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DEVELOPING METHODS TO SURVEY, COLLECT, PROCESS, AND SCREEN ENDEMIC ENTOMOPATHOGENIC FUNGI AGAINST THE ASIAN CITRUS PSYLLID IN THE LOWER RIO GRANDE VALLEY

A Thesis

by

JONATHAN CISNEROS

Submitted to the Graduate College of
The University of Texas Rio Grande Valley
In partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2020

Major Subject: Biology

DEVELOPING METHODS TO SURVEY, COLLECT, PROCESS, AND SCREEN ENDEMIC ENTOMOPATHOGENIC FUNGI AGAINST THE ASIAN CITRUS PSYLLID IN THE LOWER RIO GRANDE VALLEY

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December 2020

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ABSTRACT

Cisneros, Jonathan, Developing methods to survey, collect, process, and screen endemic entomopathogenic fungi against the Asian citrus psyllid in the Lower Rio Grande Valley. Master of Science (MS), December 2020. 68 pp., 5 tables, 7 figures, references, 121 titles.

The Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), vectors "*Candidatus* Liberibacter spp.", the causative agent of Citrus Greening Disease. Managing ACP is imperative given the continuous increase in HLB-positive trees. An aspect of integrated pest management (IPM) is the use of entomopathogenic fungi for the biological control of *D. citri*.

In order to identify endemic entomopathogenic fungi actively infecting *D. citri,* ACP were collected from local residential areas, surface sterilized, and plated on a semi-selective agar medium. Collection of over 9,300 samples from 278 sites throughout the LRGV led to the positive identification of nine entomopathogenic fungi across several genera via phylogenetic analysis. Primary and secondary acquisition bioassays revealed that both field isolated strains performed at comparable rates to *Cordyceps* (*Isaria) fumosorosea* Apopka97. In addition, thermal profiles for vegetative growth were determined. The findings revealed that the new isolates were capable of inducing mortality at rates greater than the standard, indicating that they may prove to be good candidates for the management of D. citri populations in the LRGV.

DEDICATION

First and foremost, I would like to dedicate this work to my Lord and Savior, Jesus Christ. *"To Him be the glory forever!"* The completion of my thesis would not have been possible without the unyielding love and support of my wife Rina, and my children Jonathan, Emma, and Timothy. Thank you for standing by me when I couldn't even stand myself. I am also infinitely grateful to all of my beloved friends from PIBA. You helped me up when I fell, you mentored me, and you have celebrated with me through my successes. Finally, I am indebted to my mother, Patty for all of the years of hard work and care you invested in me. Aquí hay un pequeño retorno de esa inversión. Thank you.

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I am sincerely thankful to Dr. Daniel Flores, member of my thesis committee and supervisor, for all of his mentoring and advice. His knowledge of biological control and insect rearing were essential to my success. I am also thankful to Dr. Stefan Jaronski, for his teachings and mentorship on all things entomopathogenic fungi. My thanks go to my committee chair, Dr. Christopher Vitek, and members Dr. Megan Keniry, and Dr. John Thomas for accepting the role and providing guidance and support. I am also grateful to Justin Wendel, friend and colleague. His investment of time and effort in the study was crucial to its completion.

I would also like to thank my colleagues at USDA for their help with the molecular characterization and revisions.

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CHAPTER I

LITERATURE REVIEW

Citrus greening disease or Huanglongbing (HLB) is a detrimental disease affecting many citrus tree varieties. The disease is caused by a fastidious bacteriological agent consisting of three strains; "*Candidatus* Liberibacter asiaticus (CLas), africanus (CLaf), and americanus (CLam)". The Asian strain is the most pathogenic to citrus trees (Da Graca & Korsten, 2004). The Asian and american strains are heat-tolerant forms vectored by *Diaphorina citri,* whereas the African strain is a heat-sensitive form primarily vectored by *Trioza erytreae* (Li et al., 2006). Culturing the bacterium has not yet been successful, and thus controlling the spread of the agent have been hampered. Additionally, typical bacterial concentrations in trees are often too low for traditional isolation methods or are restricted to a specific part of the tree and not systemic, leading to increased false-negative identification efforts (McClean, 1970). Identification efforts have found relative success in using real-time PCR as a means of detection and identification of CLas in citrus trees (Li et al., 2006) and other molecular techniques have seen similar success as well.

Citrus Greening Vector *Diaphorina citri*

The Asian citrus psyllid (*Diaphorina citri*) is an arthropod belonging to the order Hemiptera, in the family Liviidae. *Diaphorina citri* is typically about 4 millimeters long and mottled brown in color. The thorax of *D. citri* adults usually exhibit one of three color variants:

gray/brown, blue/green, and orange/yellow (Wenninger et al., 2009). *Diaphorina citri* nymphs are yellow with red eyespots and will molt through 5 nymph instars before reaching full maturity (Grafton-Cardwell et al., 2013). Nymphs are primarily stationary, while adults are completely motile, flying from one location to another or jumping when threatened. Adults will typically feed on both mature and young leaf flush, while nymphs develop on immature flush only (Childers & Rogers, 2005). Because the main vector of HLB is the Asian citrus psyllid, controlling this pest is one of the main methods employed in controlling disease spread.

Commonly Utilized Control Efforts

Heat therapy is among one of the various strategies used in the control of HLB incidence and transmission. Research has shown that various HLB strains have different optimum growing temperature ranges and are either negatively or positively affected in relation to their effect on host plants. Bové et al. found that the CLaf strain resulted in more severe greening results at the 22°-24°C range versus the 27°-32°C range, leading to its reference as a heat-sensitive bacterium (1974). In more recent years, researchers further tested this hypothesis by challenging host plants with heat treatments to suppress or eliminate the bacterium. In a study by Hoffman et al. (2013), researchers exposed *Citrus reticulata, C. paradisi,* and, *C. limon* citrus plants to temperatures of 40°-42°C for a period of 10 days. Results showed a significant reduction in plants after a time period of 30 days after treatment (DAT) and complete eradication (no detection) of CLas in tissues 90 DAT. Additionally, researchers also saw a regeneration of tissues in the plants, where seedlings that were completely defoliated had begun developing new growth post-treatment. Other researchers have observed similar results when performing these types of studies (Khot et al., 2014), though the findings of some of these studies were sometimes contradictory, indicating

poor control under field conditions (Kelley & Pelz-Stelinski, 2019) or failure to maintain optimal temperatures (Basunia, 2020).

Another measure in the management of HLB is the use of antibiotics. The general principle is that by administering various chemicals either topically, injected into plant tissues, or provided via liquid applications into the soil, the chemicals are expected to reduce bacterial titers, reduce the likelihood of infection, or a combination of both.

In a study seeking to identify the efficacy of antibiotics in the control of HLB in plants, researchers found that the combined use of both penicillin and streptomycin (PS) via root soaking and foliar application resulted in a significant reduction of bacterial CLas titers 90 DAT (Zhang et al., 2011). Conversely, when researchers applied tetracycline injections to trees, they found that there was only a slight reduction of apparent HLB, leading them to believe tetracycline only worked as a bacteriostatic agent (Van Vuuren, 1977). In a combination study seeking to observe the effects of both thermo- and chemotherapy, researchers subjected HLB-infected trees to sulfathiazole sodium (STZ), or sulfadimethoxine sodium (SDX) and temperatures of 40° or 45°C, then measured bacterial titers (Yang et al., 2016). Results revealed that a combination of 45°C and SDX had the highest reduction of bacterial titers 60 DAT, eliminating any detectable presence of the bacterium (Yang et al., 2016).

As in many cases, the use of chemicals, especially antibiotics, rarely comes without consequences or unexpected side effects. A comprehensive study investigating the effects of various antibiotics on the growth rate of cultured plant cells found the beta lactam class antibiotics to be the least toxic to plants, with penicillins and cephalosporins actually potentiating plant growth (Nickell, 1952). On the contrary, aminoglycosides were contraindicated in their use as plant antibiotics due to their toxic effects at even low concentrations (Pollock et al., 1983).

Given the ambiguous nature of antibiotic use in plants, care should be used when implementing them into control strategies and seeking alternatives when possible may be a more suitable option.

Chemical Control of *Diaphorina citri*

For years, chemical pesticides have been the standard for use in the control of arthropod pests. In a study performed by researchers at the Citrus Research and Education Center, Entomology and Nematology Department in Lake Alfred, Florida, several pesticides were tested by spraying trees, watering around the base of the tree, or injecting into the ground at the base of the tree. Researchers found that Agri-mek + a 435-horticultural mineral oil, Danitol, Lorsban, and Provado all resulted in high mortality 5 days after treatment, while Danitol also provided good nymph suppression of eggs 15 days post treatment (Childers & Rogers, 2005). Overall, the data suggested that horticultural mineral oils used as insecticides are a good option in the control of *D. citri*.

Similar to other insects, *Diaphorina citri* identify and seek out their hosts by means of olfaction and vision (Grafton-Cardwell et al., 2013). The presence of visual cues combined with olfactory cues often resulted in greater attraction than either of the two alone (Wenninger et al., 2009). By smelling the presence of citrus plant volatiles, *D. citri* can seek out and feed on host plants. Inversely, the use of sulfur plant volatiles, like those of *Allium* spp. (garlic, onion, etc.) have been found to repel several arthropod species. Mann et al. (2011) found that by exposing *D. citri* adults to sulfur volatiles from crushed garlic chive leaves, garlic chive essential oil, garlic chive plants, wild onion plants, and crushed wild onion leaves, they were able to significantly repel the psyllids when compared to clean air. Additionally, the presence of some of these sulfides inhibited the detection of citrus plant volatiles by *D. citri* adults. As a whole, the use of

chemical volatiles is yet another potential candidate for the control of *D. citri*, and if nothing more, a different facet of a larger IPM strategy.

Biological Control of *Diaphorina citri*

Biological control of insect pests is defined as the introduction of a natural enemy, i.e., an animal that naturally feeds or otherwise controls the target pest, to control the native and exotic species (Van Driesche & Hoddle, 2009). The practice of using natural predators as a means to control a pest spans over 100 years. A meta-analysis of over 800 studies found that in instances where biological control agents were used, pest abundance was reduced by 130%, increased parasitism by 139%, and increased overall pest mortality by over 150% (Stiling & Cornelissen, 2005). The practice of introducing foreign biocontrol agents has also been used in the past, but the introduction of these types of agents is highly regulated. These findings indicate that biocontrol strategies are capable of managing pest populations on their own, or in combination with other methods of pest control.

Natural predators of the Asian citrus psyllid are important contributors to the management of *D. citri* populations. One of the more common predators of *D. citri* is the lady beetle, *Coelophora inaequalis*. In a study seeking to elucidate the feeding behaviors of 8 different lady beetle species, researchers found that all 8 fed on *D. citri* adults, with *Coleomegilla innonata* consuming *D. citri* at a greater rate than all other lady beetle species (a mean of 1.21 *D. citri* consumed/h) (Pluke et al., 2005). These studies also showed that *Crepidodera nitidula, Chilocorus cacti, Coleomegilla innonata*, and *Cryptolaemus montrouzieri* all preferentially feed on *D. citri* over the brown citrus aphid. Other generalist predators observed to feed on *D. citri* were spiders and long-legged flies (Diptera: Dolichopodidae). During a screening of Asian citrus psyllid predators, researchers found spiders to occasionally feed on *D.*

citri, while long-legged flies were observed to flock towards infested foliage and capture *D. citri* out of the air (Chong et al., 2010).

Unfortunately, the use of chemical pesticides also poses a risk to the arthropod predators. . When insecticides were used on calendar sprays to reduce the risk of HLB infection, a significant decrease in the number of predatory insect fauna was observed, leading to reduced predation rates, and in turn, an increase in *D. citri* populations (Monzo et al., 2014). The results of this study suggest the need for a more targeted and effective method of control, while simultaneously revealing the important role that natural predators play in the control of pests.

Parasitoidism is a form of parasitism wherein an organism oviposits on or within a host. The developing larvae then feeds on the host, resulting in eventual death of the insect (Todd, 2011, Reuter, 1913). Within the Hymenoptera, parasitic wasps make up much of the order (Goulet & Huber, 1993) and are either considered endo- or ectoparasitic, depending on whether they lay their eggs in or on the host, respectively (Kapranas et al., 2012).

There are two known parasitoids of *Diaphorina citri*. *Tamarixia radiata* (Waterston), a well-studied ectoparasitic wasp, and *Diaphorencyrtus aligarhensis*, an endoparasitic wasp of moderate control of *D. citri* in Florida (Rohrig, 2014). Both *T. radiata* and *D. aligarhensis* were imported from Punjab, Pakistan, are accustomed to climates similar to that of citrus producing states like Florida and Texas (Hoddle, 2012), and have been found to successfully parasitize *D. citri*, although *T. radiata* has found greater success in doing so (Vankosky & Hoddle, 2016).

T. radiata females exteriorly oviposit on the abdomen of *D. citri* hosts, whereas *D. aligarhensis* oviposit internally within the abdomen. (Rohrig, 2014). During development of the embryo, *T. radiata* remain dorsally attached to the host and sucks fluids (Husain & Nath, 1924). This continues until the third instar, at which point the embryo moves to the ventral side of the

host and feeds. The *D. citri* nymph continues to live and feed until the nymph is consumed in its entirety, leaving behind a brown mummy (Chien et al., 1991). In *D. aligarhensis,* development from egg to adult takes approximately 16 to 18 days and includes an embryonic stage, four larval instars, a prepupal stage, and a pupal stage, all of which occur within the *D. citri* host (Rohrig et al., 2011). Upon exit of the host mummy, *D. aligarhensis* creates an exit hole on the dorsal side through the abdomen. Conversely, *T. radiata* emerges dorsally through the thorax (Hoy, 2006).

As previously mentioned, *T. radiata* has proven to be a highly successful biological control agent for suppressing *D. citri* populations in several citrus growing states. With *T. radiata* reproducing at a much higher rate than *D. aligarhensis*, shorter generation time, and almost twice the host kill rate (Skelley & Hoy, 2004), it comes as no surprise why this parasitoid has struggled to find a foothold in the current ecosystem. The availability of multiple parasitoids and the success of *T. radiata* supports the notion that biological control is a key component for suppressing *D. citri* populations.

Entomopathogenic Fungi

An entomopathogenic fungus is one that preferentially parasitizes its insect host via entry and dissemination throughout the host hemolymph. These fungi can often be found established within host populations with some variability in off target hosts. The biology of fungi has allowed them to establish themselves ubiquitously throughout various ecosystems, primarily in the soil (Zimmermann, 1986) and gives them the ability to aerially disperse with relative ease.

Entomopathogenic fungi (EPF) are found in a polyphyletic group of fungi that have evolved to parasitize insect hosts. Often times this occurs via nutrient sequestration or toxin production (Shah & Pell, 2003). These pathogens can be found within the phyla Deuteromycota,

Ascomycota, and Chytridomycota, though numerous other fungi are dispersed amongst the phylogenetic tree (Shah & Pell, 2003). In particular, *Beauveria bassiana* and *Lecanicillium muscarium* (formerly *Verticillium lecanii*) both belong to the Cordycipitaceae family (Gams & Zare, 2001, Rehner et al., 2011), whereas *Isaria fumosorosea* and *Metarhizium anisopliae* belong to the Clavicipitaceae (Cabanillas et al., 2013, Driver et al., 2000). In fact, over three quarters of the mycoinsecticides ever used have been contained within these genera (Faria & Wright, 2007).

Entomopathogenic fungi have been used as biological control agents (Goettel & Glare, 2010), although there is also research exploring their use as endophytes. Entomopathogenic fungi for use in foliar sprays are typically grown up on liquid culture and solid substrate and spores produced are mixed with water and a wetting agent or emulsifiable oils. These solutions are then sprayed onto host plants with the intention of inoculating both the insect pest and the plant surface. Spore types typically used in these formulations are either conidia from solid substrate fermentation (SSF) or blastospores from yeast-like budding in liquid culture (de Faria & Wraight, 2007). The selection of a mycoinsecticide is usually based on several characteristics like effective spore production on media, virulence, shelf life, and cost efficiency. Because of the significant differences in biology and morphology between the various genera of fungi, factors like rate of growth, virulence factors, and receptor compatibility all play important roles in the efficacy of a fungus.

Beauveria bassiana **Biology**

Beauveria bassiana is an entomopathogenic fungus with a broad host range and has been extensively studied. Host infection typically occurs via the same mechanisms as with other microorganisms; adhesion and entry must first occur, followed by invasion, establishment,

dissemination throughout the host, toxin production, and evasion of the host immune system (Bidochka & Khachatourians, 1990).

When *B. bassiana* first encounters an insect host, there are several events that must occur before infection can begin. Nonspecific binding and attachment to a host cuticle occurs via hydrostatic mechanisms (Lacey & Kaya, 2007). In a study seeking to identify the mechanisms by which this occurs, researchers found that three common morphological types of *B. bassiana* had various attachment preferences. Aerial conidia adhered poorly to weak polar surfaces while adhering well to hydrophobic and hydrophilic surfaces. Blastospores were found to poorly adhere to hydrophobic surfaces but rapidly to hydrophilic ones. In contrast, submerged conidia adhered well to all types of surfaces (Holder & Keyhani, 2005). Though further research into the subject is necessary, it is speculated that specific proteins coined 'hydrophobins' embedded in rodlet surface structures on aerial conidia are responsible for their hydrophobicity (Wessels, 2000, Holder et al., 2007). Once attachment occurs, *B. bassiana* begins to degrade the host cuticle by producing proteases and chitinases, giving the fungus access to the nutrient rich hemolymph within (Joshi et al., 1995, Fang et al., 2005).

Penetration occurs soon after, and typically consists of germination of the fungal spore on the surface of the cuticle. Hyphae work their way into openings made by proteases and chitinases, giving the fungus entry into the host hemocoel. Some conidia have been observed to form appressorial-like structures; specialized, flattened cell structures or "pressing" organs that form a peg that punch a hole into the host cell (Vey & Fargues, 1977). Penetration of the host cuticle plays an important role in the virulence of a fungus and can even be regulated to a certain extent. Researchers found that by transforming *B. bassiana* with a chitinase, binding domain, and

promoter, they were able to induce a 23% reduction in time to death of *Myzus persicae* aphids (Fan et al., 2007).

Once inside, hyphae bud into blastospores which can begin to propagate and release toxins. A well-researched toxin produced by *B. bassiana* and other entomopathogenic fungi is beauvericin. This insecticidal compound also exhibits anti-tumor and antimicrobial activity (Hamill et al., 1969). Bassiacridin, a toxic protein also produced by *B. bassiana,* was observed to cause cytotoxic effects in locust tracheae, air sacs, and melanized nodules in the fat body in contact with the cuticle. Additionally, larvae injected with a low dose of the toxin exhibited 50% mortality (Quesada-Moraga & Alain, 2004). Other toxins produced by *B. bassiana* are bassianin, bassianolide, beuverolides, and tenellin (Vey et al., 2001).

Metarhizium anisopliae **Biology**

Progression of *Metarhizium anisopliae* pathogenesis occurs in a similar fashion. When *M. anisopliae* first encounters an insect host, attachment occurs via weak hydrophobic interactions, while adherence occurs via *Metarhizium* adhesin-like protein 1 (MAD1) (Wang & St Leger, 2007). Interestingly, Wang and St Leger (2007) also found that MAD2, a similar adhesin-like protein was involved in *M. anisopliae* adhesion to plant matter, and that silencing the genes resulted in approximately 90% reduction in adherence. Mad1 silencing also resulted in delayed germination, suppressed blastospore formation, and significantly reduced virulence in caterpillars, leading them to conclude that adhesion likely plays an important role in gene expression related to the cell cycle.

Once adhered to the host, *M. anisopliae* conidia or blastospores produce a single germ tube from where an appressoria is formed and begins the process of breaking through the cuticle, eventually colonizing the hemolymph. A mucous secretion is produced at the area around the

germ tube. This secretion is suspected to facilitate cuticle breakdown as it was found to contain some of the essential cuticle-degrading enzymes like those previously mentioned (St Leger et al., 1996). According to Arruda et al. (2005), the entire process from adherence to emergence of *M. anisopliae* in *Boophilus microplus* ticks takes approximately 96 h, and similar times have been observed in other fungal genera.

Extracellular proteases and chitinases are also employed in the breakdown of insect cuticles by *M. anisopliae* similar to *B. bassiana*. Subtilisin-like proteases, trypsin-like proteases, metalloproteases, and exo-acting peptidases are some of the enzymes used in cuticle breakdown by *M. anisopliae* (St Leger et al., 1995, St Leger et al., 1996). Additionally, de Moraes et al. (2002) observed that *M. anisopliae* produced chitinases that allowed further degradation of the host cuticle once the protein constituents had been digested.

Toxin production upon entry of the host occurs, with the production of destruxins (DTX). These toxins have been found to paralyze insect hosts and reduce mortality time when compared to controls (Samuels et al., 1988). Interestingly, proteases produced by *M. anisopliae* were also found to be toxic to *Galleria mellonella* larvae, indicating that enzymes and toxins produced by the fungus do not exist in a vacuum, and could have multiple functions and even synergize in some instances (Kučera, 1980).

Conclusion

The impact of Citrus Greening on the citrus industry in the United States alone has led to the development of numerous strategies for the control of HLB and its vector, *D. citri.* As previously described, methods like the use of heat therapy to reduce bacterial titers in plants have seen success, and some researchers have developed streamlined processes to expedite the use of

these methods. Others have found success in the use of antibiotics and chemical pesticides, wherein trunk and root injections of various antibiotics saw reductions in pathogen bacterial titers, while pesticides helped reduce vector populations on trees. Some researchers have also explored the use plant sulfur volatile compounds to repel *D. citri*, though large-scale implementation has yet to be assessed. Biological control efforts like the use of ectoparasitoids and entomopathogenic fungi have also been relatively successful in controlling vector populations, and their use provides a more environmentally friendly alternative to chemical pesticides.

CHAPTER II

INTRODUCTION

Citrus greening disease, or Huanglongbing (HLB), and its vector the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) are currently found throughout all major citrus producing states in the United States (Gottwald et al., 2007, da Graça et al., 2016) and their presence and effect on citrus trees has resulted in major economic losses. Though states like Texas and California have confirmed the presence of HLB in their trees, other states like Florida have suffered greater losses in comparison. In Florida alone, it was estimated that from 2006 to 2011 approximately 5,718 jobs were lost, with an estimated revenue loss of about 1.7 million dollars (Hodges & Spreen, 2006). Given the fact that Florida is the largest producer of citrus in the United States and the second largest juice producer in the world, this information is concerning to the citrus economy in the US as a whole (Hodges & Spreen, 2006). Additionally, with an estimated value of citrus production of approximately $140 million, Texas is at risk for suffering great losses if HLB disease and vector levels rise (Kunta et al., 2014).

The presence of the Asian citrus psyllid in the Rio Grande Valley was first recorded and confirmed in September 2001 by Dr. Ru Nguyen and Dr. Susan Halbert in Weslaco, Texas (French et al., 2001) and the presence of HLB in the Lower Rio Grande Valley (LRGV) was first confirmed in San Juan, Texas on January 2012 (da Graça et al., 2015). The discovery of these vector-pests led to the implementation of several strategies in the LRGV to protect the citrus trees in the urban environment and the commercial citrus industry. Control measures included

establishing quarantine zones, planting clean nursery stock, rogueing infected trees, and vector control by means of pesticide sprays and biological control efforts (Flores & Ciomperlik, 2017).

Control of *D. citri* populations requires overcoming several challenges. Historically, Southern Texas was and still is a large producer of citrus. The LRGV was once home to thousands of acres of citrus groves. As city populations grew and the infrastructure of cities changed, more and more groves were torn down and replaced with residential lots or recreational vehicle (RV) mobile home parks. As such, many of the trees that remain in these microecosystems go untreated most of the year, allowing the establishment of *D. citri* populations to rise in these areas. Though currently many citrus producers employ the use of pesticide schedules to prevent the spread of the pest and disease, these vector insects spread via wind dispersion from residential areas to neighboring groves, putting citrus producing trees at risk of infection (Boina et al., 2009).

Because identifying and controlling the pathogen once it has established itself in trees has proven difficult and a cure has not been found to date (Bové, 2006), researchers have attempted to control vector populations as a means of impeding the spread of the disease. In Texas, researchers from the USDA APHIS PPQ S&T Mission Laboratory implemented a region-wide biological control project utilizing a small parasitoid wasp, *Tamarixia radiata* Waterston (Hymenoptera: Eulophidae).

Tamarixia radiata, the species-specific ectoparasitoid wasp responsible for a large portion of biological control in the LRGV has proven to be a very successful integrated pest management (IPM) strategy in the control of *D. citri.* Over the past several years, releases of the wasp have led to notable decreases in *D. citri* populations throughout the LRGV and Mexico, with population densities dropping from 43 immature psyllids per flush on citrus in residential

areas in 2010, to 3.8 immature psyllids found per flush in 2016; a reduction of over 91% of the psyllid population (Flores & Ciomperlik, 2017). That being said, *T. radiata* is a single tool of what should be a more complex strategy used to combat HLB and its vector. The need for other means of biological control are always present and could further reduce *D. citri* populations in the Lower Rio Grande Valley (LRGV) if implemented as part of a complete strategy. One measure of biological control that has briefly been explored in *D. citri* is the use of entomopathogenic fungi (EPF) (Subandiyah et al., 2000, Lezama-Gutiérrez et al., 2012).

Entomopathogenic fungi are a class of parasitic fungi that can invade the host and induce mortality by the production of fungal toxins, or by competition for nutrients within the host hemocoel (Roy et al., 2006). The use of EPF has been well noted in several other insects including whiteflies and grasshoppers, and the literature available for the use of EPF in *D. citri* is available from other countries as well. In a paper published by Subandiyah et al., (2000) the authors describe the finding of a colony of *D. citri* adults that appear to be mycosed by fungi. Upon culturing and identification, they were confirmed to be *Paecilomyces fumosoroseus* (currently *Isaria fumosorosea*) and *Hirsutella citriformis.* Additionally, when healthy adults were challenged with the fungus, mortality was observed within 6 days.

Although finding active entomopathogenic fungal infections in the field is possible as demonstrated in previous examples, epizootic infections are rare and difficult to find. As such, a survey of the Lower Rio Grande Valley *D. citri* population similar to studies by Jaronski et al. wherein live insects are collected, incubated, and screened for presence of entomopathogenic fungi could not only result in identifying potential EPF biocontrol candidates, but could also elucidate the rates and types of EPF found in the LRGV. It is unclear what EPF could be found

and at what rates, but previous research has shown that active EPF parasitism in live insects usually occur at low rates (Wakuma, 2016).

Seeking an entomopathogenic fungus that has an established niche within the population as a parasite could result in a good candidate strain against the pest. One of the driving mechanisms behind this idea is that finding an isolate that is well suited to the LRGV climate could mitigate the impact of negative effects caused by high temperature ranges. For example, in a study by Yeo et al. (2003), researchers found that the best suited EPF for the control of two aphid species was one where its thermal optimum growth temperature matched the temperature in the bioassay (10ºC for *Lecanicillium* sp.), indicating a strong correlation between temperature and pathogenicity. There are numerous other studies that have explored this correlation, and many researchers argue that an EPF's growth rate at a given temperature can be a strong indicator of its performance in field trials and bioassays for the control of a given pest (Davidson et al., 2003, Hong & Kim, 2007, Inglis et al., 1997, Garrido-Jurado et al., 2011) By assessing the thermal profiles, media preference, and capacity to induce mortality in dose-mortality bioassays, one could deduce whether the field isolated entomopathogenic fungi could serve as effective biocontrol agents. Ultimately, the use of entomopathogenic fungi as a means of biological control is not a silver bullet but rather, another tool to be used in the reduction of transmission of HLB and its vector, *D. citri.*

1st Hypothesis

Entomopathogenic fungi can be found within the Lower Rio Grande Valley *D. citri* population.

Rationale

Previous studies in other insects have shown that populations harbor entomopathogenic fungal infections at low rates (Wakuma, 2016), although a study in Western Asia using the 'Galleria bait method' resulted in isolation rates as high as 34% (NouriAiin et al., 2014). The behaviors of some insects also favor the persistence of chronic fungal infections in their populations, as in the case of the thermoregulating grasshopper, *Melanoplus sanguinipe* (Jaronski & Goettel, 1997).

Screening the population of *live* psyllids as opposed to only collecting dead insects will provide the opportunity to gauge the rate of entomopathogenic fungal infections in *D. citri* in the LRGV, as well as isolate any fungal pathogens for future use as biocontrol agents.

Outline

The first experiment will be to screen *D. citri* samples collected in the field from residential and RV park sites in search of an indigenous strain of fungus that proves to be more effective (than current standards) in reducing vector populations. The general outline will consist of first dividing a map of the LRGV into four main quadrants, divided by interstate 2 and interstate 69C. The reasoning behind doing so is to ensure proper distribution throughout the LRGV and avoid any type of bias in the collection site locations. Each week a trip out from Moore Airfield Base will be made to a given quadrant and visit RV parks or residential homes. Once there, collections will be made by aspirating live *D. citri* adults into dram vials labeled with a serial number that indicates the date collected. Assigned to that serial number will be a series of details like the geographical location, number of adults collected at that location (tree), and city of collection.

Isolates that were successfully identified microscopically and by means of their colony morphology will be subjected to phylogenetic studies wherein their genomic DNA will be extracted and purified. Samples will then be amplified using known nuclear gene loci commonly used in the identification of entomopathogenic fungi and these sequences will then be used in comparison to other ex-type strains to accurately identify the field isolates to the level of genus and species.

The screening of *D. citri* populations in the LRGV for any presence of EPF will elucidate the rates at which certain EPF are found in this particular population, provide insight on the distribution of the EPF in the LRGV, and most importantly provide a candidate for the control of *D. citri* populations in the LRGV. Because EPF are found ubiquitously in the wild, it is suspected that some EPF will be identified, though isolates are expected to be found at rates below 1% given past screening projects in other insect populations.

2nd Hypothesis

An entomopathogenic fungus found in the live *D. citri* population can be better suited as a biological control agent than the commercialized standard, Apopka97.

Rationale

Characterization by means of thermal profiling, media preference, and dose-mortality bioassays are important aspects of an entomopathogenic fungus that can give insight into how an isolate can be expected to perform in field conditions. Thermal profiling can help predict how an isolate can be expected to perform under various temperatures, especially in the extreme ranges. Dose-mortality bioassays are a direct measurement of the efficacy of entomopathogenic fungi against *D. citri* adults, providing insight into the field isolates' potential as a biocontrol agent.

Some of the major factors that can negatively affect fungal growth and pathogenesis are temperature, UV radiation, humidity, and nutrient preference. Therefore, seeking out a fungus that naturally grows in the LRGV should yield isolates that are acclimated to the conditions of Southern Texas and in turn should prove to be more effective than a standard.

Outline

To characterize EPF both commercially available and not, their growth kinetics are to be assessed via thermal profiles, media preference analysis, and dose-mortality bioassay screening. In the upcoming project, radial growth measurements for each of the isolates and the standard (Apopka 97) will be taken at regular time intervals to assess the rate of growth of the fungal isolates at various temperatures. Media preference analysis will also be assessed by growing up fungi on different media combinations and assess their growth rate at a standard temperature. Dose-mortality bioassays will be performed by taking any isolated fungal samples, growing them up on solid substrate, performing the dose-mortality bioassay as outlined in the Methods, and comparing their performance to a known commercial standard (Apopka97). Their performance will be quantified and ranked, allowing one to clearly identify how well the indigenous strains perform in comparison to the commercially available standard.

CHAPTER III

METHODOLOGY

Field Collections

Collections began in April 2018 by United States Department of Agriculture (USDA) Field Operations agents, in addition to making our own field collections. Field collections consisted of weekly trips to RV parks within the range of the LRGV. Once a citrus tree was identified, individual branches and leaves were inspected for presence of *D. citri*. The *D. citri* were then aspirated into vials, which were assigned an accession number that consisted of the year, month, and day collected, followed by its number in the series of vials collected for that day, e.g. *YYMMDD.01*. GPS coordinates were also annotated for each collection site, along with the number of insects collected at that site. Samples were initially sought in both residential areas and commercial citrus groves, but there was scarce *D. citri* presence in the groves, likely as a result of scheduled pesticide treatments. Additionally, the data collected at each site were reduced to the GPS coordinates, type of tree, and recently, temperature and humidity data at the time and site of collection (not shown).

Sample Processing

Samples were processed in preparation for colony characterization by surface sterilization and plating. Collected field samples were immediately stored for 24 hours at -20°C to freeze-kill

all *D. citri*. Once killed, samples were stored dry at 4°C until processed to preserve any fungal pathogens within the insects. This prevented saprophytic growth on the outside of the *D. citri*. During processing, approximately 3 mL of 10% sodium hypochlorite solution with 1 ml L^{-1} Dawn (Procter & Gamble Inc., Toronto, ON) soap as a wetting agent, were added to each individual vial and agitated via vortexer at 3,000 rpm for 30 seconds. The sample in solution was decanted onto a Buchner funnel with #1 filter (Whatman, Buckinghamshire, UK) attached to a vacuum pump, and the psyllids were rinsed thoroughly with reverse osmosis H_2O. Once the majority of the H_2O had been vacuumed off, the filter was removed from the funnel and psyllids were plated onto 100 mm selective water agar with 0.23 mg/mL dodine (Arysta LifeScience Benelux, Belgium) and 0.015 mg/mL chloramphenicol (MP Biomedicals, LLC, Ohio) (WA-DC) in rows. Plates were then incubated at 29°C and after approximately 7 days of incubation, plates were inspected for fungal growth (Figure 2). Conidia from potential candidates were subcultured onto 35 mm selective potato dextrose agar plates with 0.1% yeast extract, dodine, and chloramphenicol (PDAY-DC) by taking the back of a wooden cotton swab, gently tapping a clean agar plate to moisten the tip, gently touching the conidia on the *D. citri*, and then inoculating the center of the plate. Plates were then incubated for 7 days at 29°C in complete darkness. Inoculum from selective plates were plated onto 60 mm PDAY plates and allowed to grow until confluent. All growth on plates was then scraped off using a metal spatula and added to cryogenic vials containing 1 mL 30% glycerol and 3 glass beads. Samples were then vortexed at 3,000 rpm for approximately 30-45 seconds until homogenous and stored at -80°C for long-term preservation. Agar cultures of the isolates were submitted to the USDA Agricultural Research Service Collection of Entomopathogenic Fungal Cultures (ARSEF, Ithaca, NY) under the accession numbers listed in Table 1.

Genetic Characterization

Characterization of the field isolates to the level of genus was determined via phylogenetic studies using multiple loci. Isolates grown up on 60 mm SDAY plates were allowed to grow to the periphery of plates. Fungal mycelia were taken from plates using a sterile metal spatula and were added to 1.5 mL collection tubes. Genomic DNA was extracted using the DNEasy Plant Mini Kit (QIAGEN Inc., Germantown, MD, USA) following the manufacturers recommendations and DNA was eluted using 50 µL of EB buffer.

A fragment of four nuclear loci were amplified by Polymerase Chain Reaction (PCR) using the primers found in Table 2 (Rehner et al. 2011). PCR reactions were performed using a GeneAmp® PCR System 9700 thermocycler (Applied Biosystems, Foster, CA, USA). Amplification reactions consisted of 25 µL volumes containing 16.4 µL of water, 2.5 uL of 10X buffer, 2.6 µL of dNTPs, 0.13 µL of DNA Taq polymerase, 1 µL of each primer (10 mM), and 2 µL of DNA template. PCR reaction conditions for the B locus and EF1-α gene loci were replicated as in Rehner et al. (2006). Thermocycler reaction conditions for the remaining sequences were performed as found in Rehner et al. (2011).

PCR products were visualized on 1% agarose gels using ethidium bromide to confirm the presence of the amplified fragment and then purified using ExoSAP-IT® (Affimetrix, Santa Clara, CA) as per manufacturer instructions prior to sequencing. Samples were sent to Functional Biosciences (Madison WI, USA), for bi-directional sequencing. Sequences were edited on Sequencher 5.0 (Gene Codes Corp., Ann Arbor, Michigan).

DNA sequences were compared to public GenBank records using NCBI default BLAST search option for nucleotide sequences (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the highest genus and species matches per sequence (Table 5). The sequences were also searched

comparing them to records of *Beauveria*, *Isaria* and *Lecanicillium* to reject hypotheses of identification to these genera. Once genus-level identification was determined using the BLAST searches, a phylogenetic analysis of records was performed. An alignment was constructed for each locus using Clustal W (Thompson et al. 1994) and the program MEGA v.7.0 (Tamura et al. 2013). Aligned sequences were trimmed to the longest length of high-quality sequence reads for all specimens. Reference sequences were taken from Rehner et al. (2011) as a basis for the *Beauveria* phylogeny. In addition, the four single-locus alignments were concatenated into a multigene alignment for analysis in MEGA v.7.0. Tree reconstructions were performed for each data alignment in MEGA v.7.0 using the Neighbor-Joining algorithm, 100 bootstrap replicates, and default settings.

Determination of Thermal Profiles

Rates of vegetative growth were determined for all isolates and Apopka97 at 5, 10, 15, 20, 25, 28, 32 and 35°C to obtain a thermal profile for each strain. Aerial conidia from two-week old SDA plates were suspended in sterile 0.1% polyoxyethylene sorbitan monooleate (Tween 80) (Fisher Scientific, Fair Lawn, NJ, USA), using a sterile cotton swab. The approximate concentration of the suspensions was $5x10^7$ conidia mL^{-1} based on turbidity. To each stock suspension 20 µL of an aqueous gentamycin or chloramphenicol solution was added to inhibit any subsequent bacterial growth. Three replicate 100 mm diameter Petri plates of PDAY were inoculated with the conidial suspensions of each strain. Inoculation consisted of a 10 µL droplet placed in the center of each plate. All plate cultures were incubated for three days at 28°C, until the colonies were generally 1 cm in diameter. At this point the colony edges were marked along two perpendicular axes and the cultures transferred to the various temperatures. Colony diameters were remarked at daily (15, 20, 25, 28°C) or every other day (5, 10, 32, 35°C)

until colonies had grown to within 2 cm of the Petri dish margin, or 14 days in the case of the temperature extremes. Subsequently, the distance between opposing markings were measured and exported to an Excel file, with a Mitutoyo™ Digimatic Caliper and Input Tool (Mitutoyo Corporation, Japan).

Colony diameters were converted into radii and subjected to least squares linear regression in Microsoft Excel® to calculate growth rates in mm hr^{-1}. In addition, the growth rates at suboptimal temperatures were calculated based on the temperature having the fastest growth for each strain. Slopes of the lines for the thermal profiles were calculated using Microsoft Excel® and compared to the slopes of the lines from the first and last temperature points to identify discrepancies in the linearity of the repeated measurements. Two-Way ANOVA (α=0.05) (SigmaPlot® V11.0) was used as done in Er & Gökçe (2004) and Gabiatti et al. (2006) to determine the effects of isolate strain and temperature on growth rate. All pairwise comparisons were subjected to Tukey's multiple-comparison test in order to identify significant differences between the isolates versus the standard, Apopka97.

Media Preference Analysis

Media preferences of the first two field isolates ACP18001 and ACP18002 were determined by measuring radial growth on various media over time at a standard temperature. 60 mm plates composed of either full strength PDA, PDAY, SDA, or SDAY were inoculated with ACP18001 and ACP18002 following the steps as described in the thermal profile analysis. Plates were measured daily and datapoints were logged on an excel sheet using a digimatic caliper and graphed on their respective lines. Analysis of covariance (α=0.05) was performed on the slopes of regression lines within each group of isolates in a media type in order to identify any significant differences between the datasets.

Primary Acquisition Bioassays

The two first isolated samples that were molecularly characterized and submitted to ARSEF under the accession numbers ARSEF 13765 (ACP18001) and ARSEF 13766 (ACP18002) were cultured on solid substrate fermentation (SSF) as described in Bartlett & Jaronski (1988) and harvested spores were used in dose-mortality bioassays. Stock solutions of approximately 1×10^9 ml^{-1} were suspended in 0.1% Tween 80 using a glass tissue homogenizer and 100× dilutions were subsequently counted on a hemocytometer to determine spore concentration of the stocks using the following equation:

$$\text{spores/mL} = \left(\frac{\text{number ofspores}}{\text{number of large squares} \times 16}\right) \times (4 \times 10^6)(\text{dilution factor})$$

Spore viabilities were also determined by plating the 100× dilutions onto PDA plates and incubating at 29°C in complete darkness for 16-18 hours. Viabilities were read by staining an excised block of the inoculated PDA plates with cotton blue stain (MycoPerm; Fisher Cat # 23769320) and determining viable and non-viable spores under a microscope. At 16-18 hours, any spore that had developed a germ tube was considered viable, while any spore without a germ tube, stained or unstained was considered nonviable. Bioassays were performed by preparing spore solutions of the stock solutions at concentrations of 3×10^6, 1×10^7, 3×10^7, 1×10^8, and 3×10^8 viable spores/mL, with reverse osmosis H_2O as an untreated control for the two isolates and Apopka97. Apopka97, the *Isaria* strain found in the mycoinsecticide PFR-97® (Certis U.S.A., LLC, Columbia, MD, U.S.A.) was chosen as the standard because it is highly available and registered for use in the United States. Primary acquisition sprays were performed by taking approximately 20-35 *D. citri* in snap-cap vials and cold stunning them in a freezer at -15°C for

about 3 minutes. Stunned insects were removed from the freezer and placed onto a wax paper placed inside of the spray tower. Insects were then sprayed with 0.4 mL of a control or one of the dilutions listed above, and inoculated insects were placed in flush tubes with healthy flush and incubated for 7 days. After the 7^{th} day, flush tubes were removed from incubators and the psyllids were carefully counted to determine dead and live insects. Mortalities were counted by dividing the response (mortality) by the total number of psyllids per tube.

Mortality data was analyzed using Probit POLO JR© (LeOra Software LLC, Parma, MO, U.S.A.) to calculate the median lethal concentration (LC_{50}). 95% confidence intervals (CI) and single factor ANOVA and Tukey's post hoc test was performed using SigmaPlot® (V11.0, Systat Software Inc., San Jose, CA, U.S.A.) in order to find significant differences between the field isolates and the standard at their respective doses. Where the normality test failed, an ANOVA on Ranks was run along with a Dunn's post hoc test. The 95% CI were also compared using the confidence interval overlap test to identify if there were any significant differences. Data was displayed using a Box and Whisker graph.

Secondary Acquisition Bioassays

The two first field-isolated *B. bassiana* entomopathogenic fungal strains ACP18001 and ACP18002 were also used in secondary acquisition dose-mortality bioassays. Bioassays were performed by preparing spore solutions at concentrations of 3×10^6, 1×10^7, 3×10^7, 1×10^8, and 3×10^8 viable spores/mL for ACP18001, ACP18002, and Apopka97 following the same process previously described, with the exception that rather than spraying the *D. citri* adults directly, plant material was sprayed directly and cold stunned insects were added to the flush tubes after spraying. Plant material was pruned from 160-liter pots of orange jasmine plants, *Murraya paniculata* (L.) Jack. The flush samples measured approximately six to eight inches tall in

aquatubes (Syndicate Sales, Kokomo, IN, U.S.A.) with 0.025% MaxiGro™ hydroponic solution (General Hydroponics, Sebastopoc, CA, U.S.A.) and wrapped at the base with parafilm. Plant material was sprayed directly by placing the aquatube with plant material in a tube rack inside of the potter's spray tower and spraying each sample with 0.4 mL of the appropriate spore dilution. The dosed plant samples were added to individual acrylic tubes with mesh tops and bottoms and approximately 20-35 *D. citri* were added, capping the tube immediately after. Acrylic tubes with dosed plant material and *D. citri* were then incubated in environmental growth chambers for seven days at 29°C and approximately 80% relative humidity. After the incubation period, samples were removed from the growth chambers and mortalities were counted by removing the plant material from the tube while attempting to leave as many of the *D. citri* within the tube as possible. *D. citri* that remained on the plant material were counted and killed first, followed by those that remained inside the tube. Mortalities were counted by dividing the response (mortality) by the total number of psyllids per tube.

Mortality data was analyzed with PoloPlus using Probit analysis to calculate the median lethal concentrations (LC_{50}) where available. Given the heterogeneity of the data, LC_{50} and 95% confidence intervals could not be calculated for many of the bioassays. Therefore, box and whisker graphs were created for the data using SigmaPlot to compare the data. 95% confidence intervals (CI) and single factor ANOVA and Tukey's post hoc test were also performed using SigmaPlot in order to find significant differences between the field isolates and the standard at their respective doses. Where the normality test failed, an ANOVA on Ranks was run along with a Dunn's post hoc test.

Results

Field Collections and Processing

Since April of 2018, approximately 9,305 *D. citri* samples from 278 sites were collected and processed (Figure 1). Of these samples collected, 9 of 9,305 processed insects resulted in positive identifications for entomopathogenic fungi. Genetic characterization of the isolates was determined by means of four gene loci and phylogenetic trees were constructed based off this data. One isolate was determined to belong to the genus *Fusarium* sp. and was therefore excluded from further testing, given its frequent status as a plant, insect, and sometimes human pathogen. Based off the collected data, it was concluded that active entomopathogenic fungal infections were found in live-collected *D. citri* adults at a rate of 0.0967%. As such, I failed to reject the null hypothesis that entomopathogenic fungal infections could be found in the LRGV *D. citri* populations.

Genetic Characterization

NCBI BLAST searches for all genera in question were performed and their results could be found in Table 5. Secondary matches were provided where results of similar match percentages yielded contradictory results.

Phylogenetic trees for the concatenated alignments as well as for each individual locus for the five isolates that matched *Beauveria* spp. revealed that all five isolates belonged to the *B. bassiana* species clade. Only the tree for B locus is shown (Figure 3) because it had the highest

number of informative sites of all four loci used for this analysis yet had the same results as the other concatenated and single sequences. The BLAST results also indicated that these isolates belonged to the genus *Beauveria*. Interestingly, it was determined that isolates ACP18003, ACP18007, ACP18006, ACP18002, and ACP18008 were monophyletic, while ACP18001 belonged to a sister taxon. ACP18009, though still falling under the *Beauveria* classification, was distantly related to the other field isolates as can be observed from Figure 3.

Findings for the second tree analysis (Figure 4) revealed the two field isolates belonged to two separate genera; ACP18004 to the *Paecilomyces* spp. genus and ACP18005 to the *Isaria* spp. genus.

Thermal Profiles

Thermal profiles were used to characterize the isolates by means of radial growth bioassays, wherein the growth rates for each of the three fungal strains (Apopka97, ACP18001, ACP18002) were analyzed and compared to standard. The plots for the data are shown in Figure 5. Two-Factor ANOVA of all pairwise comparisons revealed there were significant differences between the isolates at various temperatures. As such, a Tukey post-hoc test was performed to identify any significant differences. The analysis showed that there were no significant differences between any of the isolates at the 5 and 10°C temperature points. Though no significant differenc0-e was found at the 20°C temperature point, several isolates grew at rates comparable to the standard, as can be seen in Figure 5. At the 26 and 30°C temperature points, ACP18004 grew at a rate significantly greater than the standard. Additionally, isolates ACP18002, ACP18003, ACP18004, and ACP18006 grew at rates significantly greater than the standard at the 32°C point, and little vegetative growth was observed for all isolates and the standard at the 35°C point.

Media Preference

The three groups of measurements for Apopka97, ACP18001, and ACP18002 were compared via analysis of variance and no significant differences were found (Table 3). Nevertheless, comparisons of the slopes showed a slight positive correlation between yeast extract supplementation and an increase in slope for the three fungi tested, indicating that supplementation with yeast slightly boosted growth rates for all fungi tested. Based off the data, it could be speculated that any of the media combinations tested could be used to compare growth rates as exemplified by the lack of significant differences between the groups.

Primary Acquisition (Direct Spray) Bioassays

The spray application of spores directly onto *D. citri* adults was performed in order to observe the capability of the fungus to induce mortality at various concentrations and to effectively calculate a median lethal dose (LD_{50}) for the three strains used in this experiment. The list of viable bioassays as determined by the calculation of LD_{50} and 95% confidence interval (95CI) values by PoloPlus can be found in Table 4. In addition, complete datasets including bioassays that did not produce LD_{50} and 95CI were graphed using a Bar and Whisker plot in Figure 6. Results of the Bar and Whisker graph revealed that the dataset had significant outliers, especially Apopka97. Nonetheless, an ANOVA on Ranks along with Dunn's Post Hoc test revealed that there was a significant difference between ACP18002 and the standard at the 3E8 dose, with ACP18002 inducing a higher mortality percentage at that dose.

Secondary Acquisition (Indirect Spray) Bioassays

In contrast to spraying conidial spore solutions directly onto *D. citri*, spraying flush material and then adding the insect pest to the inoculated sample allowed us to quantify the

mortality effect that secondary acquisition (spores that are picked up via adhesion to an insect appendage or body) had on *D. citri*. The list of viable bioassays as determined by the calculation of LD_{50} and 95% confidence interval (95CI) values by PoloPlus can be found in Table 4. In addition, complete datasets including bioassays that did not produce LD_{50} and 95CI were graphed using a Bar and Whisker plot on Figure 6b. The plot of this data revealed that there too were significant outliers for Apopka97 secondary acquisition data, though overall the median, upper, and lower quartile values for ACP18001 and ACP18002 were higher than Apopka97, with no overlap of the plots of the two isolates and the standard for the two highest doses. Single Factor ANOVA paired with Tukey's test, or an ANOVA on Ranks with Dunn's test where normality test failed revealed that there were significant differences between some of the doses tested (Figure 6b). ACP18001 and 02 were found to perform greater than the standard at the 3E8 and 1E8 doses, while ACP18002 alone induced higher mortality than the standard at the 3E7 and 1E7 doses.

CHAPTER IV

DISCUSSION

The purpose of this study was to seek supplemental options to currently established biocontrol tools, seeking out effective entomopathogenic fungal strains that were actively inducing infection in *D. citri* adults. The number of available mycoinsecticides in the U.S. are limited, and those that are marketed against the Asian citrus psyllid are even more scarce. Though entomopathogenic fungi are well known for being capable of infecting a wide variety of pests, research has hinted at the fact that many have host-specific binding receptors that can improve virulence, among other factors. Seeking out and isolating entomopathogenic fungi directly from the host source mitigates the issue of specificity and provides the basis for a mycoinsecticide that is evolutionarily selected for the control of the host. Testing and characterization via dose-mortality bioassays, thermal profiles, phylogenetic studies, and media preferences helped to identify the field isolates for use as potential mycoinsecticide candidates.

Field Collections and Processing

Based off collected numbers, the rate at which entomopathogenic fungi were found on live-collected *D. citri* was approximately 0.11%; a number slightly lower, but comparable to numbers found in other studies (Kalsbeek et al. 1995, Vänninen 1996). Opting to isolate entomopathogenic fungi from live insects as opposed to cadavers was a strategy that could facilitate finding active fungal infections occurring in the population of *D. citri* in the LRGV. In

addition, collecting from a live host as opposed to baiting from soil samples would also select for an isolate that could likely be acclimated to the LRGV environment. Therefore, we opted for a selective collection strategy that would give us the best chance of finding isolates that were both acclimated to the LRGV environment and could induce mortality in *D. citri*.

Genetic Characterization

The primary goal of performing phylogenetic analyses and NCBI BLAST searches of the field isolates was to genetically characterize the field isolates for identification and proper accession. Many entomopathogenic fungal genera are still currently under major revisions. Outdated classifications are also found throughout the database for some of the fungal genera. For example, the genus *Isaria,* formerly known as *Paecilomyces*, was recently revised to *Cordyceps* and includes many new taxa that were not formerly classified as such. As such, the use of both BLAST searches and a proper phylogenetic analysis were performed.

In constructing the phylogenetic tree for the *Beauveria* isolates, the data of Rehner et al. (2011) was used in large part as a reference, given that this is one of the few, if not only fully exhaustive characterizations of the genus *Beauveria*. Fortunately, this paper and their sequences were informative enough to identify our isolates to the level of species, i.e., *Beauveria bassiana* for all 7 of the isolates. Four of the five loci referenced in the Rehner et al. paper were used to characterize the isolates and construct phylogenetic trees. That study demonstrated that each locus can correctly identify Beauveria species. Therefore, multiple loci and concatenation of data are not needed. This is also seen in the current study as the separate and concatenated data sets resulted in the same identifications. The Bloc data set had the most informative sites and the phylogenetic tree was reported.

One interesting aspect of the phylogenetic analysis of the *Beauveria* isolates was the lack of diversity observed over temporal and spatial differences. Isolates ACP18003, ACP18007, ACP18006, ACP18002, and ACP18008 were genetically indistinguishable, yet these isolates were collected over the span of up to 10 months and over 20 kilometers (Fig. 1). Though further studies would be required to accurately test this hypothesis, these preliminary findings hint at the notion that at least in the *Beauveria* population in the LRGV these fungi are highly conserved and are undergoing evolutionary change at low rates.

Media Preference Analysis

The findings from this small study revealed that there were no significant differences in the growth rates of the field isolates and standard under the various media types, indicating that both SDA and PDA could be used interchangeably for the culture of entomopathogenic fungi. Though not significant, there was also an observed increase when these media were supplemented with yeast extract, a result that was to be expected given the increase in nutrient availability. Future studies will explore various concentrations to find the optimal concentrations of media in agar for the culture of the various strains of entomopathogenic fungi commonly used.

Thermal Profiles

Thermal profile characterization of fungal isolates is an indirect metric of an isolate's fitness at a given temperature point. Though cellular growth is commonly considered to be logarithmic or exponential during its growth phase, the literature commonly describes radial growth of isolates on petri dishes as a linear function of length over time (Kiewnick 2006). As such, the slope of linear regressions of the isolates radial growths were used to identify their optimal growth rates, as well as identify isolates that grew at rates greater than the standard,

Apopka97. The results of this analysis showed that there were in fact differences in the growth rates of the isolates in comparison to Apopka97, particularly at the optimal temperature range (30-32°C) for most of the fungi tested. For example, there were no significant differences at the lower extreme temperatures (5, 10°C) between the isolates and Apopka97. Conversely, at the 26, 30, and 32°C temperatures, many of the isolates grew at rates comparable to Apopka97, and 3 of the 9 isolates exhibited growth that was several magnitudes of significance greater than the standard. One explanation for the differences in growth rates between the standard and the field isolates could be their source. Apopka97 or *Isaria fumosorosea* strain Apopka97 was first isolated from a mealy bug, *Phenacocus solani* in a greenhouse in Apopka, Florida (Humber 1992). This strain was originally classified as *Paecilomyces fumosoroseus* but was later changed when the genus underwent reorganization (Rechcigl & Rechcigl 1999). By comparison, field isolates found on live Asian citrus psyllids characterized in this study belonged to the genera *Paecilomyces*, *Isaria*, and *Beauveria*. Research has shown that a fungi's host and ecological environment could play a role in their susceptibility and thermal tolerance. In a study by Fernandes et al. (2008), it was determined that there was a correlation between the climate of the isolation site of fungi belonging to the same genera and its thermal tolerance, with fungi belonging to more arid climates exhibiting higher temperature preferences. Though South Texas and Florida climates are similar, the fact that Apopka97 was isolated from a greenhouse rather than the field may have had an impact on the thermal preference of the fungus.

The findings in this study clearly showed that several isolates found in the *D. citri* population were better suited for the LRGV environment. Though there were at least three isolates that grew at rates greater than Apopka97 at varying temperatures, several of the other isolates grew at similar rates to Apopka97 with no significant differences between their growth

values (data not shown). These findings support the notion that a fungus isolated and cultured under conditions similar to its isolated environment perform better at those conditions. As such, it would be favorable to use entomopathogenic fungi for the control of pests that have a preference for similar climates.

Primary Acquisition

It was evident from the Bar and Whisker graph that there were issues with the heterogeneity of the data. As observed in figures 6a. and b, the data for the isolates and Apopka97 was highly heterogenous, with many outliers present in the dataset. One likely explanation for this was the variability in spore deposition rates when using sprayers. Due to human error during the actual spray, making of spore dilutions, among other factors, spores/ml can result in highly variable spore depositions. Some researchers have circumvented this issue by counting deposited spores on agar cubes or cover slips, essentially producing a spores/mm^2 value for each spray and adjusting the actual dose sprayed accordingly. In hindsight this would have been a more efficient way of developing and performing the bioassay. Not only would doing so have cut down on wasted replicates, but it would also provide insight into the actual spore deposition rates observed in sprayers.

Based on the statistical analyses performed in addition to the data distribution from the Bar and Whisker graph, there was comparable induction in mortality between ACP18001, ACP18002, and Apopka97, indicating that the field isolates performed at rates comparable to or greater than the standard. Further studies with an optimized bioassay protocol could possibly provide further differentiation in the performance of these organisms.

Secondary Acquisition

Secondary acquisition bioassays revealed that ACP18001 and ACP18002 were more effective at inducing mortalities in *D. citri* than the standard. Secondary acquisition bioassays assume that the target pest must come in contact with spores on a surface in order to illicit a response, and as previously mentioned this was a variable that was not controlled for in the current bioassay iteration. As could be seen from figure 6b. as well as the statistical analysis results, the response of the field isolates was more robust when compared to the standard. Interestingly, the results also revealed mortality rates for secondary acquisition that were comparable to those found in primary acquisition. Such findings indicated that ACP18001 and ACP18002 were capable of inducing mortality in *D. citri* by means of secondary acquisition of the spores by insect movement along the plant material rather than direct application as in the primary acquisition spray. These findings suggest that these field isolates could be highly successful in field trials, where direct spore deposition conditions directly onto insect pests are not optimal.

Analysis of the data showed that there was a correlation between the dose and respective response for all strains, though it was more evident for the field isolates tested. Future studies will include the testing of *D. citri* nymphs under primary acquisition bioassays to assess the extent to which ACP18001 and ACP18002 can induce mortality. There are several factors to be considered when testing these isolates, or any mycoinsecticides on nymphs for that matter. *D. citri* nymphs go through five instars, lasting approximately 2.46, 2.13, 2.39, 2.73, and 5.04 days each instar, respectively (Nava et al. 2007). In contrast, the median lethal time (LT_{50}) of a novel *I. fumosorosea* strain (*Ifr* ACP) on adult *D. citri* was approximately 102 hours, when the *D. citri* were dosed at a concentration of 1×10^8 spores/ml (Hoy at al. 2010). By taking these two factors

into consideration, one could hypothesize that this particular entomopathogenic fungus, and by extent those fungal isolates in the present study would not induce mortalities at high rates in *D. citri* nymphs due to the rapid molting that *D. citri* nymphs undergo at each stage. The results suggest that the entomopathogenic fungi would not be able to penetrate the insect cuticle and establish an infection before the nymph shed its exuviae, effectively removing the spores from its surface. As such, testing field isolates on nymphs would be crucial to further characterize the extent to which these entomopathogenic fungi could induce mortalities in the various life stages of *D. citri.* In addition, due to the substantial amount of work involved in testing field isolates under the various characterization bioassays reviewed above, only the first two underwent the majority of these tests (ACP18001 and ACP18002). This was done primarily to cut down on the amount of work involved in this thesis. Future work will also include the primary and secondary acquisition bioassays of the remaining field isolates with the implementation of spores/mm^2 as well, in order to but to exemplify the idea that field isolates taken directly from the target pest are more likely to be effective against the given pest than any random fungal entomopathogen.

As previously discussed, factors like fungal growth rate, pathogenicity, UV tolerance, among other characteristics are important when considering the commercialization of a mycoinsecticide. The fact that Apopka97 is a currently established mycoinsecticide, and that ACP18001 and ACP18002 performed comparable to or better than this strain under several circumstances indicate these two newly identified strains could be considered viable candidates for use in the management of *D. citri* and potentially other pests.

TABLES

Table 1. List of isolates with their respective accession numbers, host substrate, host plant, collection site, and collection date.

Scientific Name of Fungus	Method of ID	ARSEF Accession #	Isolate Code	Scientific Name of Host and Life Stage	Insect Host Plant	Collection Site	Collection Date
Beauveria bassiana	Bloc, EF1α, *rpbI*, *rpbII*	13765	ACP18001	*Diaphorina citri*, Adult	Grapefruit tree	Pharr, Texas	4/27/2018
Beauveria bassiana	Bloc, EF1α, *rpbI*, *rpbII*	13766	ACP18002	*Diaphorina citri*, Adult	Grapefruit tree	Pharr, Texas	5/1/2018
Beauveria bassiana	Bloc, EF1α, *rpbI*, *rpbII*	14401	ACP18003	*Diaphorina citri*, Adult	Grapefruit tree	Edinburg, Texas	11/5/2018
Paecilomyces lilacinus	EF1α, ITS	14402	ACP18004	*Diaphorina citri*, Adult	Grapefruit tree	Edinburg, Texas	11/5/2018
Isaria fumosorosea	EF1α, ITS	14403	ACP18005	*Diaphorina citri*, Adult	Grapefruit tree	Edinburg, Texas	11/5/2018
Beauveria bassiana	Bloc, EF1α, *rpbI*, *rpbII*	14404	ACP18006	*Diaphorina citri*, Adult	Grapefruit tree	Mission, Texas	1/10/2019
Beauveria bassiana	Bloc, EF1α, *rpbI*, *rpbII*	14405	ACP18007	*Diaphorina citri*, Adult	Grapefruit tree	Mission, Texas	3/7/2019
Beauveria bassiana	Bloc, EF1α, *rpbI*, *rpbII*	14406	ACP18008	*Diaphorina citri*, Adult	Grapefruit tree	Edinburg, Texas	3/21/2019
Beauveria bassiana	Bloc, EF1α, *rpbI*, *rpbII*	14407	ACP18009	*Diaphorina citri*, Adult	Grapefruit tree	Edinburg, Texas	3/21/2019

Table 2. List of primers used for the field isolates tested.

Isolate Code	Scientific Name of Fungus	EF1α	Bloc	RPB1A (RNA pol. II largest subunit)	RPB1B (RNA pol. II largest subunit)	fRPB2 (RNA pol. II 2nd largest subunit)	fRPB2 (RNA pol. II largest subunit)
ACP18001	*Beauveria bassiana*	×	×		×	×	×
ACP18002	*Beauveria bassiana*	×	×		×	×	×
ACP18003	*Beauveria bassiana*	×	×		×	×	×
ACP18004	*Paecilomyces lilacinus*	×					
ACP18005	*Isaria fumosorosea*	×					
ACP18006	*Beauveria bassiana*	×	×		×	×	×
ACP18007	*Beauveria bassiana*	×	×	×	×	×	×
ACP18008	*Beauveria bassiana*	×	×	×	×	×	×
ACP18009	*Beauveria bassiana*	×	×	×	×	×	×

Table 3. Linear Regression Coefficients for the Various Isolates on PDA, PDAY, SDA, and SDAY.

Medium	Strain	y_0	a
PDA	Apopka97	2.9051	1.9555
	ACP18001	3.3625	1.8181
	ACP18002	3.4401	1.9957
PDAY	Apopka97	3.4975	1.7905
	ACP18001	2.0821	2.6408
	ACP18002	2.0533	2.7510
SDA	Apopka97	4.3629	2.1401
	ACP18001	4.9818	2.0445
	ACP18002	6.4905	2.5654
SDAY	Apopka97	5.6399	2.2645
	ACP18001	4.0591	2.6138
	ACP18002	4.5231	2.6912

Table 4. Results of the Probit analysis indicating the LC_{50}, lower and upper 95% confidence intervals (95CI), slope, and heterogeneity values for each bioassay.

	Bioassay	LC_{50}	L95CI	U95CI	Slope ± SE	Heterogeneity
Primary Apopka97	BA 4	8.72E+08	3.82E+08	4.79E+09	0.66 ± 0.137	0.55
	BA 7	1.08E+09	4.33E+08	8.83E+09	0.66 ± 0.162	0.12
	BA 10	7.47E+08	3.44E+08	3.62E+09	0.59 ± 0.129	0.38
	BA 18	1.65E+08	7.11E+07	8.09E+08	0.44± 0.106	0.55
	BA 19	5.32E+06	3.94E+06	6.97E+06	2.35 ± 0.480	0.02
	BA 21	1.31E+08	5.25E+07	7.62E+08	0.36 ± 0.095	0.41
	BA 22	1.39E+07	1.73E+06	4.22E+07	1.382 ± 0.190	0.82
	BA 23	2.38E+09	6.14E+08	0.00E+00	0.336 ± 0.077	2.84
	BA 25	3.69E+07	7.29E+06	1.33E+08	0.89 ± 0.090	1.95
	BA 26	7.64E+04	3.10E+00	8.25E+05	0.52 ± 0.170	0.27
	BA 29	4.03E+07	2.06E+07	7.80E+07	0.577 ± 0.096	0.00
	BA 30	3.43E+06	2.35E+06	4.37E+06	2.643 ± 0.520	1.04
	BA 33	5.01E+07	2.01E+07	1.20E+08	0.450 ± 0.098	0.00
	BA 38	6.36E+06	2.54E+06	1.12E+07	1.330 ± 0.226	0.78
	BA 42	1.33E+06	9.44E+04	2.87E+06	-0.951 ± 0.314	1.35
	BA 47	5.12E+08	2.90E+08	1.32E+09	0.79 ± 0.139	0.92
	BA 51	3.76E+09	9.53E+08	0.00E+00	0.66 ± 0.180	0.85
Primary ACP18001	BA 42	1.46E+09	0.00E+00	4.11E+09	0.538 ± 0.155	0.49
	BA 44	8.34E+07	3.50E+07	1.44E+08	2.793 ± 0.376	2.19
	BA 46	1.59E+07	1.73E+06	5.69E+07	1.292 ± 0.141	2.20
	BA 47	2.78E+07	1.61E+07	4.46E+07	0.752 ± 0.092	0.20
	BA 50	4.99E+07	3.43E+07	6.83E+07	1.318 ± 0.160	0.46
	BA 51	7.99E+07	2.82E+07	3.48E+08	0.885 ± 0.093	3.08
Primary ACP18002	BA 52	4.96E+07	2.48E+07	1.06E+08	0.834 ± 0.077	1.84
	BA 53	1.10E+08	5.27E+07	2.70E+08	0.621 ± 0.129	0.86
	BA 54	1.04E+08	4.69E+07	2.65E+08	1.164 ± 0.103	3.55
	BA 95	6.80E+07	4.90E+07	9.32E+07	1.271 ± 0.145	0.53
	BA 97	1.56E+08	3.10E+07	5.44E+08	0.780 ± 0.241	0.58
Secondary ACP18001	BA 56	2.13E+08	1.41E+08	3.70E+08	1.339 ± 0.208	0.35
	BA 60	2.15E+08	1.33E+08	4.22E+08	0.888 ± 0.173	0.42
	BA 61	5.14E+08	2.99E+08	1.55E+09	1.098 ± 0.251	0.15
Secondary ACP18002	BA 57	4.85E+07	1.13E+07	2.61E+08	0.711 ± 0.091	1.35
	BA 61	1.96E+07	9.49E+06	3.40E+07	0.538 ± 0.089	0.95

Table 5. NCBI BLAST search results showing the highest identity percentage matches and their accession numbers. Alternative matches that had similar percentage similarities were also shown.

Isolate Code	Locus	BLAST Results	Query Cover	Max Identity (%)	NCBI Accession No.
ACP18001	Bloc	*Beauveria bassiana* isolate SASRI BWR1	87%	95.96%	JX110353.1
ACP18002	Bloc	*Beauveria bassiana* RCEF4425	99%	96.83%	JQ867107.1
ACP18003	Bloc	*Beauveria bassiana* strain ARSEF 2040	100%	96.61%	DQ384389.1
ACP18006	Bloc	*Beauveria* cf. *bassiana* IP-962	99%	99.52%	HQ412779.1
ACP18007	Bloc	*Beauveria bassiana* voucher CG25	100%	96.55%	MK440080.1
ACP18008	Bloc	*Beauveria bassiana* strain ARSEF 2040	100%	96.85%	DQ384389.1
ACP18009	Bloc	*Beauveria bassiana* strain RCEF1896	100%	99.87%	JQ867115.1
F181105.072	NS5	*Fusarium* sp.	99%	100.00%	GQ166777.1
	NS5	*Gibberella intermedia* isolate CA3-1	99%	100.00%	JN236216.1
	NS5	*Fusarium oxysporum* strain SP-2	99%	100.00%	HM152769.1
F181105.072	NadA	*Fusarium verticillioides* culture CBS:576.78 strain CBS 576.78	100%	100.00%	MT010915.1
	NadA	*Gibberella moniliformis*	100%	100.00%	JX910421.1
	NadA	*Fusarium musae* culture CBS:624.87 strain CBS 624.87	100%	98.60%	MT010916.1
F181105.072	Lef1	*Fusarium oxysporum* Fo5176	100%	99.62%	CP053261.1
	Lef1	*Fusarium fujikuroi* strain Augusto2	100%	99.62%	CP023090.1
	Lef1	*Fusarium proliferatum* strain ITEM2400	100%	99.62%	LT841264.1
F181105.072	ITS	*Fusarium oxysporum* f. *cucumerinum* strain WPII30-1	99%	100.00%	MK163441.1
	ITS	*Fusarium napiforme* strain CBS 748.97	100%	99.82%	MH862670.1
F181105.072	E29	*Fusarium verticillioides* strain CBS:576.78	100%	99.42%	MT010990.1
ACP18001	EF1-α	*Beauveria bassiana* strain CHE-CNRCB 161	100%	99.31%	MH203480.1
ACP18002	EF1-α	*Beauveria bassiana* strain CHE-CNRCB 161	100%	100.00%	MH203480.1

ACP18003	EF1-α	*Beauveria bassiana* strain CHE-CNRCB 161	100%	100.00%	MH203480.1
ACP18004	EF1-α	*Paecilomyces lilacinus* strain IBT27375	100%	99.78%	GU979984.1
	EF1-α	*Purpureocillium lilacinum*	100%	99.66%	MF099429.1
ACP18005	EF1-α	*Cordyceps javanica* strain BCC26304	100%	100.00%	MH521903.1
	EF1-α	*Isaria javanica* strain CHE-CNRCB 363	100%	100.00%	KY587208.1
ACP18006	EF1-α	*Beauveria bassiana* strain CHE-CNRCB 161	100%	100.00%	MH203480.1
ACP18007	EF1-α	*Beauveria bassiana* strain CHE-CNRCB 161	100%	100.00%	MH203480.1
ACP18008	EF1-α	*Beauveria bassiana* strain CHE-CNRCB 161	100%	99.65%	MH203480.1
ACP18009	EF1-α	*Beauveria bassiana* strain BCC1446	100%	100.00%	MN401499.1
ACP18001	RPB1	*Beauveria bassiana* strain ARSEF 1811	100%	99.65%	HQ880837.1
ACP18002	RPB1	*Beauveria bassiana* strain ARSEF 1811	100%	99.65%	HQ880837.1
ACP18003	RPB1	*Beauveria bassiana* strain ARSEF 1811	100%	99.77%	HQ880837.1
ACP18006	RPB1	*Beauveria bassiana* strain ARSEF 1811	100%	99.53%	HQ880837.1
ACP18007	RPB1	*Beauveria bassiana* strain ARSEF 1811	99%	99.71%	HQ880837.1
ACP18008	RPB1	*Beauveria bassiana* strain ARSEF 1811	72%	99.43%	HQ880837.1
ACP18009	RPB1	*Beauveria bassiana* strain ARSEF 1478	99%	99.77%	HQ880836.1
ACP18001	RPB2	*Beauveria bassiana* strain ARSEF 1811	96%	99.91%	HQ880909.1
ACP18002	RPB2	*Beauveria bassiana* strain ARSEF 1478	96%	99.91%	HQ880908.1
ACP18003	RPB2	*Beauveria bassiana* strain ARSEF 1478	96%	99.91%	HQ880908.1
ACP18006	RPB2	*Beauveria bassiana* strain ARSEF 1478	96%	99.73%	HQ880908.1
ACP18007	RPB2	*Beauveria bassiana* strain ARSEF 1478	96%	99.82%	HQ880908.1
ACP18008	RPB2	*Beauveria bassiana* strain ARSEF 1478	96%	99.73%	HQ880908.1
ACP18009	RPB2	*Beauveria bassiana* strain HN6	96%	99.91%	CP045883.1

FIGURES

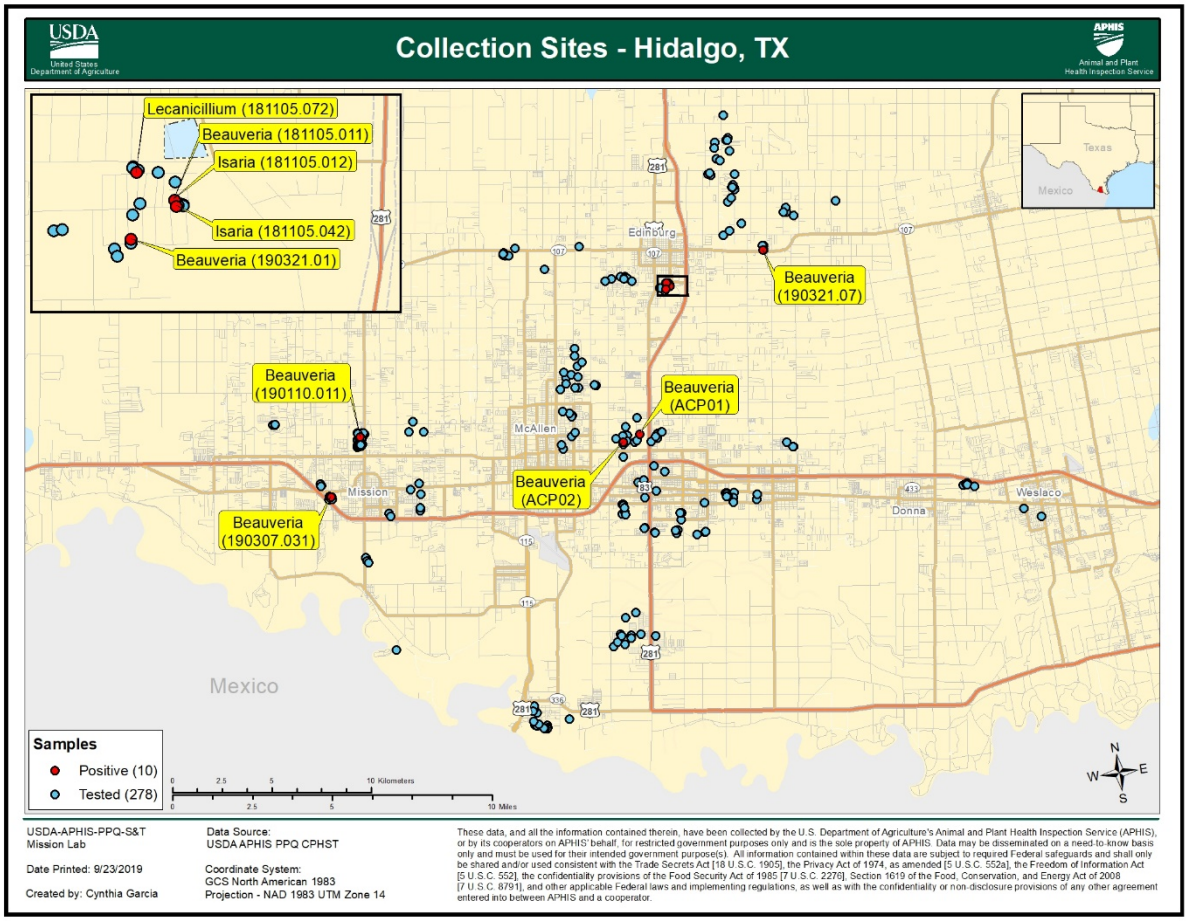

Figure 1. Map of the Lower Rio Grande Valley showing all collection sites. Red dots indicate those collection sites where *D. citri* showed entomopathogenic infection and yellow labels indicate the genus of the isolate found, as well as the original accession number related to the isolate.

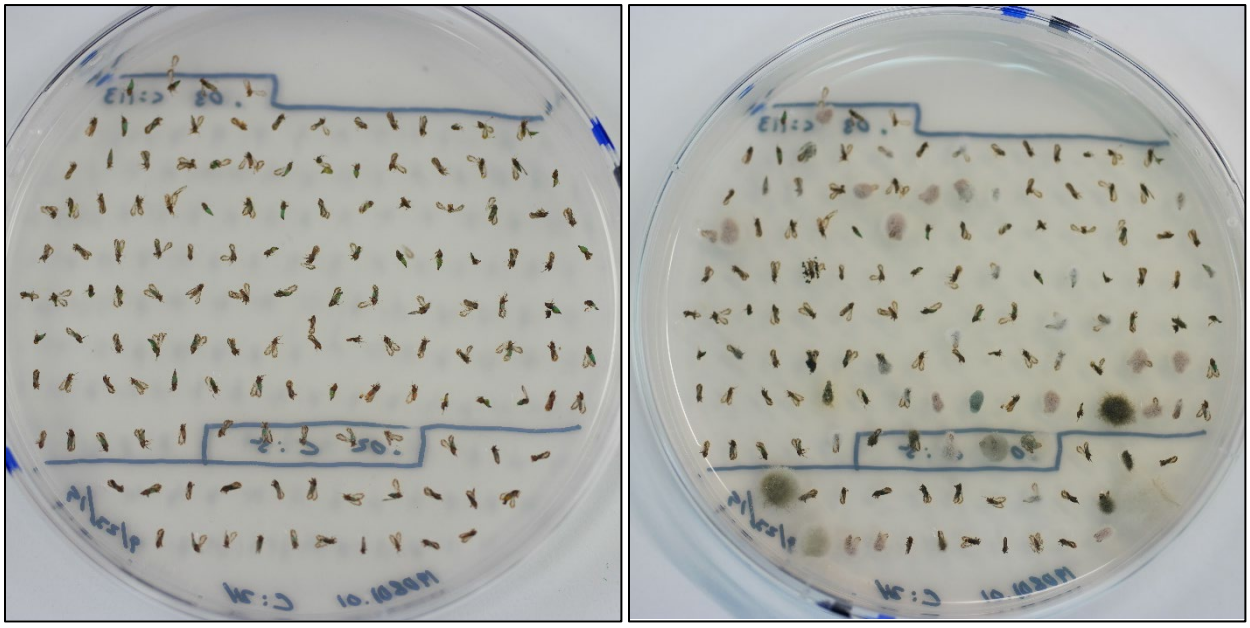

Figure 2. ACP cadavers plated on selective Water Agar (WA-DC) (a) before and (b) after the development of mycosis on cadavers.

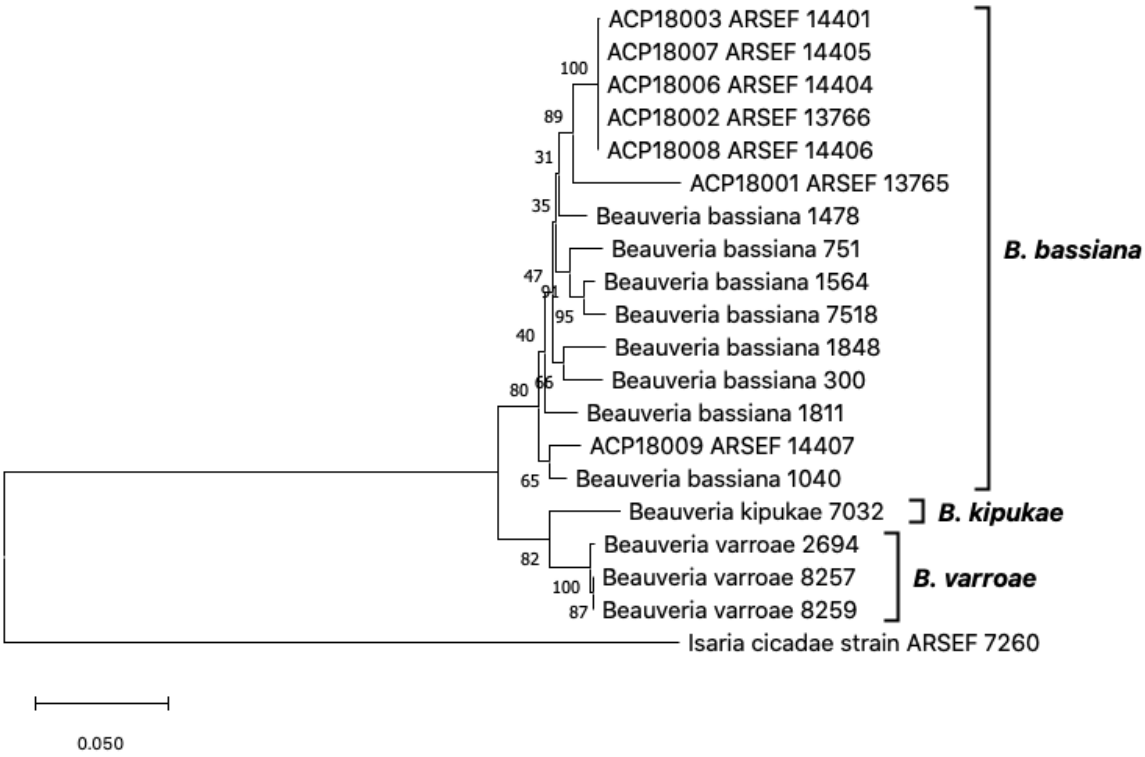

Figure 3. Phylogenetic tree of *Beauveria* illustrating species relationships inferred from Neighbor Joining (NJ) method of Bloc. Numbers above branches show boot support values for NJ methods (1,000 replicates). Species clades are in **bold** and indicated by vertical brackets.

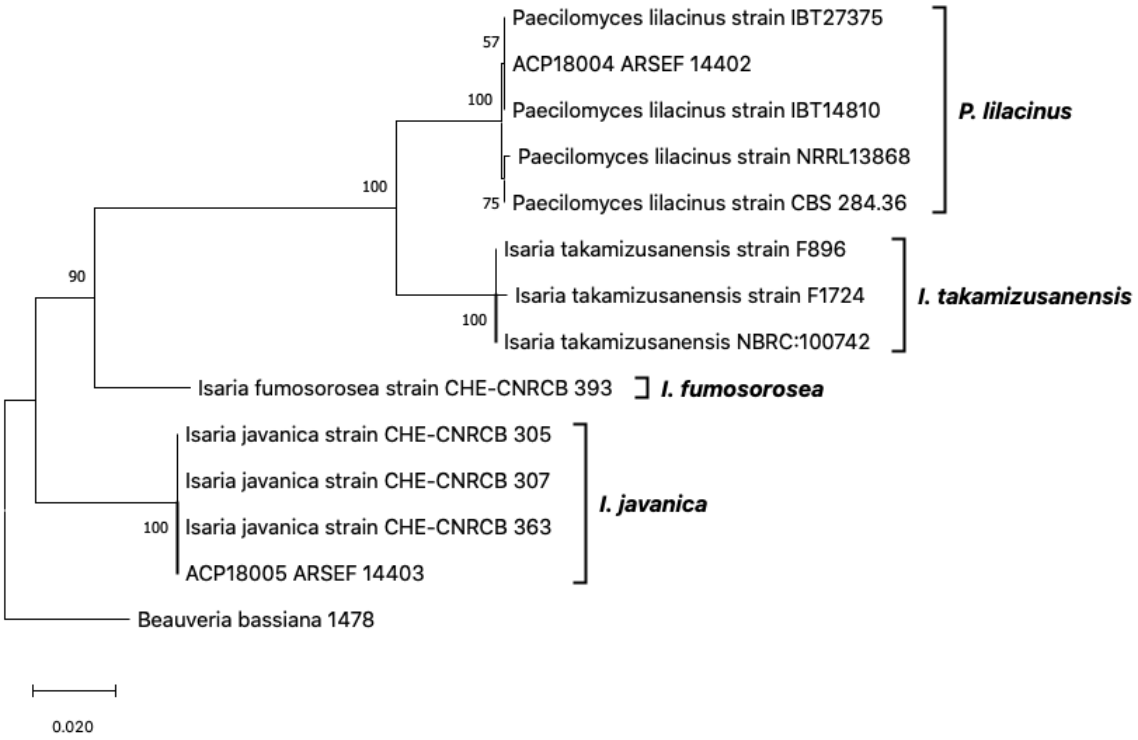

Figure 4. Phylogenetic tree of *Isaria* and *Paecilomyces* illustrating species relationships inferred from NJ method of EF1-α. Numbers above branches show boot support values for NJ methods (1,000 replicates). Species clades are indicated in **bold** and by vertical brackets.

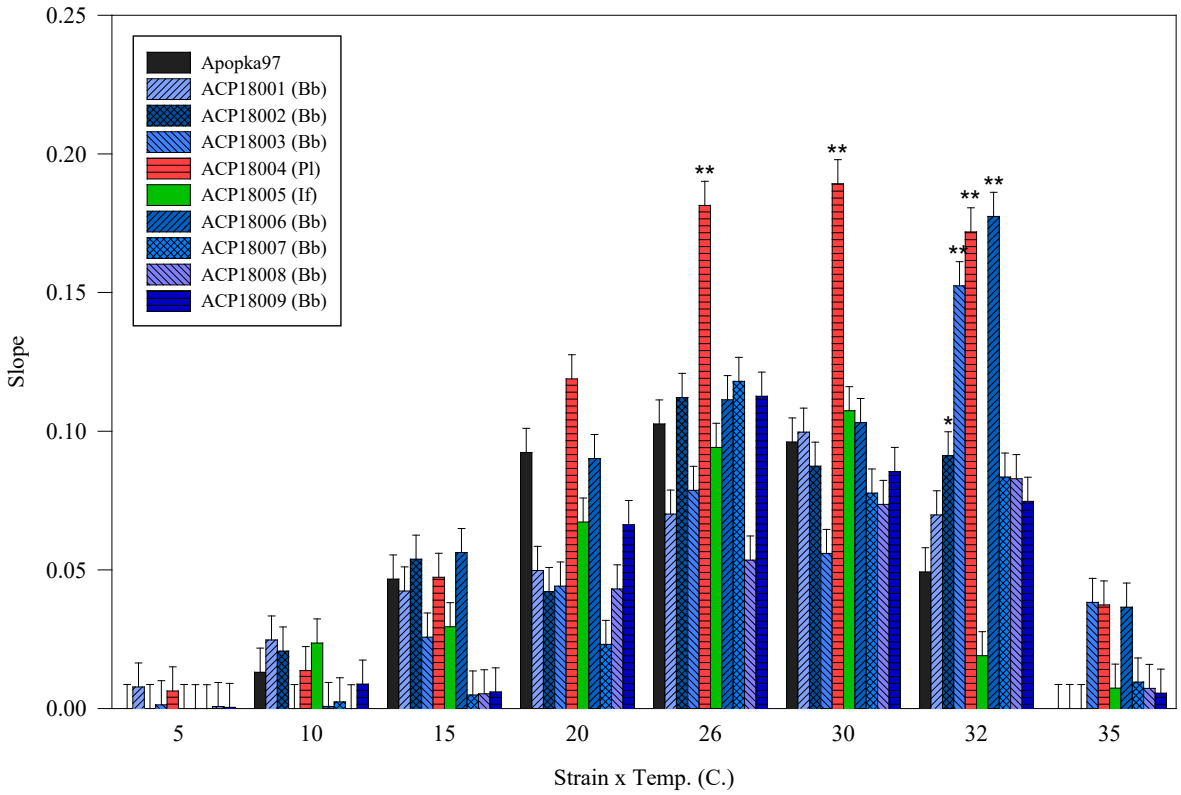

Figure 5. Bar graph with standard error of slopes of all field isolates and Apopka97 at the tested temperature points. Significance (*=*P<0.05;* **=*P<0.001*) is shown in comparison to standard, Apopka97)

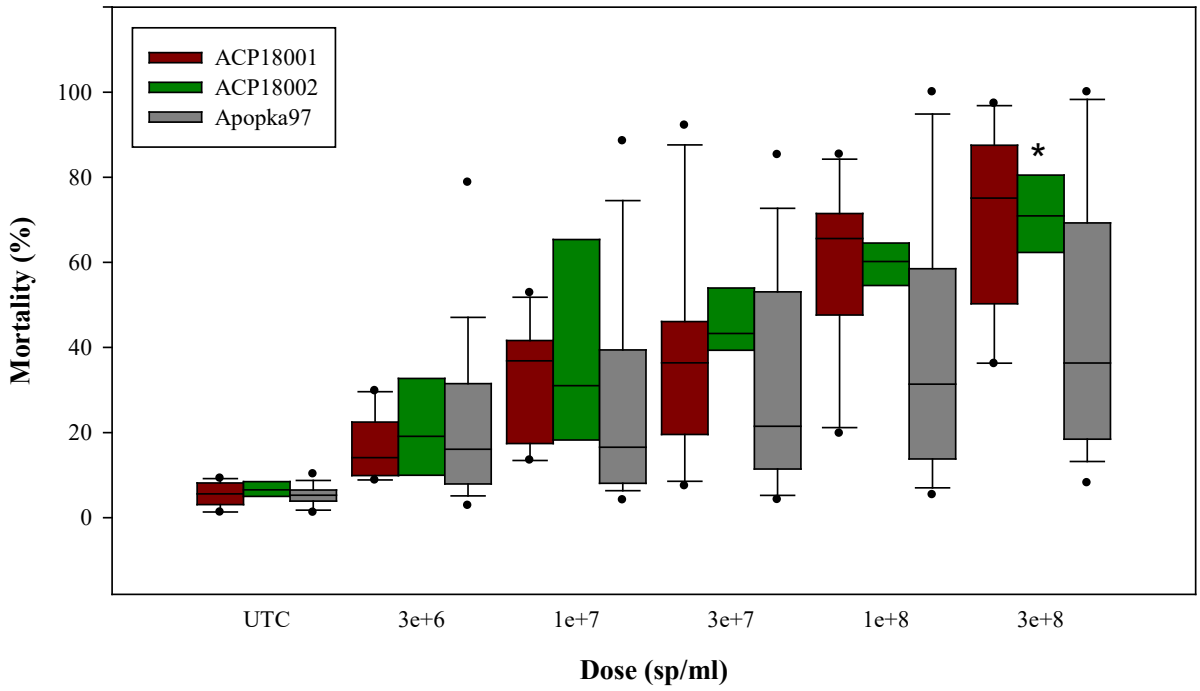

Figure 6a. Box and whisker graph of Primary Mortality Bioassays. Figure indicates the median (center line), lower and upper quartiles (box), and lower and upper extreme values (whiskers) for primary bioassay mortalities of ACP18001, ACP18002, and Apopka97. Asterisk indicates significant difference ($P<0.05$) between field isolates and Apopka97 at each dose. Single factor ANOVA and Tukey's post hoc test was performed using SigmaPlot. Where normality test failed, an ANOVA on Ranks was run along with Dunn's post hoc test.

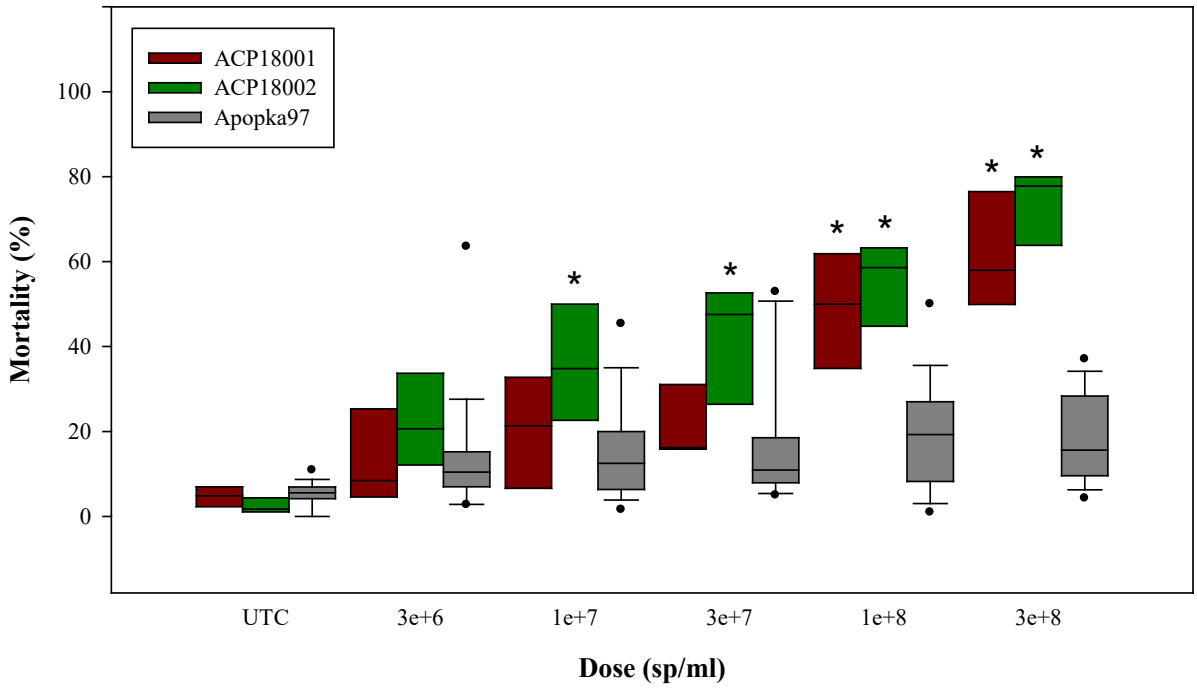

Figure 6b. Box and whisker graph of Secondary Mortality Bioassays. Figure indicates the median (center line), lower and upper quartiles (box), and lower and upper extreme values (whiskers) for secondary bioassay mortalities of ACP18001, ACP18002, and Apopka97. Asterisk indicates significant difference ($P<0.05$) between field isolates and Apopka97 at each dose. Single factor ANOVA and Tukey's post hoc test was performed using SigmaPlot. Where normality test failed, an ANOVA on Ranks was run along with Dunn's post hoc test.

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BIOGRAPHICAL SKETCH

Jonathan Cisneros earned a Master of Science in Biology in December 2020 from the University of Texas Rio Grande Valley. He began his scholastic journey at South Texas College where he completed his core, then moved on and received his undergraduate degree in Biology with a Minor in Psychology from UTRGV during the Fall of 2015. He was also employed by the same university as a Research Associate II, wherein his studies focused on various mechanisms of biological control against the citrus pest, *Diaphorina citri*. He currently resides at his permanent address: 3006 San Gabriel St., San Juan, TX 78589, and can be reached via email at jonathanpiba8@gmail.com.