentrations between 10^3 and 10^9 spores per ml. Data are mean ± SEM of 3 tests (40 psyllids per test).

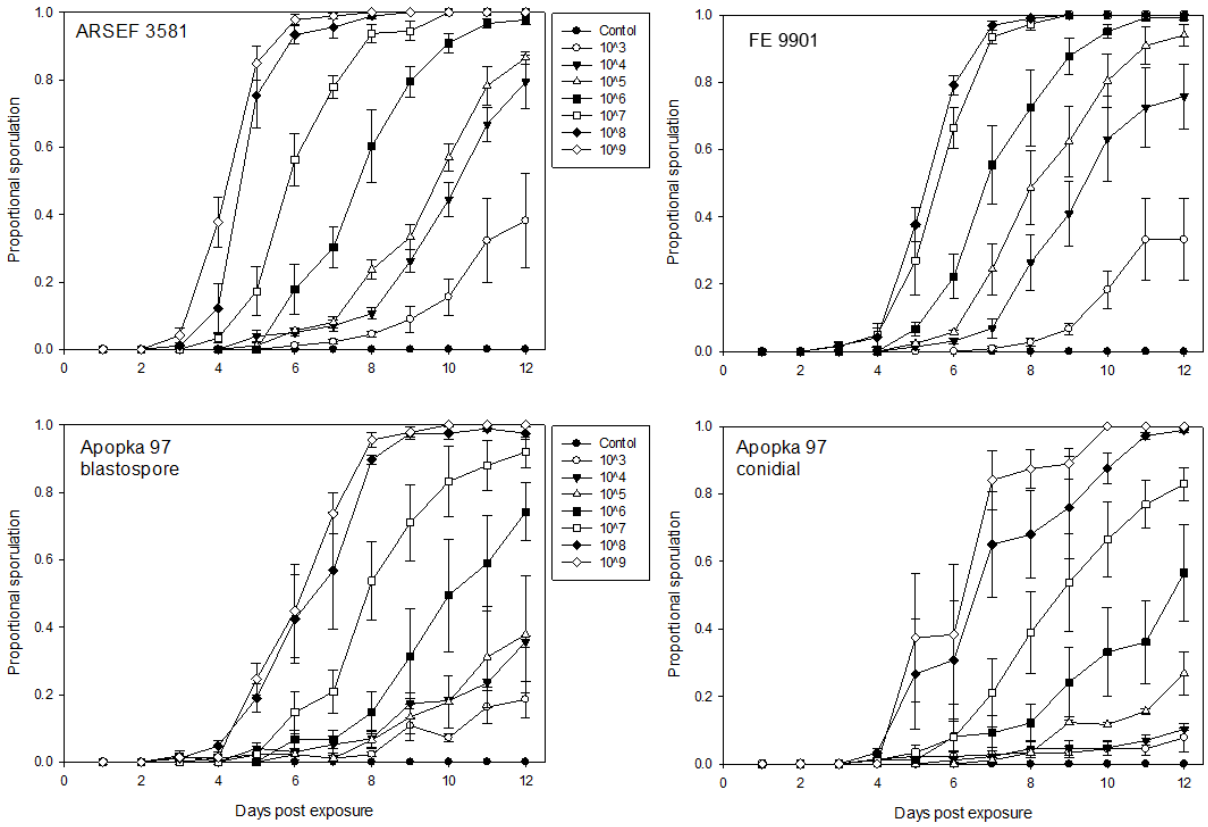

Figure 3-3. Proportion mycosis (sporulation) of adult *D. citri* following exposure to Marsh grapefruit leaves treated with different formulations of *Isaria fumosorosea* at concentrations between 10^3 and 10^9 spores per ml. Data are mean ± SEM of 3 tests (40 psyllids per test).

Figure 3-4. *Cladosporium* sp. contamination observed on Benton citrange in greenhouse cages.

Table 3-1. Average survival times (days) of adult *D. citri* exposed to 4 strains/formulations of *Isaria fumosorosea* at concentrations between 10^3 and 10^9 spores per ml.

Strain	Concentration (spores/ml)							
	0	10^3	10^4	10^5	10^6	10^7	10^8	10^9
ARSEF 3581	10.1b	9.1a	8.2a	7.1b	6.0c	4.5c	3.5d	3.1c
Apopka-97 blastospore	10.2b	9.4a	8.3a	9.1a	7.5b	6.1b	4.4b	3.9b
FE 9901	10.9a	10.2a	8.4a	7.3b	6.1c	4.6c	4.1c	NA
Apopka-97 conidial	10.3ab	10.1a	10.0a	9.4a	8.9a	6.7a	5.1a	4.7a

Means based on 3 tests (30 psyllids per test). Estimates determined by Kaplan Meier Survival analysis, with data for survivors censored at day 12. Letters in columns indicate significant differences ($P < 0.05$) between strains according to a log-rank (Mantel-Cox) test.

Table 3-2. Estimates of the median lethal concentration (spores/ml) of three entomopathogenic fungi applied against adult *D. citri* on Marsh grapefruit leaf disc assays at 7 days post inoculation.

Strain	LC_{50}	95% CL	Slope ± SEM	χ^2
ARSEF 3581	1.37×10^5	5.1×10^4 - 3.2×10^5	0.56 ± 0.15	0.21
Apopka-97	2.03×10^6	7.4×10^5 - 4.8×10^6	0.54 ± 0.12	1.18
FE 9901	1.36×10^5	5.4×10^4 - 3.0×10^5	0.32 ± 0.08	1.25
Apopka-97 conidial	1.47×10^7	5.6×10^6 - 3.4×10^7	0.32 ± 0.06	2.35

Data based on 3 tests (30 psyllids per test); all probit estimates were adjusted for control mortality.

Table 3-3. Number of infested terminals and ACP recorded on orange jessamine plants following treatment applications in the fall 2010 test.

Treatment	Rate	Infested terminals/plant				ACP adults/terminal shoot				ACP nymphs/terminal shoot			
		Pre-treat	Week1	Week2	Week3	Pre-treat	Week1	Week2	Week3	Pre-treat	Week1	Week2	Week3
Control		7.5a	10.5a	11.8a	16.8a	1.0a	0.9a	16.1a	16.2a	24.1a	30.3a	15.0a	1.8a
Merit 2F	3ml/plant	10.7a	6.2c	0.5c	0.2c	0.6bc	0.0a	0.0c	0.1d	17.2a	4.6c	0.2c	0.0b
Addit	0.25% v/v	12.0a	10.3a	10.7ab	14.0a	0.4c	0.7a	3.4b	5.5b	27.8a	16.2b	8.9ab	1.3a
PFR-97	0.28 oz/gal	10.2a	10.3a	10.2ab	15.0a	0.7abc	0.2a	5.4b	5.0b	26.7a	19.3ab	7.9ab	1.9a
PFR-97+ Addit	0.28 oz/gal + 0.25% v/v	9.0a	9.3ab	8.5ab	11.5ab	0.8ab	0.2a	3.4b	4.7b	24.8a	21.4ab	7.8b	1.1a
SuffOil-X	2% v/v	10.7a	6.8bc	7.0b	7.2b	0.6bc	0.2a	2.6bc	2.3c	30.6a	8.6c	4.4b	0.7ab

Letters in columns indicate differences ($P > 0.5$, Fisher's protected LSD). Data transformed due to unequal variances [log10 (x+1)]; non-transformed means shown.

Table 3-4. Number of infested terminals and ACP recorded on Benton citrange plants following different treatments (summer 2011 test).

		Infested terminals/plant			ACP adults/terminal shoot			ACP nymphs/terminal shoot		
Treatment	Rate	Pre-treat	Week1	Week2	Pre-treat	Week1	Week2	Pre-treat	Week1	Week2
Control		8.3a	9.5a	7.7ab	0.6a	0.7a	2.4a	36.4a	12.7a	2.8a
Merit 2F	3ml/plant	9.7a	5.6a	1.0c	0.5a	0.0a	0.0d	43.1a	1.1c	0.0c
Addit	0.25% vol/vol	8.5a	8.2a	9.7a	0.4a	0.7a	0.8bcd	32.1a	4.8b	2.1ab
PFR-97	0.28 oz/gal	9.0a	7.2a	7.7ab	0.3a	0.8a	1.1abc	38.0a	4.2b	0.6bc
PFR-97+Addit	0.28 oz/gal + 0.25% vol/vol	7.3a	5.2a	6.2b	0.5a	0.7a	1.4ab	26.2a	4.6b	1.6ab
SuffOil-X	2% vol/vol	8.3a	6.9a	6.8ab	0.3a	0.7a	0.3cd	32.9a	4.8b	0.3bc

Letters in columns indicate differences ($P > 0.5$, Fisher's protected LSD). Data transformed due to unequal variances [log10 (x+1)]; non-transformed means shown.

Table 3-5. Number of infested terminals and ACP recorded on Benton citrange plants following different treatments (fall 2011 test)

Treatment	Rate	Infested terminals/plant				Adult				Nymph			
		Pre-treat	Week1	Week2	Week3	Pre-treat	Week1	Week2	Week3	Pre-treat	Week1	Week2	Week3
Control		7.8a	11.2a	10.4a	9.4a	1.0a	0.9ab	1.2a	1.5ab	14.1a	34.1a	20.7a	6.5a
Orocit	0.25% vol/vol	7.6a	6.2bc	3.8b	4.0c	0.5a	0.2bc	0.0b	0.2c	28.7a	17.5b	3.2c	1.7b
SuffOil-X	1% vol/vol	7.0a	5.4c	4.4b	4.8bc	0.5a	0.2bc	0.1b	0.3c	18.3a	15.7b	2.1c	0.9bc
PFR-97	0.28 oz/gal	8.4a	9.0ab	7.6a	6.6ab	0.7a	1.9a	1.0a	1.7a	20.1a	19.3b	9.4b	1.7b
PFR-97+ Orocit	0.28 oz/gal +0.25%vol/vol	7.6a	7.6abc	4.6b	4.6bc	0.8a	0.0c	0.1b	0.7bc	21.3a	18.9b	3.5c	1.7b
PFR-97+ SuffOil-X	0.28 oz/gal + 1% vol/vol	7.8a	6.2bc	3.4b	4.2bc	0.5a	0.0c	0.2b	0.3c	16.7a	8.9c	1.2c	0.3c

Letters in columns indicate differences ($P > 0.5$, Fisher's protected LSD). Data transformed due to unequal variances [log10 (x+1)]; non-transformed means shown.

Table 3-6. Number of ACP on orange jessamine plants as determined by a final destructive count (fall 2010 test)

Treatment	Rate	ACP adults/plant	ACP nymphs/plant	Total/plant
Control		292.7a	11.3a	304.0a
Merit 2F	3ml/plant	0.2d	0.0c	0.2d
Addit	0.25% vol/vol	130.3b	3.5ab	133.8b
PFR-97	28 oz/100 gal	137.0b	8.3a	145.3b
PFR-97+ Addit	28 oz/100 gal + 0.25% vol/vol	144.0b	8.5a	152.5b
SuffOil-X	2% vol/vol	41.5c	2.2bc	43.7c

Letters in columns indicate differences (P > 0.5, Fisher's protected LSD). Data transformed due to unequal variances [log10 (x+1)]; non-transformed means shown.

CHAPTER 4
DISCUSSION

Renewed interest in the use of entomopathogenic fungi to manage insect pests and the discovery of a strain of *I. fumosorosea* (=*Paecilomyces fumosoroseus*) naturally infecting *D. citri* in a Florida citrus grove led to this study to further assess the potential of *I. fumosorosea* for use to manage *D. citri*. Presently three strains of *I. fumosorosea* are available for research as blastospore formulations in the USA—Apopka-97, which is formulated as a commercial material PFR-97 WDG (Certis USA) for control of soft-bodied insects including aphids, mites and whiteflies; FE 9901, which is supplied by Natural Industries Inc. as NoFly® bioinsecticide with registration status pending; and ARSEF 3581, supplied by USDA-ARS, NCAUR, Peoria, Illinois in diatomaceous earth formulation (Jackson *et al.* 1997). A simple method to quantify the pathogenicity and virulence of *I. fumosorosea* strains under optimum conditions was used. In developing this method, we found that the use of disposable opaque cups successfully maintained high, humid conditions necessary for the fungal isolate's development and sporulation. Additionally, the cups supported a detached leaf diet sufficient enough to keep five adult psyllids alive for a period of up to twelve days while maintaining leaf turgor. Control mortality remained low (< 20%) after 7 days, although it increased to higher than preferred levels (30–45%) after 12 days. The sealed cups minimized contamination and standardized light and temperature conditions throughout the bioassay.

Laboratory conditions are artificial, thus, it was important to quantify the impact of the fungus under more realistic conditions. We noted that all of the fungal strains reduced the survival of adult psyllid by up to 100%. We selected a commercial strain (Apopka-97) for greenhouse tests. The Apopka-97 blastospore strain was originally

isolated from mealybugs at MREC (Osborne and Landa 1992) and is now commercially marketed as PFR-97 WDG by Certis, USA (Menn 2003)—registered for control of soft-bodied insects including aphids, mites and whiteflies on food crops including citrus. Since various petroleum and organic derived oils are used in citrus groves in Florida for control of soft-bodied insects and disease management (Rogers *et al.* 2011), we also chose to include them in this research. The oils used in this study are used by growers in Europe and United States. In the first greenhouse test, we observed that PFR-97 reduced psyllid populations by approximately 50% over 3 weeks while the oil treatments ranged from 56–85% mortality. Additionally in the 2010 test, the petroleum-based oil (Suffoil-X®) was significantly more effective at reducing psyllid numbers when compared with the vegetable-based oil (Addit®). The reason for these different impacts are unknown, but may relate to the higher rates applied for the former, as well as the superior coverage of the highly refined paraffinic material.

It was hypothesized that formulating blastospores with emulsifiable oils might improve control compared with the fungus alone. Previous studies have highlighted several benefits of formulating entomopathogenic fungi with oils; these benefits enhanced not only deposition on the insect cuticle, but also germination rate and rain fastness (Inglis *et al.* 2002). The effectiveness of Apopka-97 in our tests was not enhanced by adding emulsifiable oils and did not increase psyllid mortality compared with oils used alone. The reasons for the lack of synergy are unknown. However one possibility is that the oils negatively affected germination or penetration of blastospores. Unfortunately this possibility was not specifically tested in this study. In 2011, Kim *et al.* assayed carriers to enhance persistence of *Isaria fumosorosea*. They found that a corn

oil carrier was superior in maintaining germination rates of *I. fumosorosea* when compared with other oils such as soybean oil, cotton seed oil, paraffin oil, and methyl oleate. The variability in different oil traits needs to be studied further with combined fungus and oil treatments. Entomopathogenic fungi effectiveness may also be inconsistent due to abiotic factors, such as temperature and humidity, and biotic factors like interactions of antagonistic microorganisms (Ferron 1978; Villani *et al*. 1992). It can be speculated that increasing the period of hydration of blastospores for several hours prior to application may improve the germination success on the target insect.

In follow up tests conducted in 2011, there were several difficulties in assessing the fungus under greenhouse conditions, mainly because of high mortality in all treatments and the widespread contamination with *Cladosporium* sp. that was observed several weeks into the study. Previous studies have shown that adult and nymphal mortality rates are higher where a minimum daily relative humidity is high (Étienne *et al.* 2001; Hall *et al*. 2008; Meyer *et al*. 2008). It may be that high mortality in controls was due to the spreading or natural occurrence of *I. fumosorosea* in the greenhouse, which was encouraged by the very high humidity (i.e. in summer 2011 tests r.h. was > 90% in > 50 % of hourly recordings). However, this hypothesis could not be confirmed since *Cladosporium* sp. grew on almost all insect cadavers and honeydew deposits. This growth was thought to be saprophytic since *Cladosporium* sp.did not kill psyllids at 10^7 conidia/ml in the laboratory. There is a previous report that *Cladosporium* sp. nr. *oxysporum* Berk. Infected *D. citri* in Réunion Island (Aubert 1987). Avery *et al.* (2004) isolated *Cladosporium* sp. on whitefly nymphs infected with Trinidadian strains of *I. fumosorosea*.

PFR is a known entomopathogen of ACP (Hoy *et al.* 2010; Hunter *et al.* 2011) and other insects including whiteflies, aphids, thrips and spider mites. Avery *et al.* in 2011 studied the effects of two PFR 97 formulations (blastospores and conidia) on the ACP. They monitored feeding rates and adult mortality through a laboratory bioassay and discovered that after 7 days post-exposure, total mortality occurred from both isolates. The blastospore formulation caused a significantly higher mortality than conidia within the first 3 days. They also documented that infected adult psyllid produced less honeydew than healthy psyllids, suggesting that a reduction in feeding activity could potentially reduce the spread of huanglongbing.

Avery *et al.* (2011) speculated that the use of *Isaria*, which is specific to insects, is valuable because it reduces feeding by healthy psyllids, prevents infected psyllids from spreading the disease and allows infected psyllids to serve as inoculums and spread the fungus throughout the citrus groves, thus killing even more psyllids. He developed an 'autodissemination system' in the laboratory that increased horizontal transfer of PFR and suggested that its adaptation for use in the grove could reduce HLB dissemination and potentially reduce costs to growers (Avery *et al.* 2009).

Several additional entomopathogenic fungi are known to infect *D. citri*, including *Lecanicillium lecanii* (= C*ephalosphorium lecanii*) (Rivero-Aragon and Grillo-Ravelo 2000; Xie *et al.* 1988), *Beauveria bassiana* (Bals.) Vuill. (Rivero-Aragon and Grillo-Ravelo 2000), *Capnodium citri* Berk and Desm (Aubert 1987) and *Hirsutella citriformis* Speare (Étienne *et al.* 2001; Meyer *et al.* 2007; Rivero-Aragon and Grillo-Ravelo 2000; Subandiyah *et al.* 2000). A previous report, dating back to 1987, indicates that

Cladosporium sp. nr*. oxysporum* Berk. Infect *D. citri* in Reunion Island (Aubert 1987). Entomopathogens are considered important factors of psyllid mortality.

Potato psyllid, *Bactericera cockerelli*, is a serious pest of potato and other solanaceous vegetables also transmit a bacterium, *Candidatus Liberibacter solanacearum* that causes a disease known as “zebra chip.” Lacey *et al.* (2009) observed under ideal laboratory conditions that two isolates of *Metarhizium anisopliae* and two isolates of *Isaria fumosorosea* caused > 95% mortality of adult potato psyllid. A *Beauveria bassiana* isolate provided 53% mortality after 2–3 days. The mortality rate of *Isaria* isolates in the laboratory was similar to our findings. In further field tests in Texas, treatment with *M. anisopliae* (F 52) , PFR-97 and abamectin (Agri-Mek) significantly decreased plant damage and zebra chip symptoms in a potato field (Lacey *et al.* 2011). Casique-Valdes *et al.* (2011) confirmed in their laboratory studies that *Hirsutella* cf. *citriformis* fungal isolates provide a viable component for an integrated pest management (IPM) strategy for control of *D. citri*. They successfully isolated *H. citriformis* from ACP and found it to be pathogenic to adults of *D. citri*, the potato psyllid, *B. cockerel and Nilaparvata lugens* (brown leafhopper).

Tamarixia radiata (Waterston) is a classical biologically introduced ectoparasitoid of ACP from India (Étienne *et al.* 2001; Hoy and Nguyen 2001; Hoy *et al.* 2006; Waterston 1922). It is one of many predators and parasitoids that keep psyllid nymphs in check (Chien *et al*. 1991; Étienne *et al.* 2001; Halbert and Manjunath 2004; Hoy and Nguyen 2001; León and Sétamou 2010; Michaud 2004; Pluke *et al.* 2008; Waterston 1922). However the amount of psyllid control provided by introduced parasitoids has been insufficient to slow disease spread.

Michaud (Michaud and Olsen 2004) found that other predators are equally efficient in controlling psyllids. Through the use of field cages, he found that, although *T. radiata* contributed to mortality of the psyllids, coccinellid beetles, such as *Harmonia axyridis* Pallas, were more important biological control agents in high-density *D. citri* populations in central Florida.

In an IPM program, a priority should be placed on natural mortality of the ACP wherever possible. Many pesticides have been shown to be highly toxic to psyllid predators and parasitoids (Michaud and Grant 2003). Toxicology studies from Hall and Nguyen (2010) indicated that carbaryl, chlorpyrifos and fenpropathrin were lethal to adult *T. radiata* as late as 3 days after application. Systemic insecticides, such as imidacloprid, are an important part of psyllid control (Rogers *et al.* 2010; Boina *et al.* 2009). Imidacloprid (Merit) provides persistent systemic activity against citrus psyllids. Used as a drench or foliar spray, it functions as a broad spectrum insecticide that is active on various Homoptera and leaf beetles (Rogers *et al.* 2010). Unfortunately, imidacloprid also causes mortality on beneficial insects, including *T. radiata*, through chemical residual effects (Cocco and Hoy 2008). Broad spectrum pesticides have a place in an IPM program; however, current recommendations limit applications to late spring and early fall when psyllid populations are actively reproducing on new flush (Rogers *et al*. 2010).

The persistence of *Paecilomyces fumosoroseus* is significantly affected by relative humidity of the drying air. Desiccation and shelf-life of blastospore preparations are improved when the relative humidity of the drying air exceeds 50% (Jackson and Payne 2007). High temperatures during storage results in a reduction of shelf-life of *Isaria.* In

order for living biological control agents to be useful, they must be stable, economical and provide viable fungal isolates in field conditions.

CHAPTER 5
CONCLUSIONS

All three *Isaria* isolates were pathogenic to ACP in the laboratory. Apopka-97 provided 50% or higher mortality on mixed age psyllid populations under greenhouse conditions. Foliar applications of oils (SuffOil-X®, Addit® and Orocit®) were effective in reducing psyllid populations by a range of 56-85% over three weeks. It did not appear as if the oils acted synergistically with the *Isaria*, although further research is needed in this area. Imidacloprid applied as a drench killed 100% of psyllid within 3 weeks in greenhouse tests. There are several factors that can determine how well *Isaria* would work in a commercial grove setting. Important factors will include ambient air temperatures and relative humidity within the citrus grove, air circulation within the canopy, accurate spray equipment and thorough coverage of the foliage during spray applications. Continued research is needed on effective, non-toxic environmentally friendly insecticides that can help with the fight against the ACP. These tools are vital in an integrated crop management program against HLB.

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BIOGRAPHICAL SKETCH

Karen Palanuk Stauderman was born in Springfield, Oregon and graduated from Thurston Senior High school in 1979. She earned a B. S. in plant pathology and a B. S. in horticulture from Oregon State University. She attended graduate school at the University of Nevada-Reno and Oregon State University, majoring in plant science.

In 1988, Karen relocated to Florida where she began work with the University of Florida as a biological scientist at the Central Florida Research & Education Center in Apopka, FL. Karen worked in the Entomology Department led by Dr. Lance Osborne and assisted in the initial culturing of *Isaria*. After a year, she was promoted to a biological scientist II in the Plant Pathology Department and was relocated to the UF Sanford Research Center (1989) where she remained for 10 years, managing laboratory investigations in cut foliage, carrots and the cabbage leaf curl virus.

Karen left UF in 2000 to expand a family farm along with her husband Harry and daughter Lynn (then age 5). Their venture, Oak Haven Farms of Mount Dora, LLC (established 1996) and Oak Haven Winery is an agri-tourism farm offering U-pick strawberries, restaurant, vineyard and winery.

Upon completion of her M. S. program, Karen will continue her current position with UF as a Horticulture Extension agent with Volusia County in Deland, FL where she has been employed since 2007.