University of Texas at Tyler

Scholar Works at UT Tyler

Biology Theses

Biology

Summer 6-14-2016

EXPLORATION OF CHEMICAL AND BIOLOGICAL MANAGEMENT STRATEGIES FOR DIAPHORINA CITRI THE PRIMARY VECTOR OF CANDIDATUS LIBERIBACTER ASIATICUS

Gretta Marie Sharp
University of Texas at Tyler

Follow this and additional works at: https://scholarworks.uttyler.edu/biology_grad

Part of the Agricultural Science Commons, Bioinformatics Commons, Biosecurity Commons, Entomology Commons, Food Microbiology Commons, Fruit Science Commons, Plant Pathology Commons, and the Toxicology Commons

Recommended Citation

Sharp, Gretta Marie, "EXPLORATION OF CHEMICAL AND BIOLOGICAL MANAGEMENT STRATEGIES FOR DIAPHORINA CITRI THE PRIMARY VECTOR OF CANDIDATUS LIBERIBACTER ASIATICUS" (2016). *Biology Theses.* Paper 37.
http://hdl.handle.net/10950/395

This Thesis is brought to you for free and open access by the Biology at Scholar Works at UT Tyler. It has been accepted for inclusion in Biology Theses by an authorized administrator of Scholar Works at UT Tyler. For more information, please contact tgullings@uttyler.edu.

EXPLORATION OF CHEMICAL AND BIOLOGICAL MANAGEMENT STRATEGIES FOR *DIAPHORINA CITRI* THE PRIMARY VECTOR OF *CANDIDATUS* LIBERIBACTER ASIATICUS

by

GRETTA MARIE SHARP

A thesis submitted in partial fulfillment
of the requirements for the degree of
Masters of Science of Biology
Department of Biology

Blake Bextine, Ph.D., Committee Chair

College of Arts and Sciences

The University of Texas at Tyler
April 2016

This is to certify that the Master's Thesis of

Gretta Marie Sharp

Has been approved for the thesis requirement on
May 17, 2016
For the Master's of Biology degree

Approvals:

Thesis Chair: Blake Bextine, Ph.D.

Member: Kate Hertweck, Ph.D.

Member: Dustin Patterson, Ph.D.

Member: Riqing Yu, Ph.D.

Chair, Department of Biology: Srini Kambhampati, Ph.D.

Dean, College of Arts and Sciences: Martin Slann, Ph.D.

Acknowledgements

I would like to thank my husband who has been an invaluable source of steadfast support and encouragement. I would also like to thank my parents and grandparents whose personal investment in my education has instilled in me the abilities required to accomplish my goals. Without the unwavering support of my family I would not have had been able to continue this challenging and rewarding endeavor.

To my colleagues in the Bextine lab, I offer more gratitude than I can express in words. You have been a source of knowledge and support that I will continue to appreciate as we depart on separate career paths. A special thank you to Chris Powell who has guided me from day one on this journey and who has always been willing to help regardless of how many projects he is working on. I would also like to thank Patrick Rydzak has been a great friend who has was willing to journey to Florida to assist with my project. To Megan Rudolph I would like to say that I will never forget the many hours that we agonized over class projects together. Thank you for your support. You have all been wonderful friends and I wish each one of you happiness and fulfillment.

Blake Bextine, I have appreciated the opportunity to work in your lab. You have helped me to develop academically and professionally by encouraging a high work ethic and independent inquiry. I have undergone a great amount of personal and professional growth in your lab and I attribute that to the opportunities I have been given while working for you. You have opened doors for me that have helped to shape my career and for that I am eternally grateful. Thank you for your mentorship.

I would like to thank the Department of Chemistry and Biochemistry at the University of Texas at Tyler for welcoming and mentoring me as a scientist and academic. As a graduate

adjunct I felt immediately welcomed into the family. I am sad to leave the wonderful mentors and friends that I have had the pleasure of knowing during the past two years. Working in such a positive and supportive environment gave me a wonderful feeling of belonging.

I would also like thank my committee members Dr. Kate Hertweck, Dr. R. Yu, and Dr. Dustin Patterson. Interacting with you has broadened my academic knowledge and encouraged me to pursue further education. I appreciate your guidance and support while I have been working on my thesis.

A special thank you to the labs of Dr. Stelinski and Dr. Pelz-Stelinski who gave me access to their labs and equipment in order to complete my thesis project. In addition to their professional generosity, Dr. Dr. Stelinski and Dr. Pelz-Stelinski opened their home to me and gave me wonderful professional advise that I will take with me into the rest of my career. I was humbled to feel so welcomed by each member of their labs.

Thank you to the lab of Dr. Setamou. It was at his lab were I completed my first bioassays and gained special insight into *Diaphorina citri*. It was wonderful to work with all of the students and professionals at the citrus center.

I would like to thank the Biology department of the University of Texas at Tyler for allowing me to enter this program and supporting me to its completion. I have enjoyed taking classes and making friends who are as excited about science as I am. Thank you for putting so much time into the education and development of your students.

Finally, I would like to thank Sean Whipple and the Ishihara Sangyo Kaisha, LTD (ISK) for providing supplies, funding and support for this project. It was there support that got this project off the ground. Thank you for choosing the Bextine Lab for this project.

Thank you,

- Gretta Marie Sharp

Table of Contents

List of Tables……………………………………………………………………………...………ii

List of Figures………………………………………………………………………………………iii

Abstract ……………………………………………………………………………………………...v

Chapter One: Overview of chemical and biological management of *D. citri*
Introduction………………………………………………………………………………..1

References……………………………………………………………………...……...…11

Chapter Two: *Diaphorina citri* response to cyclaniliprole
Introduction ………………………………………………………………………...…17
Materials and Methods……………………………………………………………...21
Results……………………………………………………………………………….....25
Discussion……………………………………………...……...32
References……………………………………...……...35

Chapter Three: *Diaphorina citri* response to flonicamid
Introduction…………………………………………………………………………38
Materials and Methods………………………………………………………...…40
Results…………………………………………………………………48
Discussion…………………………………………………………..51
References………………………………………………..53

Chapter Four: Identification of a *Diaphorina citri* alimentary tract associated bacterium
Introduction…………………………………………………………………………56
Materials and Methods……………………………………………………...…59
Results…………………………………………………………………63
Discussion……………………...…………………………...…69
References……………………………………..............70

Chapter Five: Conclusions and Future Research
Conclusion……………………………………………………………………………72

List of Tables

Table 1.1 Chemical application timeline for the management of *D. citri* in Texas………………9

Table 2.1 Probit transformation data…………………………………………………..……30

Table 4.1 Nucleotide BLAST using the NCBI database…...……………………………………64

List of Figures

Figure 1.1 Global Distribution of Three Economically Important Psyllid Species………………4

Figure 1.2 Adult *D. citri* with two late stage instar nymphs……….……………….………..……5
.
Figure 1.3 systemic symptoms of HLB in citrus……………………………………….…………7

Figure 2.1 Insect RyR receptor diagram…………………………………………………………19
.
Figure 2.2 Line structure of Cyclaniliprole vs. Ryanodine………………………………………20

Figure 2.3 Wild caught *D. citri* undergoing CO_2 anesthesia and plate transfer…….….…………21

Figure 2.4 Experimental setup for the Cyclaniliprole mortality bioassay…...…………………..22

Figure 2.5 *D. citri* percent mortality…………………………………………………………..…26

Figure 2.6 *D. citri* feeding at 24 hrs……………………………………………………….…….27

Figure 2.7 Dose response curves from the cyclaniliprole bioassay…………………………..….…28

Figure 2.8 Probit transformation modeled using SPSS……………………………….....……29

Figure 2.9 Detection of C. L. asiaticus from *D. citri* in cyclaniliprole bioassay…………..……31

Figure 2.10 Detection of C. L. asiaticus from leaves in cyclaniliprole bioassay……….………32
..
Figure 3.1 Line structure of flonicamid…………………………………………………………40

Figure 3.2 Wild caught *D. citri* undergoing CO_2 anesthesia and plate transfer………..…..……41

Figure 3.3 Petri plates with *Citrus sinensis* and *D. citri*…………………………………………42

Figure 3.4 Experimental set up for the systemic and translaminar Flonicamid assays on *D. citri*………………………………………………………………………………………..…..43

Figure 3.5 Close-up of the experimental set-up for fecal output behavioral bioassay for *D. citri* with *Citrus sinensis*..……..………………………………………………………………………44

Figure 3.6 Experimental bioassay set up for the foliar fecal output behavioral assay for *D. citri*……………………………………………………………………………………………45

Figure 3.7 *D. citri* bioassay with Flonicamid time lapse graphs………………………….………48

Figure 3.8 Foliar vs. Systemic flonicamid 1000ppm and 500ppm……………………………..48

Figure 3.9 *D. citri* fecal output in response to Flonicamid treatments…………………..…………49

Figure 3.10 Detection of *C*. L. asiaticus……………………..…………………..…………………50

Figure 4.1 The plasmids isolated from a *D. citri* bacterial culture were run on a gel……...……62

Figure 4.2 Contig 1 of the bacterium isolated from *D. citri* and identified as *Enterobacter cloacae*..………………………………………………………………………………………65

Figure 4. 3 Contig 2 of the bacterium isolated from *D. citri* and identified as *Enterobacter cloacae*..………………………………………………………………………………………66

Figure 4.4 Contig 3 of the bacterium isolated from *D. citri* and identified as *Enterobacter cloacae*..………………………………………………………………………………………67

Figure 4.5 Contig 4 of the plasmid isolated from *D. citri* and identified as *Enterobacter cloacae*..………………………………………………………………………………………68

Abstract

EXPLORATION OF CHEMICAL AND BIOLOGICAL MANAGEMENT STRATEGIES FOR *DIAPHORINA CITRI* THE PRIMARY VECTOR OF *CANDIDATUS* LIBERIBACTER ASIATICUS

Gretta Marie Sharp

Thesis Chair: Blake Bextine, Ph.D.

The University of Texas at Tyler
April 2016

Diaphorina citri (Hemipteran: Psyllidae), the Asian citrus psyllid, is a phloem-feeding, invasive species endemic to southern Asia that was first identified in the United States in Florida in 1998. Since introduction, *D. citri* has spread across the major citrus growing regions of the United States. This is of great concern to the citrus industry because *D. citri* is the primary vector of *Candidatus* Liberibacter asiaticus the causal agent of Haunglongbing (HLB). Haunglongbing is the most economically devastating disease of citrus causing an estimated 8.92 billion dollars lost in revenue and 4.62 billion dollars lost in gross domestic product between 2006-2007 in Florida alone (Hodges and Spreen, 2012). Current management strategies have had some effect on the spread of *Candidatus* Liberibacter asiaticus; however, due to concerns about insecticide resistance and the effect on non-target organisms, new pest management strategies need to be considered. Most of the current insecticides used to treat psyllids are neuromuscular toxins which affect the nervous system and muscles. Cyclaniliprole is an anthranilic diamide that acts on the endoplasmic reticulum's ryanodine receptor in insects. When *D. citri* were exposed to citrus leaf substrate containing cyclaniliprole significant mortality was observed. Flonicamid is an antifeedent currently used on sap feeding insects like aphids. *D. citri* exposed to leaves treated with flonicamid had an alteration in feeding behavior. Flonicamid also has very low toxicity to

humans. Possible biological management strategies were also considered. Paratransgenesis is a strategy that attempts to remove a pathogens transmission ability from a vector by transforming a symbiont of the vector that would produce a protein that is toxic to the pathogen. In this case, the paratransgeneic model would be to remove *Candidatus* Liberibacter asiaticus from *D. citri* using an alimentary associated bacterium isolated from *D. citri* an alimentary canal associated bacterium that was isolated from *D. citri*. This bacterium was identified as *Enterobacter cloacae* and the bacterium and associated plasmid were annotated for use in a future paratransgeneic management strategy for *D. citri*.

Chapter One

Exploring Chemical and Biological Management Strategies of *Diaphorina citri* the Primary Vector of *Candidatus* Liberibacter asiaticus

1.1 Introduction

The Asian citrus psyllid, (*Hemiptera; Homoptera*) *Diaphorina citri*, jumping plant lice are phytophagous, true bugs, taxonomically located in the class *Insecta*, order *Hemiptera*, suborder *Sternorrhyncha*, superfamily *Psylloidea* and family *psyllidae* (Burckhardt et al., 2012). Psyllids are included in the most primitive suborder of *Hemiptera, Sternorrhyncha* (*Homoptera*), due to the distal position of their mouth parts with respect to their head (Burckhardt et al., 2012). Other members of this phytophagous suborder include aphids, whiteflies and scale insects. The family psyllidae of which *D. citri* is a member, tend to be host specific, and include members that are classified as monophagous or oligophagous. Fossils of psyllids have been dated to the Permian (252-299 million years ago), which predates the appearance of angiosperms (100-125 million years ago) in the fossil record (Hodkinson, 1980). It is hypothesized that psyllids of that time would have fed from gymnosperms or lycopods (Hodkinson, 1980). After the emergence of angiosperms in the Cretaceous, many members of psyllidae shifted away from their previous food sources to angiosperms (Mitter, Farrell and Wiegmann, 1988). This relationship has been used to support the hypothesis that plant/insect interactions may not be phylogenetically fixed traits and are instead a shifting mosaic influenced by the environment, as well as, the local genetic and demographic structure of a population (Thompson, 1994). This could give insight into *D. citri's* relationship with citrus, as well as its acquisition of bacterial symbionts. Bacterial symbionts play a key role in an insects ability to adapt to new environments and food sources

(Planck et al., 2015). The majority of bacterial symbionts are vertically inherited, however some can be horizontally inherited (Ferrari and Vavre, 2011). Three bacterial endosymbionts commonly associated with *D. citri* are *Wolbachia sp.* from the Alphaproteobacteria group often found with arthropods, *Canadidatus* Carsonella ruddii a species of Gammaproteobacterium that is associated with nutritional acquisition for the host, and *Candidatus* Profftella armature a species of Betaproteobacterium that produces the defensive polyketide disphorin (Chu et al., 2016). If the pathogen *Canadidatus* Liberabacter asiaticus is acquired by *D. citri*, a change in the relative abundance of *Wolbachia*, *Canadidatus* Carsonella ruddii and *Candidatus* Profftella armature will correlate with the relative abundance of *Canadidatus* Liberabacter asiaticus. This indicates a possible relationship between these bacterial symbionts and the pathogen. Other bacterial symbionts that have been associated with *D. citri* are *Nitrosospira multiformis*, *Alkanindiges illinoisensis*, *Buchnera aphidicola* and *Enterobacter cloacae*. *Enterobacter cloacae* is a Gammaproteobacteria that has been completely sequenced and extensively studied in both insects and humans due to its abundance (Li et al., 2006). These bacterial symbionts aid in the reproduction, nutritional acquisition and acquired defenses of *D. citri* and contribute to the overall fitness of the psyllid.

Approximately 40 species of psyllid are known plant pests (Percy et al., 2005). Adult psyllids feed using a thin stylet to penetrate parenchyma cells surrounding the vascular bundles of the host plant and feed from the phloem of the plant (Eyer and Crawford, 1933). The adult psyllids lay eggs on newly emerged foliage, and after emergence the nymphs feed on the phloem fluid from the tender leaves. Due to the high sugar content of their diet, the nymphs produce a sugary, waxy, solid excrement known as “honeydew” or “psyllid sugars”.

The three most economically relevant psyllid species that affect food crop production are: *Bactericera cockerelli* (potato psyllid), *Diaphorina citri* (Asian citrus psyllids), and *Trioza apicalis* (carrot psyllid). These three psyllid species are competent vectors of the phytopathogen *Candidatus* Liberibacter. *Trioza apicalis* populations are consolidated in Northern Europe and are not of economic concern in the United States. *Bactericera cockerelli* populations are spread throughout North America. *D. citri* originated in eastern Asia and now thrives in southeast Asia, the subcontinent of India, the Reunion and Mauritius islands, Saudi Arabia, Iran, Brazil, Venezuela, Argentina, Guadeloupe and other major citrus growing regions (Lin, 1956; Mead, 1977) (Cermeli et al., 2000; Mead, 1977) **(Figure 1.1)**. In 1998 *D. citri* was identified in Florida, and since then populations have spread across United States affecting the major citrus growing states of Florida, Texas, Arizona and California (Grafton-Cardwell et al. 2006). *Diaphorina citri* and *B. cockerelli* populations overlap in the southern United States but cold winters in the mid to northern states have slowed the cold sensitive *D. citri* population's progression northward. This could be one reason why the citrus production in California has been less affected by *Candidatus* Liberibacter asiaticus compared to Florida.

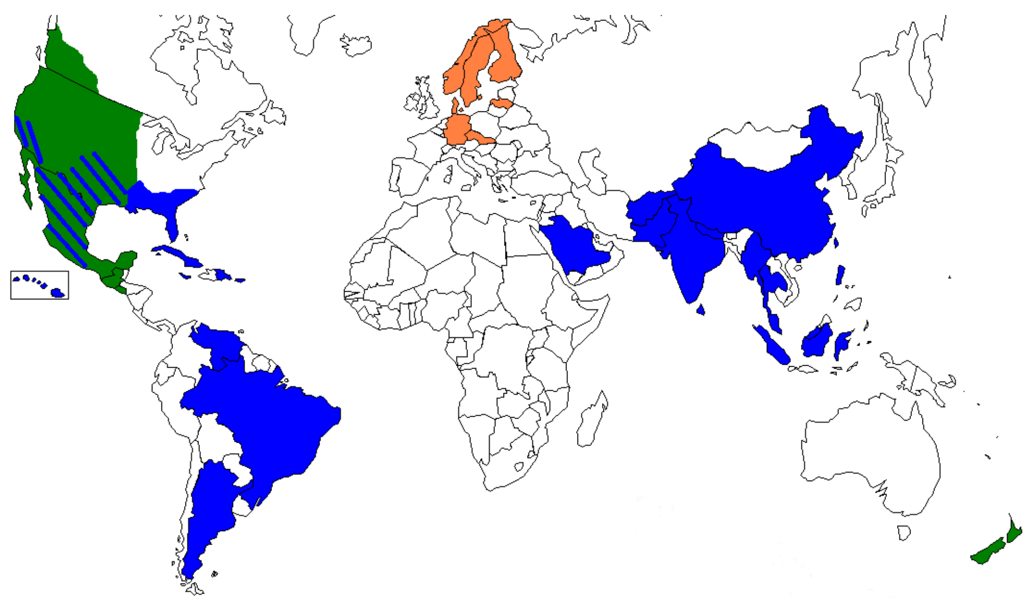

Figure 1.1 Global Distribution of Three Economically Important Psyllid Species (Daymon Hail, 2008) Green: *Bactericera cockerelli*, Orange: *Trioza. apicalis*, Blue: *Diaphorina. citri*. Green and Blue stripes indicate overlapping populations of the stripe colors. (Hail, 2011)

Diaphorina citri Kuwayama (Hemiptera: Liviidae) feeds on *Citrus sp.* and related species (Burckhardt et al., 2012). It has been determined that there are two major haplotypes of *D. citri* by analysis of the mitochondrial cytochrome oxidase I (Boykin et al., 2012). The two haplotypes are southwestern Asia (SWA) *D. citri,* and southeastern Asian (SEA) *D. citri;* predominate haplotype found in North America is SWA, while both SWA and SEA are found in South America (Boykin et al., 2012). *Diaphorina citri* undergoes a temperature dependent 30-day life cycle, and is hemi-metabolus. The optimum temperature for rearing *D. citri* is 25-28°C (Liu and Tsai, 2000). The nymph stage includes five instars; the first instar measures 0.25 mm in length and will reach to 1.7 mm in length in the fifth instar. Wing pads become visible during the second instar as well as abdominal filaments around the apical plate (Hall et al., 2013)**(Figure 1.2)**. When *D. citri* is not transmitting a pathogen, it is considered a minor plant pest; however, it is economically important as the primary vector of the pathogen *Candidatus* Liberibacter

asiaticus (Jagoueix et al., 1994). This pathogen is one of the Liberibacter species known to cause Huanglongbing (HLB), a greening disease of citrus (Jagoueix et al., 1994).

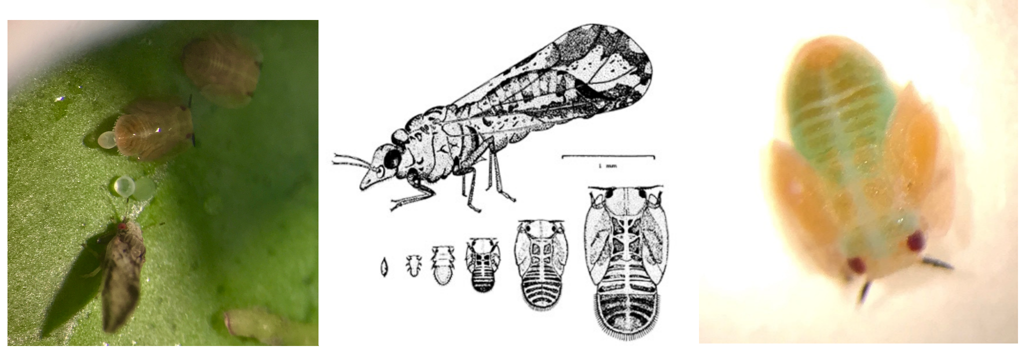

Figure 1.2 (A) **Adult *D. citri* with two late stage instar nymphs**. “Honeydew” excreted from the left most nymph above the adult psyllid. (C) V instar nymph with prominent wing pads. (B) Adult female *D. citri* with egg and five nymphal instars. (Illustration from the UF IFAS Division of Plant Industry)

Bacteria of the genus *Candidatus Liberibacter* are known to be causal agents of greening diseases: *C.* L. africanum, *C.* L. asiaticus, *C.* L. solanacearum, *C.* L. americanus, *C.* L. europaeus, *C.* L. psyllaurous and *C.* L. crescens (Garnier et al., 2000; Planet et al., 1995; Crosslin et al., 2009). All species are fastidious phloem-restricted gram-negative bacteria that have not been successfully cultured *in vitro* (Garnier et al., 2000; Planet et al., 1995; Crosslin et al., 2009). Despite the inability to culture *Candidatus* Liberibacter sp. *in vitro,* the genomes of *C.* L. asiaticus strains can be sequenced directly from psyllid vectors or host plants using next generation sequencing (Wu et al., 2015). This satisfies molecular Koch’s postulates, which are a series of experimental criteria used to show that an organism is the causal agent of a disease (Falkow, 1988). *Candidatus* Liberibacter africanum, *C.* L. asiaticus, and *C.* L. americanus are the known causal agents of Haunglongbing, an economically destructive disease of citrus (Kuwayama, 1908). Similar management strategies have been employed against *B. cockerelli*

and *D. citri* because of the analogous manner in which *C.* L. solanacearum and *C.* L. asiaticus are transmitted to their respective hosts. This common management strategy is helpful when studying *C.* L. asiaticus because of its status as a restricted agent due to the economic devastation it has caused to the Florida citrus industry. Since *C.* L. asiaticus and *D. citri* are so restricted, experiments are performed on *B. cockerelli* with *C*. L. solanacearum and then applied to *D. citri*.

Huanglongbing, Likubin, and citrus greening all refer to the same systemic disease of citrus. HLB is considered to be the most economically detrimental and lethal disease of citrus. Two species of *Candidatus* Liberibacter are known to cause HLB (Bove et al., 1974). The first is *C.* L. africanum transmitted *D. citri*, requires temperatures below 30°C for the symptoms of HLB to develop (Tsai and Liu, 2000). This temperature requirement has helped to limit the range of this heat-sensitive bacterium to southern Africa (Tsai and Liu, 2000). The second species of interest is *C.* L. asiaticus, this species is more tolerant of high temperatures (Tsai and Liu, 2000). *Canadidtus* Liberibacter asiaticus is transmitted by *D. citri* and can be found in Asia and the Americas (Garnier et al., 2000; Planet et al., 1995). Early symptoms of this disease are characterized by leaf shoot yellowing and leaf mottling (Garnier et al., 2000; Planet et al., 1995). *Canadidtus* Liberibacter asiaticus spreads throughout the phloem of the plant from the inoculation site causing stunting and dieback (Garnier et al., 2000). As the disease progresses, the fruits from affected trees are asymmetrical in shape, size and color; bitter in taste and fail to ripen (Garnier et al., 2000)**(Figure 1.3)**. The perpetually green fruit gave rise to the colloquialism citrus greening.

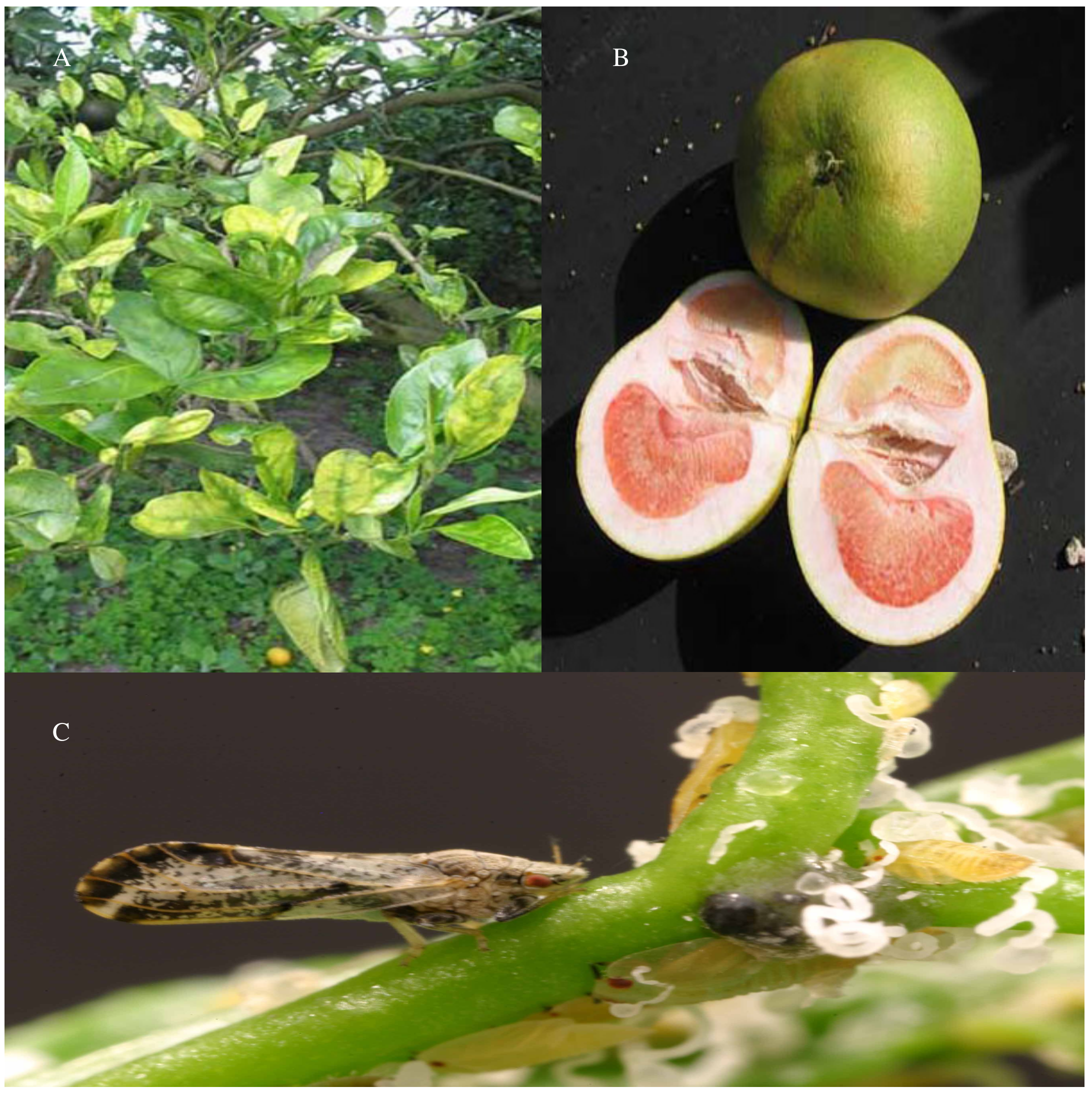

Figure 1.3 (A) S**ystemic symptoms of HLB in citrus**. (B) Depiction of asymmetry and greening in citrus fruit due to Liberibacter sp. (C) Adult *D. citri* feeding, and *D. citri* nymphs feeding and producing “honeydew” tubes. (Warnert 2013 ANR UC Divison)

Conventional chemical management of *D. citri* populations involves the use of multiple insecticides, no single insecticide has proven to adequately manage *D. citri* populations (Tansey et al., 2015). In part, this is because a single insecticide is not effective against all the life stages of *D. citri* (Tansey et al., 2015). Many insecticides have difficulty in reaching the egg stage. Currently a “layered” management approach is used, which involves the use of different insecticides at different times of the year when they are most effective **(Table 1.1)**(Boina, 2015).

In Texas, current recommended chemical management strategy is a dormant spray of Pyrethroids, Organophosphates (OP), and Neonicotinoids treatments January through March. Treatments with Neonicotinoids, lipid biosynthesis inhibitors (LBI), Spinosyns, Diflubenzurons, Pyrethroids and Carbamates are recommended April through September. Another dormant spray is advised October through December with Ops and Pyrethroids **(Table 1.1)**. These recommendations for Texas are based on psyllid population fluctuation in response to flush shoot cycles in citrus. In Texas, citrus flush shoots emerge in late September to early October, there is a direct correlation between flush emergence and psyllid populations, this increase in *D. citri* population coincides with an increase in HLB. Neonicotinoid insecticides are commonly used but require root activity for effective uptake, which limits their application to June through September (Boina, 2015). Insecticides that require root uptake for systemic movement within the plant are limited to growing months. If an insecticide is not taken up by the plant it will not be affective against piercing and sucking insects like psyllids. Neonicotinoid insecticides which act as a Nicotinic acetylcholine receptor (nAChR) antagonists have been scrutinized for the possible negative effect they may have on non-target insects, particularly important pollinators like *Alpis mellifera* (European honey bee) and mammals (Boina, 2015). Currently, there has been a push for the development of targeted long term insecticides and growth regulators (IGRs) to combat the *D. citri* insecticide resistance and reduce the effect on non-target organisms like *Alpis mellifera* and mammals (Boina, 2015). Diamides are a group of insecticides that target insect ryanodine receptors (RyR) and are inactive against cells that do not express insect RyRs. Diamides are potent and the specificity of the target site reduces target-site cross resistance with other chemotypes (Qi, 2014). The specificity of the target site also reduces the toxicity to mammals (Qi, 2014). Cyclaniliprole is an anthranilic diamide insecticide, which acts as a RyR

allosteric (Mustafa et al., 2015). RyR receptors are specialized intercellular rapid release Ca^{2+} channels (Mustafa et al., 2015). Diamide insecticides disrupt the muscle contraction process using an allosteric mechanism (Mustafa et al., 2015). It is hypothesized that this disruption of the muscle contraction process will inhibit *D. citri* feeding. Another insecticide of interest is flonicamid (ISK Biosciences Corporation, Concord, OH). This systemic compound works as an antifeedant in sap sucking insects with a novel mode of action that has no statistically significant impact on non-target insects (Black et al., 2007).

Table 1.1 A current chemical application timeline for the management of *D. citri* in Texas (Biona et al., 2015). Root uptake from the citrus plant is important for some compounds and it is therefore recommended to apply those compounds only when citrus is in its active growing stage. Other compounds are recommending during the dormant stage of citrus to management *D. citri* populations.

Treatment Compounds	**Time of Year Corresponding to Citrus Growth Cycle**		
	Dormant Treatment (Jan-March)	Active Uptake Treatment (April-Sept)	Dormant Treatment (Oct-Dec)
Pyrethroids class insecticides	X	X	X
Organo Phosphate class insecticides	X		X
Portal (Fenpyroximate)	X		X
Neonicotinoids class insecticides	X		
Movento (Spirotetramat)	X	X	
Imidacloprid compound insecticides		X	
Thiamethoxam compound insecticides		X	
Delegate (Spinetoram)		X	
Micromite (Diflubenzuron)		X	
Carbamates class insecticides		X	
Leverage (Imidaclopride + beta-cyfluthrin)		X	

Current biological management strategies of *D. citri* have had limited effect (Hoddle and Hoddle, 2013). In California in 2012, two species of parasitic wasps, *Tamarixia radiate* and *Diaphorencyrtus aligarhensis* were released in the hopes that the wasps would parasitize *D. citri* nymphs (Hoddle and Hoddle, 2013; Hoddle, 2015). In an experimental setting, *D. citri* nymphs were parasitized at a rate as high as 70%; however, in the field, there has been no statistically significant affect on *D. citri* populations (Hoddle and Hoddle, 2013; Hoddle, 2015).

Paratransgenesis has been considered as a possible biological management strategy for *D. citri*. This strategy is used for the management of vector-borne diseases by transforming a symbiont of the vector (Durvasula et al., 1997). *Diaphorina citri* has many bacterial symbionts permanently residing in specialized cell bundles called bacteriomes (Baumann et al., 1995). The main issue with these symbionts from *Candidatus* is that they are not able to be cultured and therefore are not a viable option for transformation at this time. The primary symbiont in *D. citri* is *Candidatus* Carsonella ruddii; this symbiont supplements nutrients and metabolites like amino acids that are missing from the diet of *D. citri*. The genome of the endosymbiont *Candidatus* Carsonella ruddii is organelle like in its simplicity (Nakabachi et al., 2006). *Wolbachia sp.* is an important insect endosymbiont that has been identified as a sex ratio influencer in *B. cockerelli* (Liu et al., 2006). The identification and annotation of a *D. citri* symbionts gives a clearer picture of the symbiont community of D. citri, but also drive toward a more long term management strategy for *D. citri*.

References Cited

Baldo, L., Hotopp Dunning, J., Jolley, K., Bordenstein, S., Biber, S., Choudhury, R., Hayashi, C., Maiden, M., Tettelin, H., and Werren, J. 2006. Multilocus sequence typing system for the endosymbiont Wolbachia pipientis. Appl Env. Microbiol., 72(11): 7098–110.

Baumann, P., L. 1995. Genetics, physiology, and evolutionary relationships of the genus *Buchnera*: intracellular symbionts of aphids. Annual Reviews in Microbiology 49(1): 55-94.

Boykin, L. M., De Barro, P., Hall, D. G., Hunter, W. B., McKenzie, C. L., Powell, C. A., and Shatters, R. G. 2012. Overview of worldwide diversityof Diaphorina citri Kuwayama mitochondrial cytochrome oxidase 1haplotypes: two Old World lineages and a New World invasion. Bull. Entomol. Res., 102(05): 573–582.

Burckhardt, D., and Ouvrard, D. 2012. Article A revised classification of the jumping plant-lice (Hemiptera : Psylloidea). 1(34): 1–34.

Davidson, E. W., Segura, B. J. 1994. Microorganisms influence the composition of honeydew produced by the silverleaf whitefly, *Bemisia argentifolii*. Journal of Insect Physiology 40(12): 1069-1076.

Davidson, M. M., Teulon, D.A.J., Scott, I.A.W. and Workman, P. 2008. A review of the potato psyllid (*Bactericera cockerelli*); a new insect pest of potato in New Zealand - A report prepared for Horticulture New Zealand. New Zealand Institute for Crop & Food Research Limited 12(13): 24-27.

Duan, Y., Zhou, L., Hall, D. G., Li, W., Doddapaneni, H., Lin, H., Liu, L., Vahling, C. M., Gabriel, D. W., Williams, K. P., Dickerman, A., Sun, Y., and Gottwald, T. 2009.

Complete Genome Sequence of Citrus Huanglongbing 38 Bacterium, 'Candidatus Liberibacter asiaticus' obtained through metagenomics. MPMI 22: 1011–1020.

Eyer, J. and Crawford, R. 1933. Observations on the Feeding Habits of the Potato Psyllid (*Paratrioza cockerelli* Sulc.) and the Pathological History of the" Psyllid Yellows" Which It Produces. Journal of Economic Entomology 26(4): 846-850.

Gai, C. S., Lacava, P. T. 2009. Transmission of *Methylobacterium mesophilicum* by *Bucephalogonia xanthophis* for paratransgenic control strategy of Citrus Variegated Chlorosis. The Journal of Microbiology 47(4): 448-454.

Goolsby, J., Adamczyk, J. 2007. Development of an IPM program for management of the potato psyllid to reduce incidence of zebra chip disorder in potatoes. Subtropical Plant Science 59: 85-94.

Halbert S. E. 2005. The discovery of huanglongbing in Florida, presentation H3. Proceedings of 2nd International Citrus Canker and Huanglongbing Research Workshop. (1) 7-11.

Hall, D. G., Hentz M. G., Adair R. C. 2008. Population ecology and phenology of Diaphorina citri (Hemiptera: Psyllidae) in two Florida citrus groves. Environ Entomol. 37(4):914-24

Hall, D. G., Richardson, M. L., Ammar, E.-D., and Halbert, S. E. 2013. Asian citrus psyllid, Diaphorina citri , vector of citrus huanglongbing disease. Entomol. Exp. Appl., 146(2): 207–223.

Hodges, A. W., and Spreen, T. H., 2012, Economic Impacts of Citrus Greening (HLB) in Florida. Appl Micro 2(13): 1-6.

Hunter, W., Dowd, S. 2009. Psyllid biology: expressed genes in adult Asian citrus psyllids, *Diaphorina citri* Kuwayama. Open Entomology Journal 3: 18-29.

Jagoueix, S., Bove, J.-M., and Garnier, M. 1994. The Phloem-Limited Bacterium of Greening Disease of Citrus Is a Member of the α subdivision of the proteobacteria, Int. J. Syst. Bacteriol., 44(3): 379–386.

Kumagai L. B., LeVesque C. S., Blomquist C. L., Madishetty K., Guo Y., Woods P. W., Rooney-Latham S., Rascoe J., Gallindo T., Schnabel D., Polek M. 2013. First report of "Candidatus Liberibacter asiaticus" associated with citrus huanglongbing in California. Plant Dis 97:283. http:// dx.doi.org/10.1094/PDIS-09-12-0845-PDN.

Kuwayama, S. 1908. Die Psylliden Japans. I," Trans. Sapporo Nat. Hist. Soc., II, pp. 149–189.

León, J. H. De, Sétamou, M., Gastaminza, G. a., Buenahora, J., Cáceres, S., Yamamoto, P. T., Bouvet, J. P., and Logarzo, G. A. 2011. Two Separate Introductions of Asian Citrus Psyllid Populations Found in the American Continents. Ann. Entomol. Soc. Am., 104(6), pp. 1392–1398.

Liefting, L., Ward, L. 2008. A New '*Candidatus* Liberibacter' Species in *Solanum betaceum* (Tamarillo) and *Physalis peruviana* (Cape Gooseberry) in New Zealand." Plant Disease 92(11):1588-1588.

Liefting, L. W., Weir, B. S. 2009. *Candidatus* Liberibacter solanacearum associated with plants in the family Solanaceae. International Journal of Systematic and Evolutionary Microbiology 59(9):2274.

Liu, D., Trumble, J. T. 2006. Genetic differentiation between eastern populations and recent introductions of potato psyllid (*Bactericera cockerelli*) into western North America. Entomologia experimentalis et applicata 118(3):177-183.

Li W., Hartung J.S., Levy L. 2006. Quantitative real-time PCR for detection and identification of Candidatus Liberibacter species associated with cit rus huanglongbing. J Microbiol Methods 66:104 –115. http://dx.doi.org/ 10.1016/j.mimet.2005.10.018.

Manjunath, K., S. Halbert, et al (2008). "Detection of *'Candidatus* Liberibacter asiaticus' in *Diaphorina citri* and Its Importance in the Management of Citrus Huanglongbing in Florida." Phytopathology 98(4):387-396.

Marutani-Hert, H. Hunter W. B., Dowd, S. 2008. Endosymbiotic microbiota of Asian Citrus Psyllid (Diaphorina citri) IRCHLB Proceedings 6.6 224-227.

Munyaneza, J., Crosslin, J. 2007. Association of *Bactericera cockerelli* (Homoptera: Psyllidae) with Zebra Chip," a New Potato Disease in Southwestern United States and Mexico. Journal of Economic Entomology 100(3):656-663.

Nakabachi, A., Yamashita, A. 2006. The 160-kilobase genome of the bacterial endosymbiont *Carsonella*. Science 314(5797): 267.

Pearson, W. R. 1990. Rapid and Sensitive Sequence Comparison with FASTP and FASTA. Methods Enzymol. 183: 63–98.

Sandström, J., Moran M. 1999. How nutritionally imbalanced is phloem sap for aphids? Entomologia Experimentalis et Applicata 91(1): 203-210.

Setamou, M., J. V. Da Graca. J. L. Sandoval. 2015. Siutability of native North American Rutaceae to serve as host plants for the Asian citrus psyllids. (Hemiptera: Liviidae). Journal of Applied Entomology. 4(8): 6-12.

Reese, J., Christenson, M. K., Leng, N., Saha, S., Cantarel, B., Tamborindeguy, C., Maccarthy, J., Weaver, D., Trease, A. J., Steven, V., Davis, V. M., Mccormick, C., Haudenschild, C., Han, S., Shannon, L., Shelby, K. S., Huang, H., Bextine, B. R., Shatters, R. G., Hall, D.

G., Paul, H., and Hunter, W. B. 2014. Characterization of the Asian Citrus Psyllid Transcriptome. Genomics 2: 54–58.

Rogers, M. E., Stansly, P. A., Stelinski, L. 2014. Florida Citrus Pest Management Guide: Asian Citrus Psyllid and Citrus Leafminer. IFAS Extension 1: 1-9.

Rosenthal, G., McNally, A. 2010. USDA Announces Quarantine to Prevent Spread of Citrus Disease. 1: 1-9.

Teixeira, D., Saillard C., Eveillard S., Danet J., Costa P., Ayres A., Bové J. 2005. Managing the Spread of HLB. Int J Syst Evol Microbiol 55(5):1857-1862. doi:10.1099/ijs.0.63677-0.

Thao, M. L. L., Clark M. A. 2001. Phylogenetic Analysis of Vertically Transmitted Psyllid Endosymbionts (*Candidatus* Carsonella ruddii) Based on atpAGD and rpoC: Comparisons with 16S–23S rDNA-Derived Phylogeny. Current Microbiology 42(6): 419-421.

Tiwari, S., Gondhalekar, A. D., Mann, R. S., Scharf, M. E., and Stelinski, L. L. 2011. Characterization of five CYP4 genes from Asian citrus psyllid and their expression levels in Candidatus Liberibacter asiaticus-infected and uninfected psyllids. Insect Mol. Biol. 20(6): 733–44.

Yang, Y., Huang, M., Beattie, G. A. C., Xia, Y., Ouyang, G., and Xiong, J. 2006. Distribution, biology, ecology and control of the psyllid Diaphorina citri Kuwayama, a major pest of citrus : A status report for China. Int. J. Pest Manag. 52(12): 343–352.

Zheng Z, Deng X, Chen J. 2014. Draft genome sequence of “Candidatus Liberibacter asiaticus” from California. Genome Announc 2(5):e00999-14. http://dx.doi.org/10.1128/genomeA.00999-14.

Zheng Z., Deng X., Chen J. 2014. Whole-genome sequence of “Candidatus Liberibacter asiaticus” from Guangdong, China. Genome Announc 2(2): e00273-14. http://dx.doi.org/10.1128/genomeA.00273-14.

Chapter 2

Diaphorina citri response to Cyclaniliprole

2.1 Introduction

Diaphorina citri, the Asian citrus psyllid, is a phloem feeding pest of citrus and primary vector of the phloem limited bacteria *Canadidatus* Liberabacter asiaticus, the most devastating pathogen of citrus and causal agent of Haunglongbing (HLB) (Hall et al., 2008). The south Asian native *D. citri* was discovered in the United States in 1998, in Florida (Grafton-Cardwell et al., 2006). Since its introduction to the United States, *D. citri*, along with *C.* L. asiaticus has spread through the citrus growing regions of the United States (Grafton-Cardwell et al., 2006). Adult *Diaphorina citri* feed on the phloem of citrus by piercing the parenchyma cells around the vascular bundles of the host plant (Eyer and Crawford, 1933). The pathogen *C.* L. asiaticus can be transmitted vertically from adult female *D. citri* to her eggs, as well as horizontally transmitted from exposed to unexposed insect. When the nymphs emerge, they can begin to transmit the pathogen to their host plant (Hodges and Spreen, 2012). Once inoculated, *C.* L. asiaticus spreads through the phloem of the citrus plant (Kumagai et al., 2013). The citrus plant will begin to show symptoms of Haunglongbing, which include mottled leaves, stunted growth, asymmetrical bitter fruit and death (Hall et al., 2008). The spread of *C.* L. asiaticus has devastated citrus orchards and caused considerable economic damage in States, like Florida, where citrus industries once thrived (Hodges and Spreen, 2012).

Current chemical management strategies for *D. citri* rely on neonicotinoid class insecticides which require systemic root uptake to be effective and are therefore limited to summer applications (Halbert, 2005). Pesticides in the neonicotinoid class include: acetamiprid, clothianidin, thiacloprid and imidacloprid. Imiacloprid is one of the most widely used

insecticides worldwide (Cressey, 2013). However, imiacloprid and other compounds of this kind have been highly criticized for the negative affect they could have on non-target insects like *Apis mellifera* and small mammals (Chao et. al., 1997). This is because the mode of action for imiacloprid blocks nicotinic acetycholine receptors preventing impulses between nerves causing paralysis and/or death. While imiacloprid has the greatest impact on insects due to the fact that it binds most strongly to insect neuron receptors, scientists have concluded from studies that imidacloprid can affect the neural receptors of mammals (Tomizawa et al., 2004). The limitations of current chemical management and a lack of specificity has prompted the inquiry into new chemical management strategies.

Diamide insecticides disrupt the muscle contraction process using an allosteric mechanism that targets insect ryanodine receptors and locks the Ca^{2+} channel in the semi-open position, continuously triggering muscle contraction (Mustafa et al., 2015). Ryanodine is a plant alkaloid and ligand is used to purify and characterize the ryanodine receptor (RyR**)(Figure 2.1 and Figure 2.2)**. Ryanodine receptors are a specialized class of ligand gated Ca^{2+} channels located along the endoplasmic reticulum of neurons and sarcoplasmic reticulum of muscles (Sattelle et al., 2008). These channels are responsible for controlling the release of Ca^{2+} from intracellular stores.

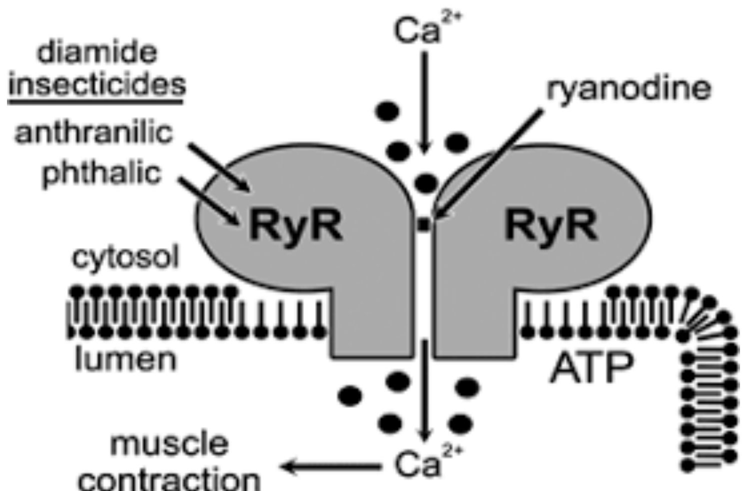

Figure 2.1. Insect RyR receptor diagram. Two of the four total RyR receptor units are shown in this schematic. The diagram shows the mode of action for a diamide insecticide. The calcium channel is locked in the semi-open position by the diamide, allowing calcium to flow freely from the endoplasmic reticulum causing continuous muscle contraction (Sattelle et al., 2008).

Cyclaniliprole **(Figure 2.1)**, IUPAC 2',3-dibromo-4'-chloro-1-(3-chloro-2pyridyl)-6'-{[(1RS)-1-cyclopropylethyl]carbamoy}pyrazole-5-carboxanilide is an anthranilic diamide insecticide, which acts as an insect RyR allosteric by binding to a target site on the ryanodine receptor (Mustafa et al., 2015). Diamides have been on the market in the United States since 2008 and the Insecticide Resistance Action Committee (IRAC) lists them as nerve and muscle target group 28. The target site of cyclaniliprole on the RyR receptor is highly specific, meaning that this diamide has no known effect on mammals, and no significant impact on the mortality of beneficial arthropods like *Harmonia ayridis, phytoseiulus persimilis and Aphidius colemani* (Tomizawa et al., 2003; Tomizawa, 2004). Cyclaniliprole is an allosteric diamide that is specific to insect RyR receptors (Teixeira and Andalora, 2013). It is hypothesized that this disruption of the muscle contraction process will inhibit *D. citri* feeding and result in increased mortality when compared to a control treatment. Bioassays were performed in order to assess Cyclaniliprole's effectiveness in affecting the mortality of psyllids. Bioassays on *D. citri* were performed at the

University of Florida due to the quarantine of *C*. L. *asiaticus* positive tissue. The mortality of *D. citri* in response to cyclaniliprole treatments were observed using single leaf bioassays. A Two-Way ANOVA was performed on the mortality data from the bioassay.

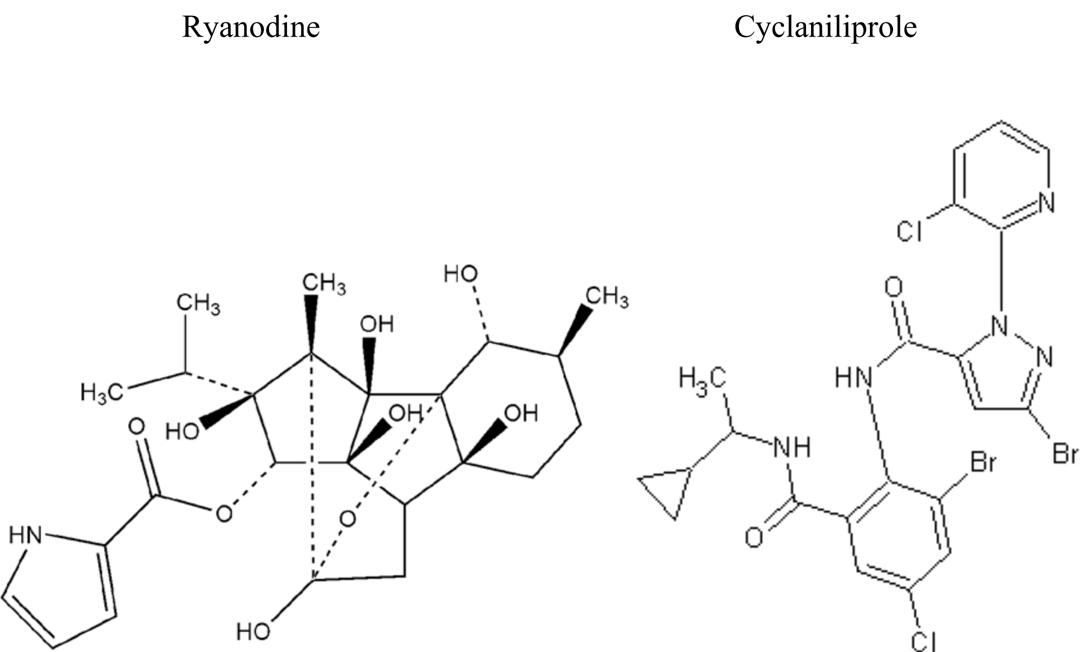

Figure 2.2 Line structure of Ryanodine ($C_{25}H_{35}NO_9$) vs a line structure of Cyclaniliprole ($C_{12}H_{17}Br_2Cl_2N_5O_2$). The structure of cyclaniliprole shows the overall compound and the diamide region.

2.2 Materials and Methods

2.2.1 *D. citri* collection

Diaphorina citri were collected from a citrus grove at the University of Florida extension center under the supervision of Dr. Lukaz Stelinski and were kept in a temporary colony. The *D. citri* population from the field was determined to have a *C*. L. asiaticus colonization rate of fifty percent using PCR (Stelinski, 2013). The *D. citri* were anesthetized using CO_2 and placed in a 100mm x 15mm petri plate containing a *Citrus sinensis* (Valencia orange) leaf **(Figure 2.3)**.

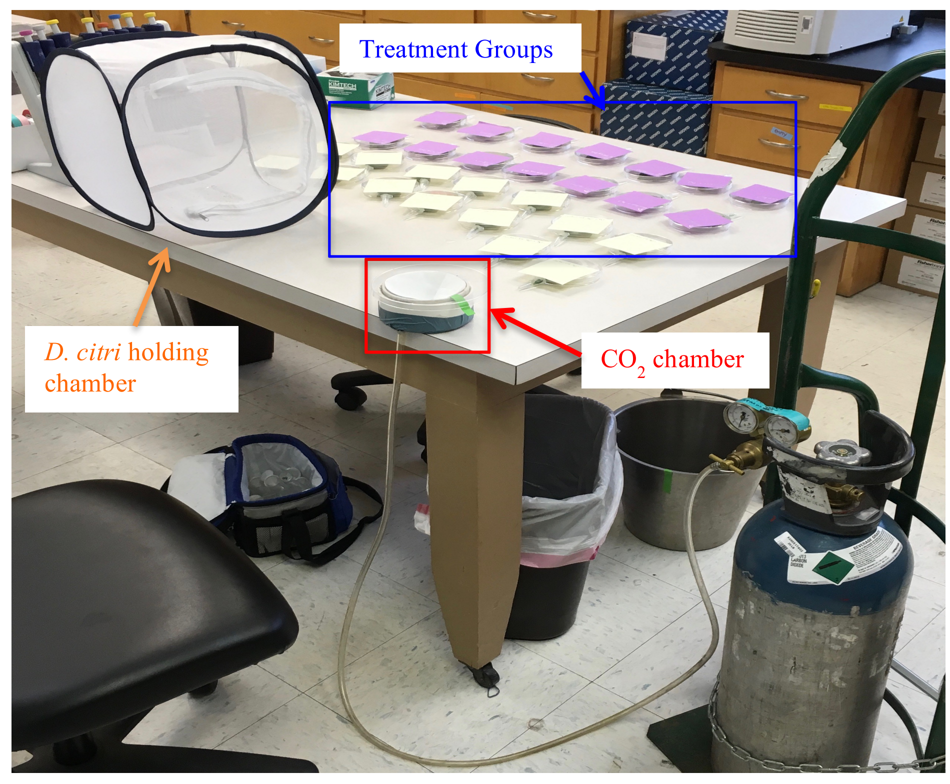

Figure 2.3 Wild caught *D. citri* undergoing CO_2 anesthesia and plate transfer.

The *C. sinensis* had been confirmed to be free of *C.* L. *asiaticus* using PCR (Stelinski, 2013). Each of the petri plates containing *D. citri* and *C. sinensis* had a small hole melted into the side to permit the petiole of the *C. sinensis* to fit into a 1.5 mL tube filled with Nanopur water. The experimental layout including replications can be seen in **Figure 2.4**.

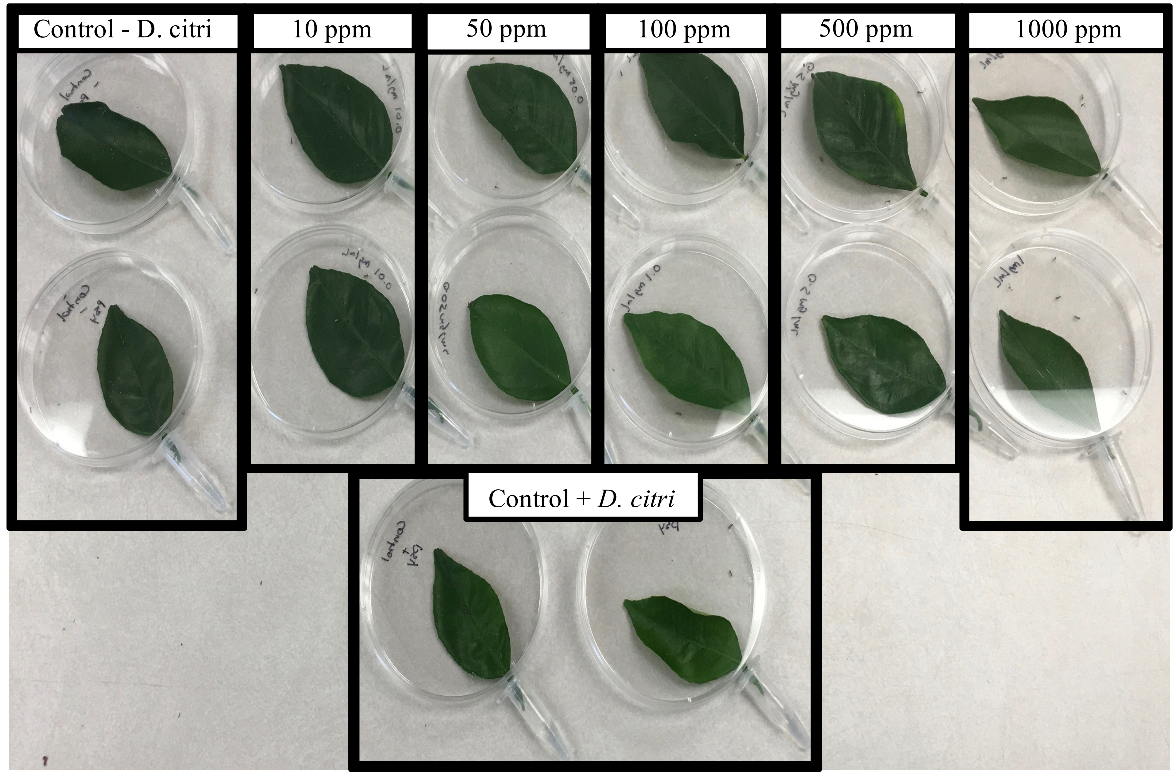

Figure 2.4 the experimental setup for the Cyclaniliprole mortality bioassay.

2.2.2 Cyclaniliprole mortality bioassay

Dilutions were performed using a stock solution of 1000 ppm of cyclaniliprole and sterile water. The treatment concentrations of cyclaniliprole used were 1000 ppm, 500 ppm, 100 ppm, 50 ppm, 10 ppm and a control group treated with sterile water. Ten *D. citri* were placed in each dish. Each treatment had two replications per concentration. The leaf dipping method was used (Cuthbertson et al., 2009). The leaves were allowed to dry for 12 hours to ensure that *D. citri* would not encounter water droplets and skew the mortality data. The experimental set-up was covered with a layer of paper to mimic the shaded feeding environment of *D. citri* and reduce psyllid loss due to escape. Mortality was assessed every 12 hours for 4 days. The complete

paralysis of *D. citri* was used to assess mortality. A Two-Way ANOVA was completed using Prism (Graphpad, La Jolla CA, USA) .

2.2.3 DNA isolation from *D. citri*

DNA was extracted from 3 whole adult *D. citri* by CTAB method (modified from Ruangwong and Akarapisan, 2006). The psyllid bodies in centerfuge tubes were ground finely with 125 µl of cold phosphate buffered saline (PBS) buffer (137 mmol/L NaCl: 2.7 mmol/L KCl: Na_2HPO_4: 1.8 mmol/L KH_2PO_4) and additional 125 µl of cold grinding buffer. The extract was centrifuged at 10,000 x g for 5 minutes at 4°C and the supernatant was collected. After centrifugation at 14,000 x g for 25 minutes, the pellets were re-suspended in 250 µl of CTAB buffer (2% CTAB: 100 mM Tris: 100 mL 20 mM EDTA) and incubated at 60°C for 30 minutes. Then, 250 µl of chloroform/isoamyl alcohol (24:1) was added to the mixture and centrifuged at 7,000 x g for 5 minutes. The aqueous supernatant phase was collected and combined with an equal volume of isopropanol, followed by centrifugation at 14,000 x g for 15 minutes. The pellets were washed with 200 µl of 70% ethanol, dried, and re-suspended in 20 µl of sterile water.

2.2.4 DNA isolation from *C. sinensis*

Genomic DNA was isolated from individual *C. sinensis* leaves using a plant genomic DNA CTAB protocol (Stelinski, 2010). Two hundred mg of mid rib tissue was ground into a fine paste. The ground tissue was transferred into a centrifuge tube and 500 ul of CTAB buffer was added. The CTAB/plant extract was incubated in a 60°C water bath for 15 min. The CTAB/plant extract was centrifuged at 12000 x g for 5 min. The supernatant was pipetted into new centerfuge tubes, and the infranatant was discarded. 250 ul of Chloroform: iso amyl alcohol (24:1) was

added to each centerfuge tube and incorporated by inversion. The centerfuge tubes were centrifuged at 13000 rpm for 1 min. The aqueous phase supernatant was pipetted into new centerfuge tubes and the organic phase was discarded. 50 ul of 7.5 M Ammonium Acetate and 500 ul of cold ethanol was added to each sample. The centerfuge tubes were then inverted to precipitate the DNA. The DNA was pelleted by centrifuging at 13000 rpm for 1 minute. The supernatant was discarded and the pellets were allowed to air dry. The dry pellets were sent, on ice, to Texas where they underwent re-suspention in DNase free water. The quality of the DNA extraction was assessed by NanoDrop ND 1000 (Thermo Scientific, Waltham MA, USA).

2.2.5 *Canadidatus* Liberabacter asiaticus Transmission using 16S rDNA Real Time PCR

To detect *C*. L. asiaticus in both *D. citri* and *C. sinensis,* a real time PCR was performed. The primers targeted 16S in *C*. L. asiaticus and design was provided by the Stelinski lab. The forward primer (F1- TGACGTTGGAAGATGTTTGTAGC) and the reverse primer (R1- ACGCAGGCTCATCTCTCTCC) yielded an amplicon of 263 bps. The samples and reagents were stored at -20°C prior to the PCR. The DNA samples examined for purity using the NanoDrop and the concentration of nucleic acid were diluted to 50 ng/uL. The master mix was created consisting of 12.5 ul of Amplitaq Gold Taq., 1 uL GC, 1 uL Forward primer (F1- TGACGTTGGAAGATGTTTGTAGC), 1 uL Reverse primer (R1- ACGCAGGCTCATCTCTCTCC), 1.25 uL Eva Green dye, 6.25 uL sterile H_2O. The total volume per reaction was 25 uL (Stelinski, 2010). After the initial real time PCR a nested-qPCR was performed using a *C*. L. asiaticus 16S primers that were found within the original primer set. The forward internal primer was (F- TCGAGCGCGTATGCGAATAC) and the reverse internal primer was (R-GCGTTATCCCGTAGAAAAAGGTAG). The internal primer set yielded an

amplicon of 79 bps. The total volume per reaction was 25 uL. An agarose gel was run using electrophoresis in order to visualize the *C.* L. asiaticus bands.

2.2.6 Bioassay to determine Lethal Concentration, 50% (LC_{50})

Dose response curves were calculated from the mortality bioassay for 24 hours, 60 hours and 84 hours due to the levels of *D. citri* mortality (Prism Graphpad, La Jolla CA, USA). The lethal concentration that resulted in 50% mortality (LC_{50}) was calculated from the 84-hour time point because all cyclaniliprole treatments had exhibited an effect at this time point. A probit analysis was performed on the 84-hour dose response curve (SPSS: IBM Corp. Armonk, NY)

2.3 Results

2.3.1 Cyclaniliprole mortality bioassay

Diaphorina citri that received 50 ppm of cyclaniliprole exhibited significant mortality over the 96-hour study period **(Figure 2.5)**. The highest concentration of cyclaniliprole (1000 ppm) led to significant mortality at 24 hrs. and led to 100% mortality at 72 hrs. The second highest concentration of cyclaniliprole (500 ppm) led to significant mortality ($P<0.001$) at 48 hrs. Cyclaniliprole at 100 ppm led to significant mortality ($P<0.001$) at 60 hrs. and 50 ppm led to significant mortality ($P<0.005$) at 72 hours. All of the treatment concentrations of cyclaniliprole exhibited an effect on *D. citri* mortality by 84 hrs. The largest gap in mortality was between 50 ppm and 100 ppm. The *D. citri* feeding on treated leaves at the time point prior to the significant mortality ($P<0.001$) time point for a concentration of cyclaniliprole exhibited a change in feeding behavior when compared to the control groups **(Figure 2.6)**. The observed alteration in feeding

behavior of *D. citri* in response to cyclaniliprole concentrations was observed as reduced *D. citri* feeding at the mid rib of the leaves and a decrease in *D. citri* on the leaves **(Figure 2.6)**.

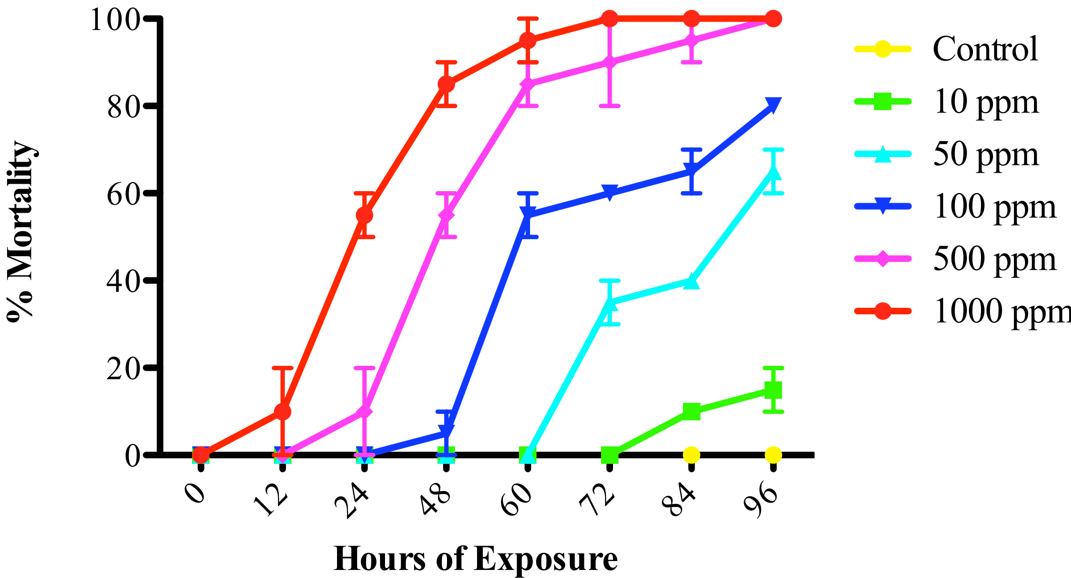

Figure 2.5. *D. citri* percent mortality observed over a period of 4 days every 12 hours. The x-axis represents the hours of exposure the *D. citri* had the treatment concentrations of cyclaniliprole. The y-axis represents the percent mortality of the *D. citri* over the 4 day bioassay.

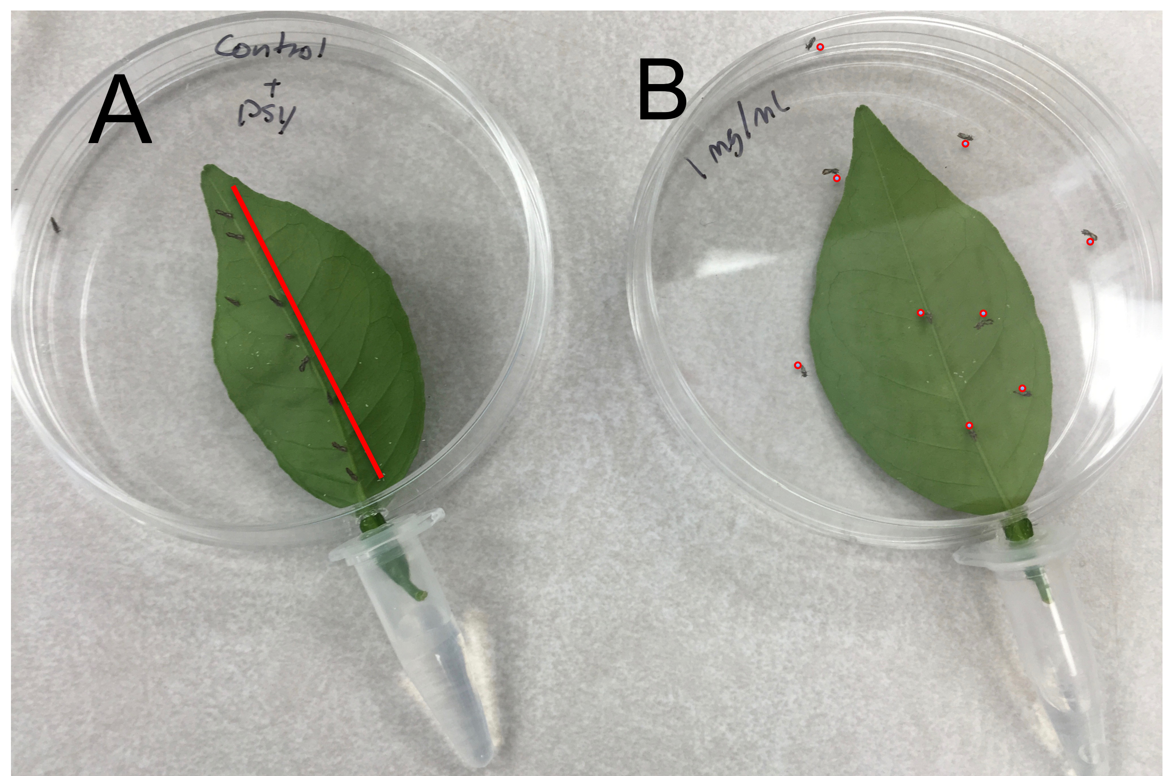

Figure 2.6 The *D. citri* feeding at 24 hrs. on the underside of the control leaf (A) are gathered along the midrib of the leaf and can be seen actively feeding on the leaf. The D. citri with leaf (B) are spread out around the petri dish.

2.3.2 Bioassay to determine Lethal Concentration, 50% (LC_{50})

Diaphorina citri that received cyclaniliprole treatments exhibited significantly higher mortality compared to control groups that received sterile water **(Figure 2.5)**. Psyllids that received 500 ppm of cyclaniliprole or above reached 100% mortality at or before 96 hours **(Figure 2.5)**. Dose response curves were calculated for 24, 60, and 84-hour time points **(Figure 2.7)**. The probit transformation modeled using SPSS and *D. citri* data after 84 hours of exposure to 10, 50, 100, 500, or 1000 ppm of cyclaniliprole was can be visualized in **Figure 2.8**. The results of the probit analysis are summarized in **Table 2.1**. The LC_{50} was determined to be 95 ppm after 84 hours of exposure **(Figure 2.8 and Table 2.1)**.

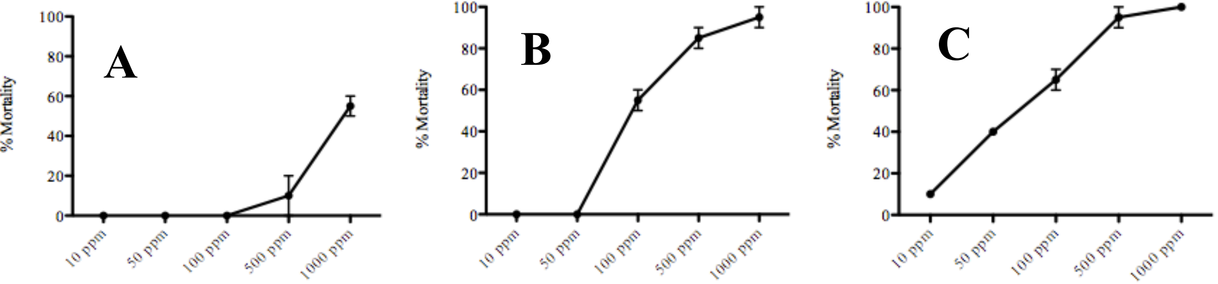

Figure 2.7 Dose response curves from the cyclaniliprole bioassay for (A) 24, (B) 60 and (C) 84-hour time points. The x-axis represents cyclaniliprole concentration in mg/mL and the y-axis represents the percent mortality of *D. citri.*

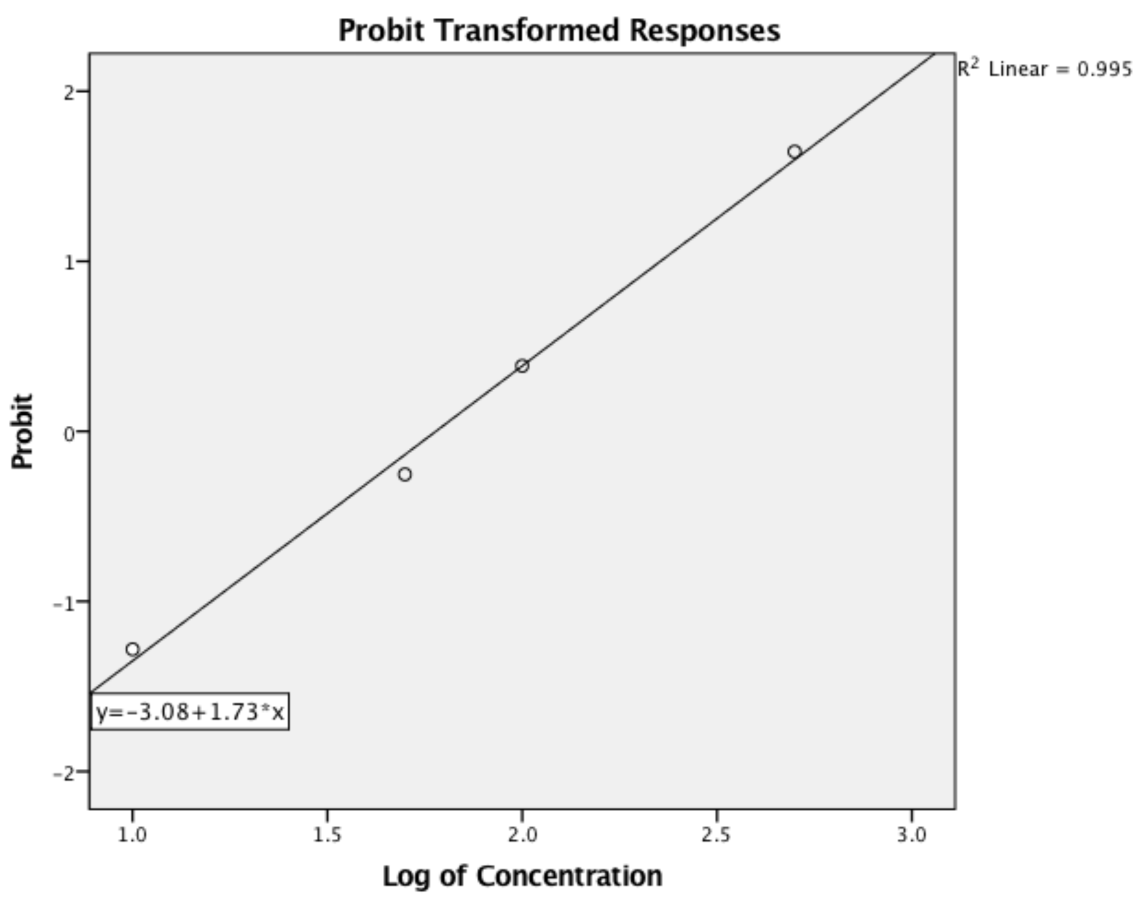

Figure 2.8 Probit transformation modeled using SPSS and *D. citri* data after 84 hours of exposure to 10, 50, 100, 500, or 1000 ppm of cyclaniliprole.

Table 2.1. Probit transformation data. VAR00003 values are the log (cyclaniliprole concentrations), number of subjects corresponds to the total number of *D. citri* for each concentration. The observed responses correspond to the total number of deaths for each concentration. The LC_{50} was found by taking the natural log of Probit concentration 5 (3.000).

	Number	VAR00003	Number of Subjects	Observed Responses	Expected Responses	Residuals	Probability
PROBIT	1	1.000.	20	2	1.530.	0.470.	0.077.
	2	1.699.	20	8	8.807.	-0.807	0.440.
	3	2.000.	20	13	13.113.	-0.113	0.656.
	4	2.699.	20	19	19.069.	-0.069	0.953.
	5	3.000.	20	20	19.743.	0.257	0.987.

2.3.3 *Canadidatus* Liberabacter asiaticus transmission from *D. citri* to *Citrus sinensis*

No transmission was detected from *D. citri* to *C. sinensis* in the 96-hour mortality bioassay. *Diaphorina citri* tested positive via qPCR; however, the *C. sinensis* that they were exposed to for 4 days did not test positive for *C.* L. asiaticus **(Figure 2.9 and Figure 2.10)**.

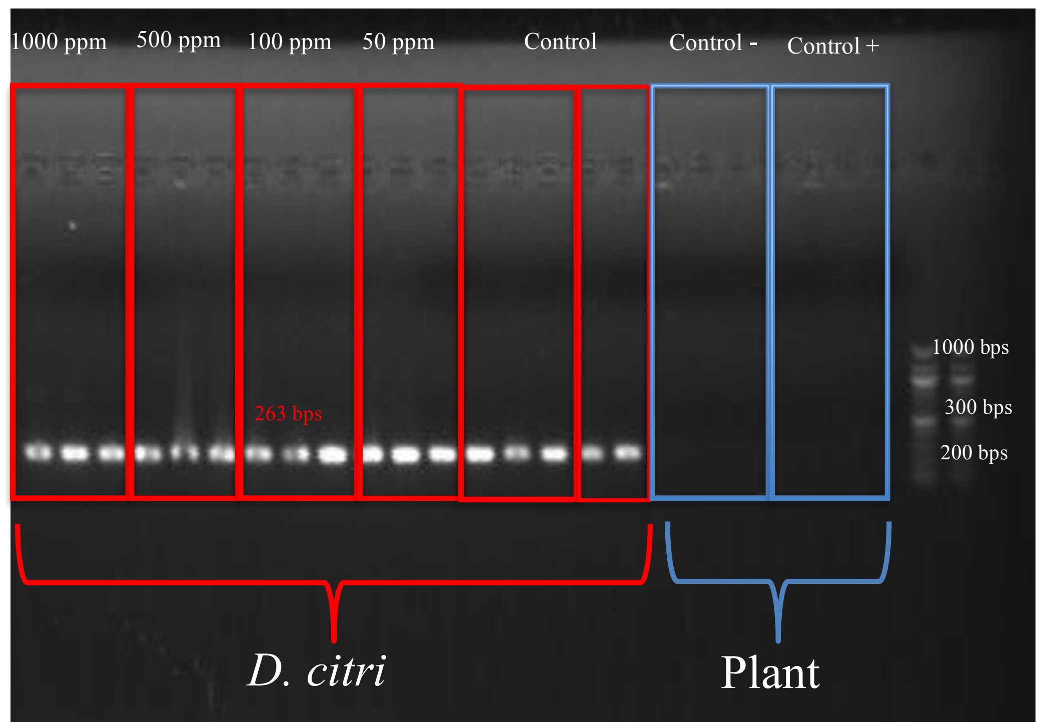

Figure 2.9 Detection of C. L. asiaticus from *D. citri* in cyclaniliprole bioassay. (1000 ppm) *D. citri* with 1000 ppm cyclaniliprole. (500 ppm) *D. citri* with 500 ppm cyclaniliprole. (100 ppm) *D. citri* 100 ppm cyclaniliprole (50 ppm) *D. citri* with 50 ppm cyclaniliprole (Control) *D. citri* colony control (Control -) *C. sinensis* control with no *D. citri* (Control +) *C. sinensis* sterile water control with psyllids.

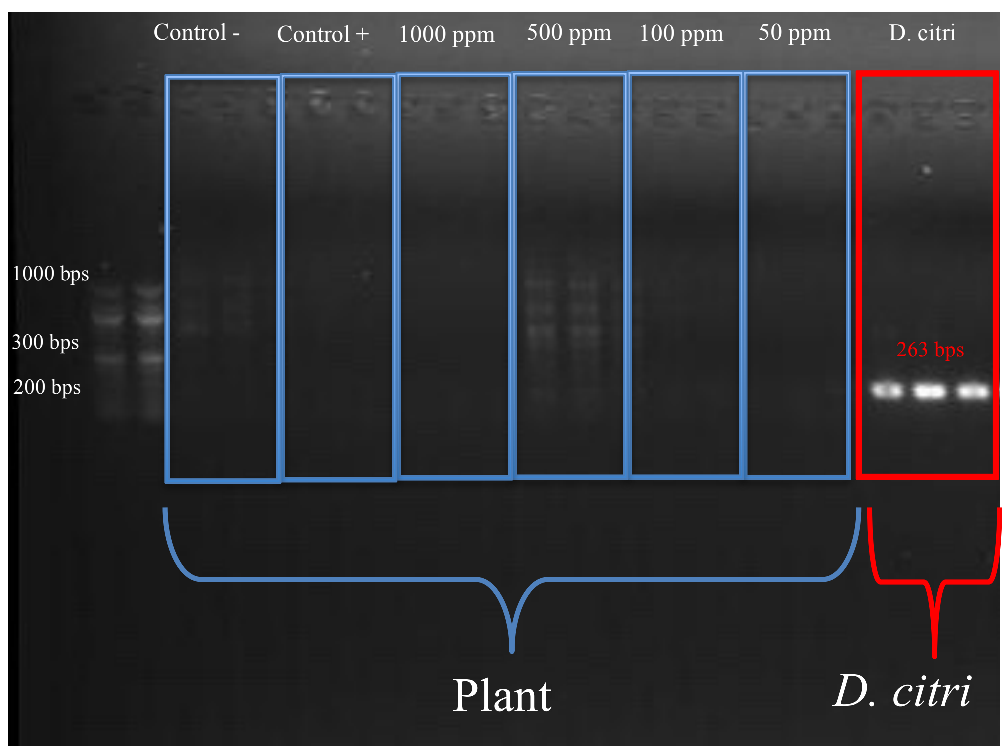

Figure 2.10 Detection of C. L. asiaticus from *C. sinensis* in cyclaniliprole bioassay. (Control -) *C. sinensis* sterile water control. (Control +) *C. sinensis* control with *D. citri* (1000 ppm) *C. sinensis* with 1000 ppm cyclaniliprole (500 ppm) *C. sinensis* with 500 ppm cyclaniliprole (100 ppm) *C. sinensis* with 100 ppm cyclaniliprole (50 ppm) *C. sinensis* with 50 ppm cyclaniliprole (D. citri) *D. citri* colony control.

2.4. Discussion

2.4.1 Cyclaniliprole mortality bioassay

Diaphorina citri that received 50 ppm of cyclaniliprole or above exhibited significant mortality compared to the control group. The high rate of mortality exhibited by *D. citri* can be attributed to the insect exposure to cyclaniliprole. The highest rates of *D. citri* mortality were seen in the highest concentration of groups of cyclaniliprole. This can be explained by the mode of action of cyclaniliprole and other diamide insecticides. Diamide insecticides act on the RyR receptor causing a continuous outflow of Ca^{2+} ions from the endoplasmic reticulum, resulting in

continuous muscle contraction. The higher rates of *D. citri* mortality at higher concentration of cyclaniliprole are consistent with the hypothesis that the disruption of muscle contraction by the diamide insecticide would result in high *D. citri* mortality.

2.4.2 Bioassay to determine Lethal Concentration, 50% (LC_{50})

Diaphorina citri that received cyclaniliprole treatments exhibited significantly higher mortality compared to control groups that received sterile water. *D. citri* that received 500 ppm of cyclaniliprole or above reached 100% mortality at or before 96 hours **(Figure 3.1)**. This is likely due to the diamide insecticide working on the RyR receptor. The LC_{50} was determined to be approximately 95 ppm further experimentation with a larger concentration range and sample size is advised in order to gain a greater specificity.

2.4.3 *Canadidatus* Liberabacter asiaticus transmission from *D. citri* to *C. sinensis*

Transmission of *C.* L. asiaticus transmission from *D. citri* to *C. sinensis* was not detectible in the *D. citri* mortality bioassay in both a traditional real time PCR or a nested real time PCR. This could be due to the fact that *C.* L. asiaticus levels in the *C. sinensis* were not at detectable levels after only 4 days of exposure. It is also possible that the 50% positive *C.* L. asiaticus *D. citri* used in this study did not have high enough concentrations of *C.* L. asiaticus to inoculate the *C. sinensis*. *D. citri* are always a vector for *C.* L. asiaticus; however, the highest rates of *C.* L. asiaticus in the *D. citri* population occur in summer and coincide with the *C. sinensis* bloom.

References

Cordova, D., Benner E. A., Sacher M. D., Rauh J. J., Sopa J. S., Lahm G. P., Selby T. P., Stevenson T. M., Flexner L., Gutteridge S., Rhoades D. F., Wu L., Smith R. M., Tao Y. 2006. Anthranilic diamides: a new class of insecticides with a model mode of action, ryanodine receptor activation. Pesticide Biochemistry and Physiology. 84(3): 196-214.

Cressey, D. 2013. Europe debates risk to bees. Nature 496 (7446):408.

Halbert, S.E. 2005. The discovery of huanglongbing in Florida, presentation H3. Proceedings of 2nd International Citrus Canker and Huanglongbing Research Workshop. 1: 7-11.

Hall, D. G., Hentz M. G., Adair R. C. 2008. Population ecology and phenology of Diaphorina citri (Hemiptera: Psyllidae) in two Florida citrus groves. Environ Entomol. 37(4):914-24.

Hall, D. G., Richardson, M. L., Ammar, E.-D., and Halbert, S. E. 2013. Asian citrus psyllid, Diaphorina citri , vector of citrus huanglongbing disease. Entomol. Exp. Appl. 146(2): 207–223.

Hodges, A. W., Spreen, T. H. 2012. Economic Impacts of Citrus Greening (HLB) in Florida. Applied Micro 2: 1-4.

Hunter, W., Dowd S. 2009. Psyllid biology: expressed genes in adult Asian citrus psyllids, *Diaphorina citri* Kuwayama. Open Entomology Journal 3: 18-29.

Jagoueix, S., Bove, J. M., Garnier, M. 1994. The Phloem-Limited Bacterium of Greening Disease of Citrus Is a Member of the α subdivision of the proteobacteria. Int. J. Syst. Bacteriol. 44(3): 379–386.

Kumagai, L. B., LeVesque C. S., Blomquist C. L., Madishetty K., Guo Y., Woods P.W., Rooney-Latham S., Rascoe J., Gallindo T., Schnabel D., Polek M. 2013. First report of

"Candidatus Liberibacter asiaticus" associated with citrus huanglongbing in California. Plant Dis 97:283. http://dx.doi.org/10.1094/PDIS-09-12-0845-PDN.

Kuwayama, S. 1908. Die Psylliden Japans. I. Trans. Sapporo Nat. Hist. Soc. 2:149–189.

El Hassani, A., Dacher M., Gauthier M., Armengaud C. 2005. Effects of sub lethal doses of fipronil on the behavior of the honeybee (Apis mellifera). Pharmacology Biochemistry and Behavior. 82(1): 30- 39.

Lee Chao, S., Casida, J. E. 1997. Interaction of Imidacloprid Metabolites and Analogs with the Nicotinic Acetylcholine Receptor of Mouse Brain in Relation to Toxicity. Pesticide Biochemistry and Physiology 58: 77–88. doi:10.1006/pest.1997.2284.

Li W, Hartung J. S., Levy L. 2006. Quantitative real-time PCR for detection and identification of Candidatus Liberibacter species associated with citrus huanglongbing. Microbiol. Methods 66:104 –115. http://dx.doi.org/ 10.1016/j.mimet.2005.10.018.

Manjunath, K., Halbert S. 2008. Detection of '*Candidatus* Liberibacter asiaticus' in *Diaphorina citri* and Its Importance in the Management of Citrus Huanglongbing in Florida. Phytopathology 98(4):387-396.

Marutani-Hert, H., Hunter W. B., Dowd, S. 2008. Endosymbiotic microbiota of Asian Citrus Psyllid (Diaphorina citri) IRCHLB Proceedings 6.6 224-227.

Sattele, D.B., Cordova D., Cheek T.R. 2008. Insect ryanodine receptors: molecular targets for novel pest control chemicals. Invertebrate Neuroscience. 8:107-119.

Teixeira L. A., Andaloro J.T. 2013. Diamide insecticides: global efforts to address insect resistance stewardship challenges. Pesticide Biochemistry and Physiology. 106(2013):76-78.

Tomizawa, M. 2004. Neonicotinoids and Derivatives: Effects in Mammalian Cells and

Mice. Journal of Pesticide Science 29 (3): 177–172. doi:10.1584/jpestics.29.177.

Tomizawa, M., Casida, J. E. 2003. Selective toxicity of neonicotinoids attributable to specificity of insect and mammalian nicotinic receptors .Annual Review of Entomology 48: 339–364.

Chapter Three

Diaphorina citri response to Flonicamid

3.1 Introduction

Diaphorina citri also known as the Asian citrus psyllid is a phloem feeding Hemipteran native to southern Asia. *Candidatus* Liberibacter asiaticus is a phloem limited bacteria, which is the primary causal agent of the devastating disease of citrus, Haunglongbing (Hall et al., 2008). *Diaphorina. citri* is the primary vector for *C.* L. asiaticus and can be found throughout Asia, the Middle East, South America and the south portion of North America (Halbert, 2005). *Diaphorina citri* was first observed in the United States in 1998, in Florida (Hall et al., 2008). *Diaphorina citri* can acquire the pathogen *C.* L. asiaticus through vertical transmission, horizontal transmission, or feeding on infected citrus (Chu et al., 2016). Once a plant is inoculated with *C.* L. asiaticus, it will begin to show symptoms of Haunglongbing, which include mottled leaves, plant dieback and bitter asymmetrical fruit (Kuwayama, 1908). *Candidatus* Liberibacter asiaticus is a gram negative bacteria that is limited to the phloem of a plant and moves systemically through the plant using the phloem. As *C.* L. asiaticus multiplies in the phloem tissues, it begins to restrict the movement of nutrients throughout the plant causing the symptoms associated with Huanglongbing.

Diaphorina citri undergoes a 30-day temperature dependent life cycle that includes 5 instars (Liu and Tsai, 2000; Hall et al., 2013). After emergence, the nymphs feed on the newly emerged leaves of the host plant, called the flush. *Diaphorina citri* feed by puncturing the epidermis of the leaf with their stylet and sucking the sugar-laden juices from the phloem (Hall et

al., 2013). During the feeding process, *C.* L. asiaticus can be transmitted from *D. citri* to the host plant.

Existing chemistries for the management of *D. citri* populations cannot adequately target the *D. citri* populations during the whole year (Goolsby and Adamczyk, 2007). Neonicotinoid insecticides like acetamiprid, clothianidin, thiacloprid and imidacloprid are only effective when actively taken up by plant roots in the growing months (Goolsby and Adamczyk, 2007). The most widely used neonicotinoid, imidacloprid has been criticized for the lack of specificity of its target site which could lead to negative effects on mammals as well as insects (Chao et al., 1997). Insect resistance to current chemical insecticides is also a concern; therefore, the development and testing of new compounds is in very high demand. These compounds would ideally be effective through both foliar and systemic uptake and have a limited effect on non-target insects.

Flonicamid (IUPAC N-cyanomethyl-4-(trifluoromethyl) nicotinamide) is an anti-feedant and has been used globally since the late 1990's (Morita et al., 2014) **(Figure 3.1)**. Anti-feedants reduce or alter the feeding behavior of the target organism. Flonicamid is a white, odorless crystalline powder that acts as a chordotonal organ modulator with an undefined target site. This compound has a molecular weight of 229.2 g/mol, vapor pressure of 9.43×10^{-4} mPa (20°C) and water solubility of 5.2 g/L (20°C). Flonicamid has a novel mode of action that has no known cross resistance with common insecticide classes like neoicotinoids, pyrethroids, carbamates or organophosphates. Flonicamid has been used in the management of aphids Hemipterains, which gives justification for the use of this compound on other Hemiptera. Flonicamid has systemic and translaminar activity that promotes long-term management of an insect population.

Figure 3.1 Line structure of Flonicamid ($C_9H_6F_3N_3O$)(sigmaaldrich)

Behavioral bioassays were performed in order to assess flonicamid's effectiveness in managing psyllids. The feeding behavior of *D, citri* was observed using time-lapse photography over a 4-day period. The reduction of feeding duration was observed which could have led to the higher rates of mortality. Bioassays on *D. citri* were performed at the University of Florida due to the quarantine of *C.* L. *asiaticus* positive tissue. The behavioral changes of *D. citri* in response to flonicamid treatments were observed in two ways. The first way was through time- lapse photography. The second way was by utilizing pH sensitive cellulose to quantify the change in fecal output from *D. citri* in response to flonicamid treatments.

3.2 Materials and Methods

3.2.1 *Diaphorina citri* collection

Diaphorina citri were collected from a citrus grove at the University of Florida extension center under the supervision of Dr. Lukaz Stelinski. The *D. citri* population from the field was determined to have a *C.* L. *asiaticus* colonization rate of fifty percent using PCR (Stelinski, 2013). The *D. citri* were anesthetized using CO_2 and placed in a 100mm x 15mm petri plate containing a *Citrus sinensis* (Valencia orange) leaf **(Figure 3.2)**.

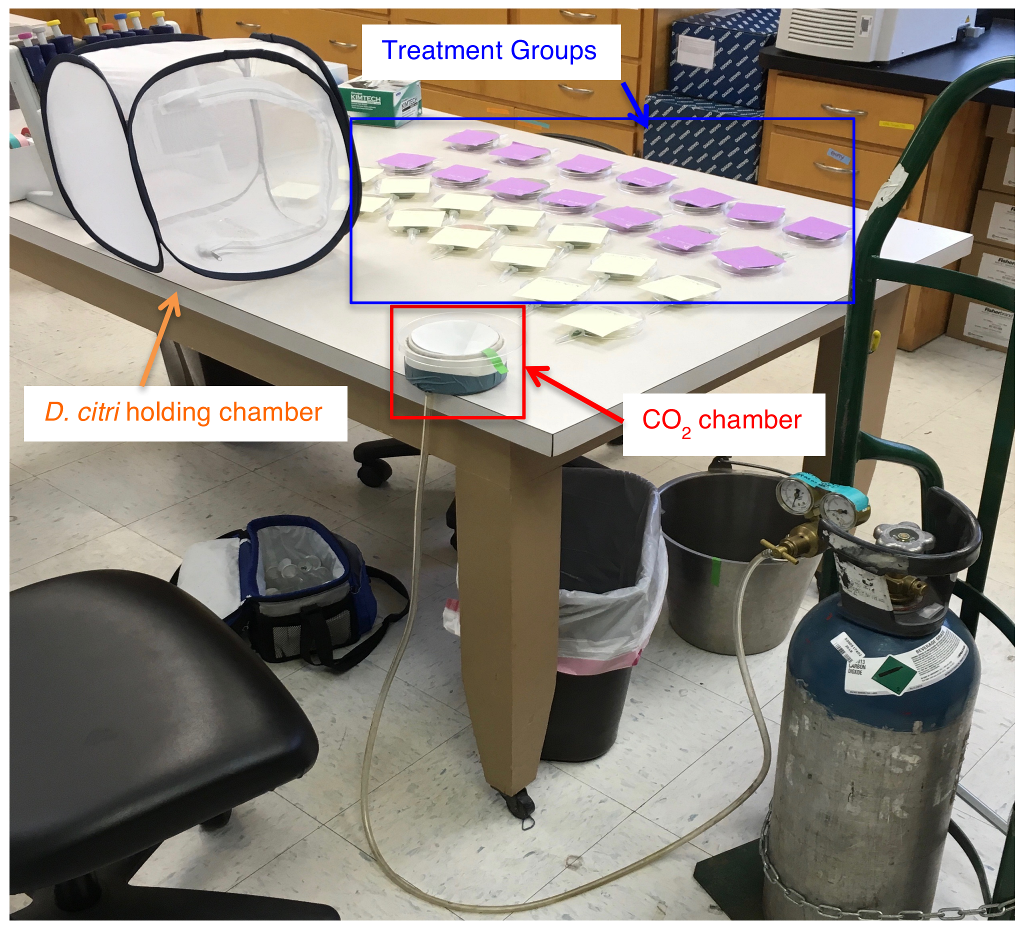

Figure 3.2 Wild caught *D. citri* undergoing CO_2 anesthesia and plate transfer. The holding chamber labeled in orange is the chamber used to transition *D. citri* from the wild caught population to the anesthesia chamber. The CO_2 chamber labeled in red was used to anesthetize 10 *D. citri* for each of the petri dishes for the bioassay. The treatment groups labeled in blue received 10 D. citri per petri dish and remained covered in order to minimize *D. citri* escape.

The *C. sinensis* had been confirmed to be free of *C.* L. *asiaticus* using PCR (Stelinski, 2013). Each of the petri plates containing *D. citri* and *C. sinensis* had a small hole melted into the side to permit the petiole of the *C. sinensis* to fit into a 1.5 mL tube **(Figure 3.3)**.

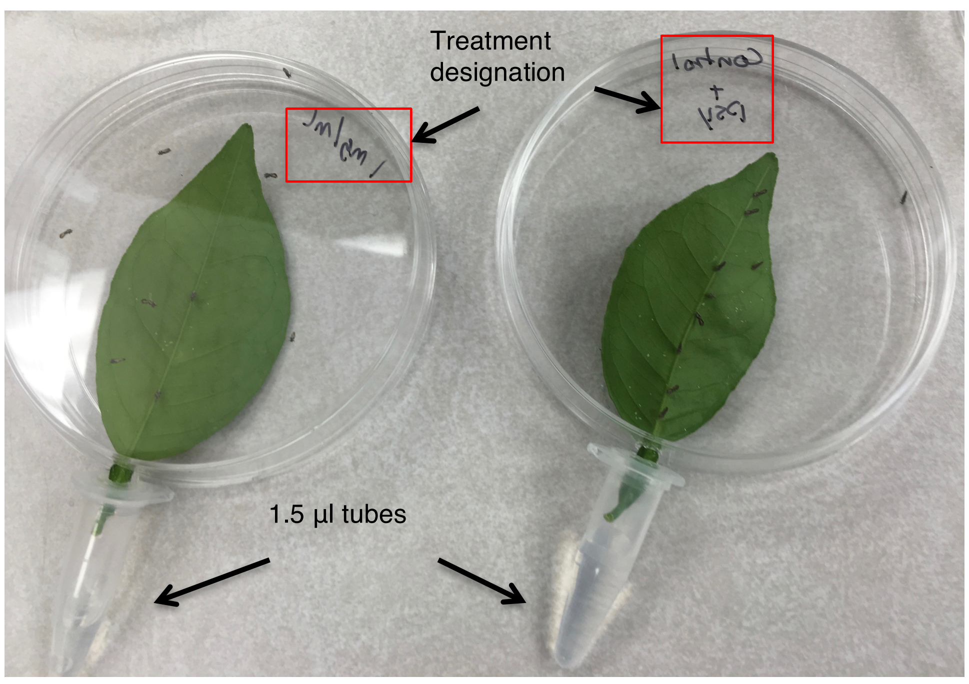

Figure 3.3 100mm x 15mm petri plates with *C. sinensis* and *D. citri.*

3.2.3 Foliar/Systemic Flonicamid behavioral assay

To test the systemic and translaminar activity of flonicamid, two different time-lapse photo bioassays were performed. The first bioassay tested the systemic activity of flonicamid **(Figure 3.4)**. From a stock solution of 1000 ppm, four treatment concentrations were created. The concentrations used for the study were 1000 ppm, 500 ppm, 100 ppm and 50 ppm. Each concentration had two replications. This bioassay also included a positive control (*D. citri* present) and a negative control (no *D. citri* present). The second bioassay tested the translaminar aspect of flonicamid on *D. citri*. The concentrations of flonicamid were used and introduced to leaf via the leaf dipping method (Cuthbertson et al, 2009). After dipping, the leaves were allowed to dry for 12 hours and two replications were completed for each concentration **(Figure 3.4)**.

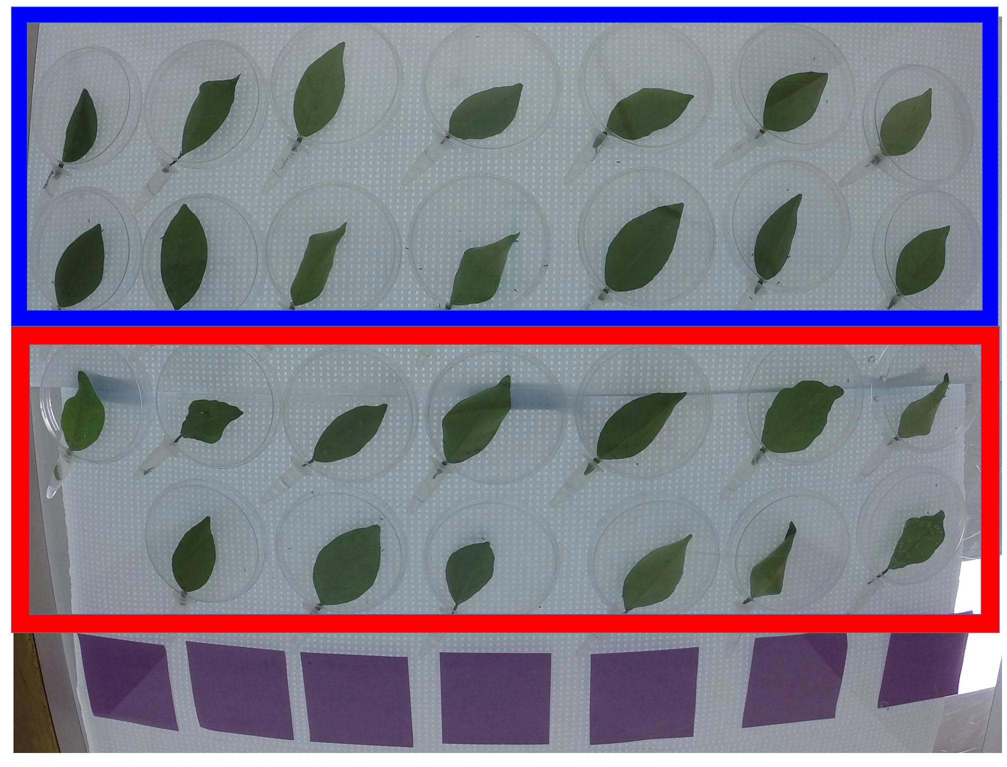

Figure 3.4 Experimental set up for the systemic and translaminar flonicamid assays on *D. citri*. The red box indicates the systemic time-lapse assay. The blue box indicates the translaminar
time-lapse assay.

Feeding behavior and mortality were monitored using time-lapse photography. A photograph was taken by a GOPRO Hero 3 camera every sixty seconds for five days. Feeding behavior was quantified by counting the number of actively feeding *D. citri* every five minutes. ImageJ was used to analyze the photographs. The impact of systemic flonicamid treated *C. sinensis* on *D. citri* was assessed by performing a linear regression on the mean functions to compare between the treatment groups. A pairwise test was completed to compare between foliar and systemic applications.

3.2.4 Foliar Fecal output Bioassay

The second *D. citri* behavioral bioassay used pH sensitive cellulose (Educational Innovations Inc. Bethel, CT) to measure fecal output in *D. citri* in response to flonicamid treatments **(Figure 3.5 and Figure 3.6)**. Each leaf was treated with a concentration (1000 ppm to 50 ppm). The leaves were dipped in their respective concentrations and allowed to dry for 12 hours. ImageJ was used to qualify fecal out-put (Schneider et al, 2012). The images were collected and the movement of the *D. citri* was analyzed by counting the number of movements of the *D. citri* between images.

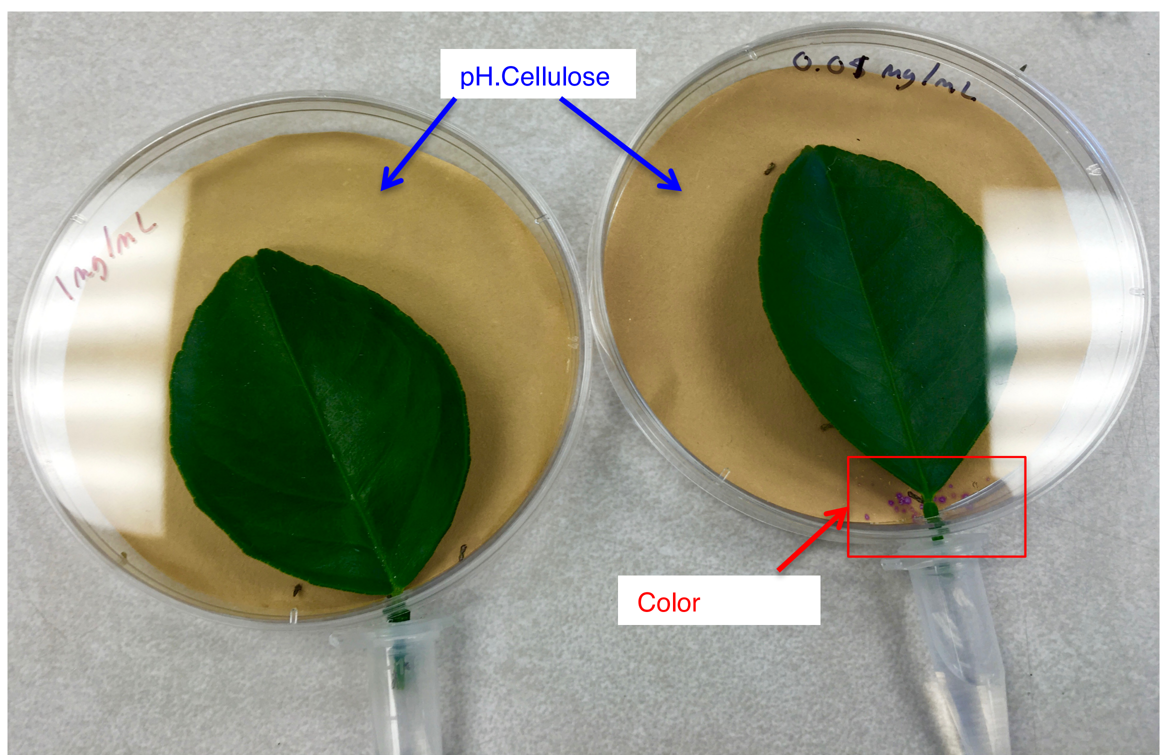

Figure 3.5 Close-up of the experimental set-up for fecal output behavioral bioassay for *D. citri* with *C. sinensis.* Red box shows a color change from tan to purple in response to *D. citri* detection.

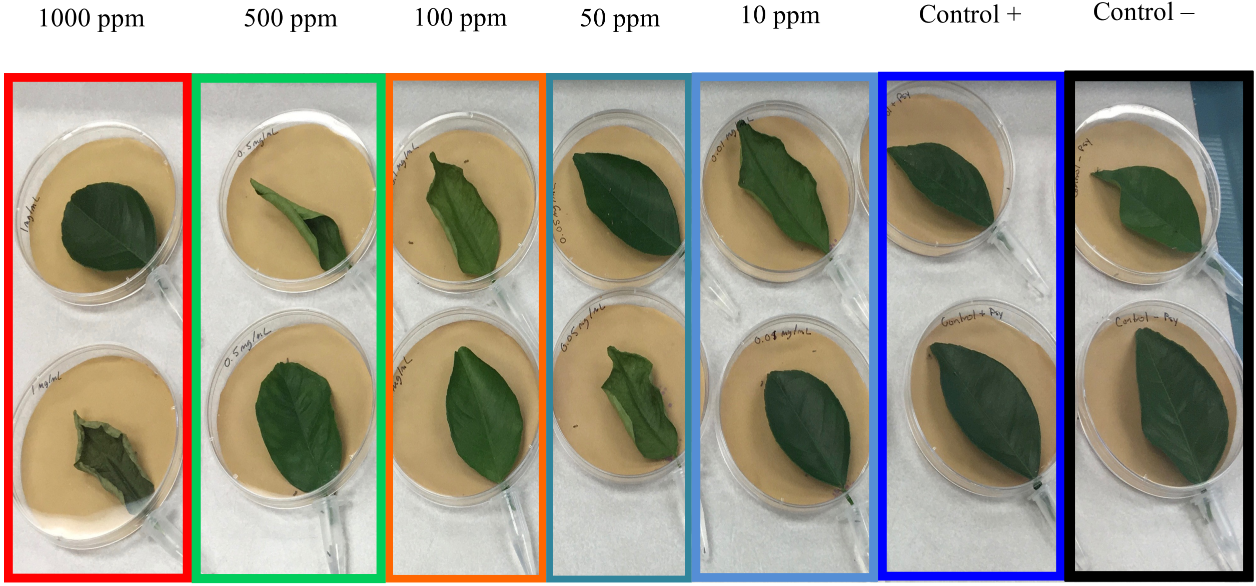

Figure 3.6 Experimental bioassay set up for the foliar fecal output behavioral assay for *D. citri*. Each color shows the replication for each concentration. The 4 concentrations of flonicamid are labeled highest to lowest 1000 ppm, 500 ppm, 100 ppm and 50 ppm. Two controls were used. The first control designated in blue contained 10 *D. citri* per plate. The control designated in purple contained no D. citri and was used as a transmission control.

3.2.5 . *C.* L. asiaticus detection in *C. sinensis* treated with flonicamid

Transmission of *C.* L. asiaticus from *D. citri* to flonicamid treated *C. sinensis* was assessed using nested qPCR (Stelinski, 2010). The foliar and translaminar treatments of flonicamid were assessed separately.

3.2.6 DNA isolation from *D. citri*

DNA was extracted from adult *D. citri* bodies by CTAB method (modified from Ruangwong and Akarapisan, 2006). The psyllid bodies in centerfuge tube were ground finely with 125 µl of cold buffer and additional 125 µl of cold buffer. The extract was centrifuged at 10,000 x g for 5 minutes at 4°C and the supernatant collected. After centrifugation at 14,000 x g for 25 minutes, the pellets were re-suspended in 250 µl of CTAB buffer and incubated at 60°C for 30 minutes. Then, 250µl of chloroform/isoamyl alcohol (24:1) was added to the mixture and

centrifuged at 7,000 x g for 5 minutes. The aqueous supernatant phase was collected and combined with an equal volume of isopropanol, followed by centrifugation at 14,000 x g for 15 minutes. The pellets were washed with 200 µl of 70% ethanol, dried, and re-suspended in 20 µl of sterile water. The quality of the DNA extract was assessed using NanoDrop ND 1000 (Thermo Scientific, Waltham MA, USA).

3.2.7 DNA isolation from *Citrus sinensis*

Genomic DNA was isolated from individual *C. sinensis* leaves using a plant genomic DNA CTAB protocol (Stelinski, 2010). Then, 200mg of mid-rib tissue was ground into a fine paste. The ground tissue was transferred into a centerfuge tube and 500 µl of CTAB buffer was added. The CTAB/plant extract was incubated in a 55°C water bath for 15 min. The CTAB/plant extract was centrifuged as 12000 x g for 5 minutes. The supernatant was pipetted into new microfuge tubes, and the infranatant was discarded. 250 µl of Chloroform: iso amyl alcohol (24:1) was added to each microfuge tube and incorporated by inversion. The centerfuge tubes were centrifuged at 13000 rpm for 1 minute. The aqueous phase was pipetted into new microfuge tubes and the organic phase was discarded. 50:l mixture of 7.5 M ammonium acetate and 500 µl of cold ethanol was added to each sample. The microfuge tubes were then inverted to precipitate the DNA. The DNA was pelleted by centrifuging at 13000 rpm for 1 minute. The supernatant was discarded and the pellets were allowed to air dry. The dry pellets were sent on ice to Texas where they underwent re-suspending in DNase free water. The quality of the DNA extraction was assessed by NanoDrop ND 1000 (Thermo Scientific, Waltham MA, USA).

3.2.8 *C.* L. asiaticus Transmission using 16S rDNA Real Time PCR

In order to detect *C* L. asiaticus in both *D. citri* and *C. sinensis,* a real time PCR was performed. The primers targeted 16S in *C.* L. asiaticus and design was provided by the Stelinski lab (Stelinski, 2010). The forward primer (F1- TGACGTTGGAAGATGTTTGTAGC) and the reverse primer (R1- ACGCAGGCTCATCTCTCTCC) yielded an amplicon of 263 bps. The samples and reagents were stored at -20°C prior to the PCR. The DNA samples were examined for purity using the NanoDrop ND 1000 (Thermo Scientific, Waltham MA, USA) and the concentration of nucleic acid was diluted to 50 ng/µl. The master mix was created consisting of 12.5 µl of Taq., 1 µl GC, 1 µl Forward primer (F1- TGACGTTGGAAGATGTTTGTAGC), 1 µl Reverse primer (R1- ACGCAGGCTCATCTCTCTCC), 1.25 µL Eva Green dye, 6.25 µl sterile H_2O. The total volume per reaction was 25 µl. The total volume per reaction was 25 µl. An agarose gel was run using electrophoresis in order to visualize the *C.* L. asiaticus bands.

2.5.6 Nested qPCR for the detection of *C.* L. asiaticus in *D. citri* and *C. sinensis*

A nested quantitative polymerase chain reaction (qPCR) was performed. First, PCR was performed using the external forward primer Clas-16S-Ex-F1: TGACGTTGGAAGATGTTTGTAGC and the external reverse primer Clas-16S-Ex-R1: ACGCAGGCTCATCTCTCTCC. The amplicon sequence for this primer set is 263 bp. The nested qPCR forward primer was 16S F- TCGAGCGCGTATGCGAATAC and the nested qPCR reverse primer was 16S R- GCGTTATCCCGTAGAAAAAGGTAG. The amplicons for the nested qPCR primers were 79 bp. An agarose gel was run using electrophoresis in order to visualize the *C.* L. asiaticus bands

3.3 Results

3.3.1 Foliar and systemic flonicamid behavioral assay results

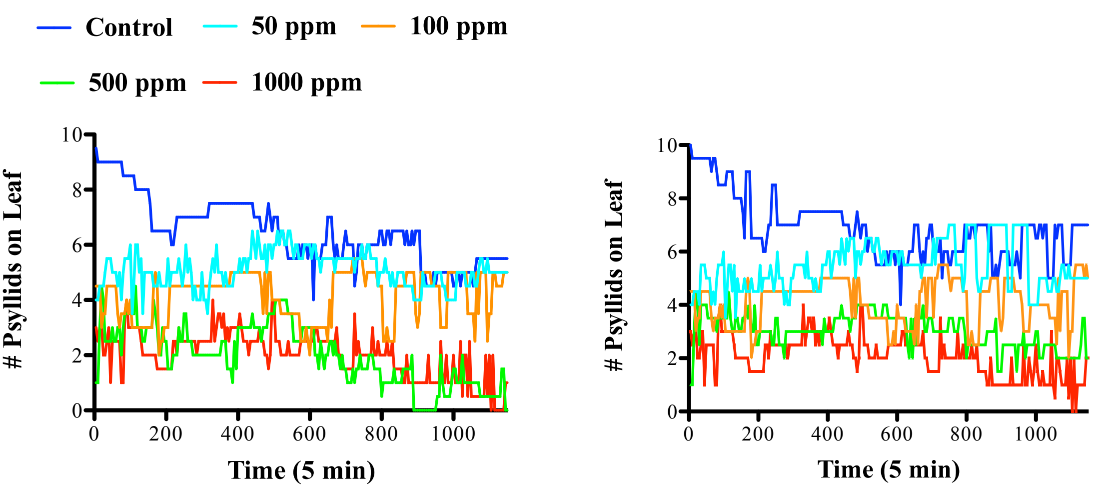

Figure 3.7 The graph on the left is of the *D. citri* systemic bioassay with flonicamid. The graph on the right is of the *D. citri* foliar bioassay with flonicamid. For both graphs (A) Control with D. citri, (B) 1000 ppm, (C) 500 ppm, (D) 100 ppm, (E) 50 ppm flonicamid. Both the systemic (left graph) and foliar (right graph) bioassays follow the same pattern. The control groups had the least amount of *D. citri* mortality while the higher the concentration of flonicamid the greater the *D. citri* mortality.

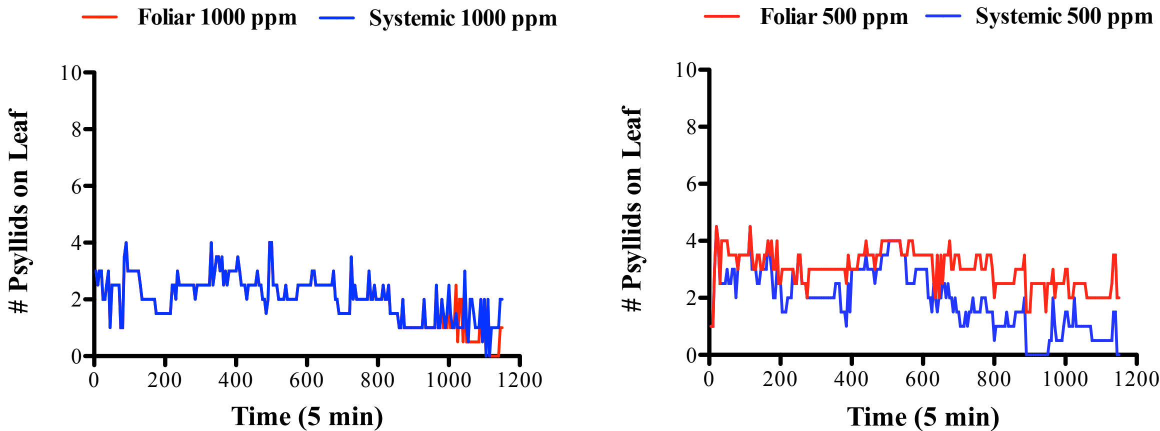

Figure 3.8 Foliar vs. Systemic application of flonicamid. (Left) Shows foliar (red) vs. systemic (blue) applications of flonicamid at 1000 ppm. (Right) Shows foliar (red) vs. systemic (blue) applications of flonicamid at 500 ppm.

The feeding behavior of *D. citri* was significantly altered (P<0.001) on *C. sinensis* leaves treated with the two highest concentrations (1000 ppm and 500 ppm) of foliar flonicamid compared to the control groups **(Figure 3.7)**. The feeding behavior of *D. citri* was significantly altered (P<0.001) on *C. sinensis* leaves treated with the two highest concentrations (1000 ppm and 500 ppm) of systemic flonicamid compared to the control groups. Both the foliar and systemic applications of flonicamid resulted in a similar alteration of *D. citri* feeding behavior **(Figure 3.8)**. In both applications of flonicamid the two highest concentrations (1000 ppm and 500 ppm) significantly altered *D. citri* feeding behavior (P<0.001). The pairwise test done to compare between the foliar and systemic applications resulted in a significant similarity between the two applications at the two highest concentration of flonicamid (1000 ppm and 500 ppm). When the highest concentration of flonicamid (1000 ppm) was compared between the foliar and systemic applications the correlation was high (P<0.024). The correlation between foliar and systemic applications in the second highest concentration (500 ppm) was also significant

(P<0.002). While the similarity between the effect of foliar and systemic applications on *D. citri* feeding behavoir was great, systemically applied flonicamid resulted in less *D. citri* on the leaves at 500 ppm compared to the foliar application at that same concentration **(Figure 3.8)**.

3.3 Fecal output assay

The *D. citri* exposed to leaves treated with the two highest concentrations of flonicamid, 1000 ppm and 500 ppm had a significantly deceased (P <0.001) fecal output compared to the control group **(Figure 3.9)**. The third highest concentration of flonicamid (100 ppm), that the *D. citri* were exposed to also resulted in a significant decrease in fecal output (P<0.005). The other two concentration of flonicamid 50 ppm and 10 ppm had less fecal matter on the plate when compared to the control; however, this was not statistically significant.

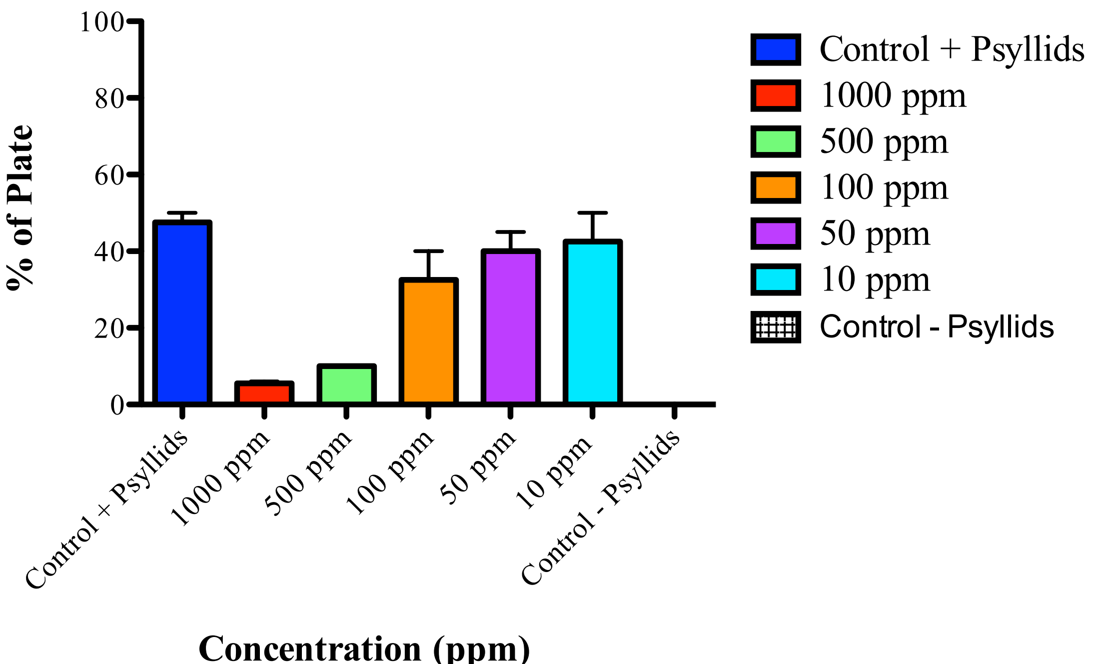

Figure 3.9 *D. citri* fecal output in response to flonicamid treatments.
The fecal output of *D. citri* was significantly altered (P<0.001) on *C. sinensis* leaves treated with the two highest concentrations (1000 ppm and 500 ppm) of foliar flonicamid compared to the control groups.

3.3.2 *C.* L. asiaticus in *C. sinensis* treated with foliar or systemic flonicamid

No transmission was detected from *D. citri* to *C. sinensis* in the 96-hour mortality bioassay. The *D. citri* that had been confirmed to be 50% positive for *C.* L. asiaticus (Stelinski, 2010) was reconfirmed to be positive for *C.* L. asiaticus in *D. citri*, but *C.* L. asiaticus was not detected in *C. sinensis* **(Figure 3.10)**. Neither a traditional qPCR or a nested qPCR was able to detect *C.* L. asiaticus from *D. citri* to *C. sinensis.*

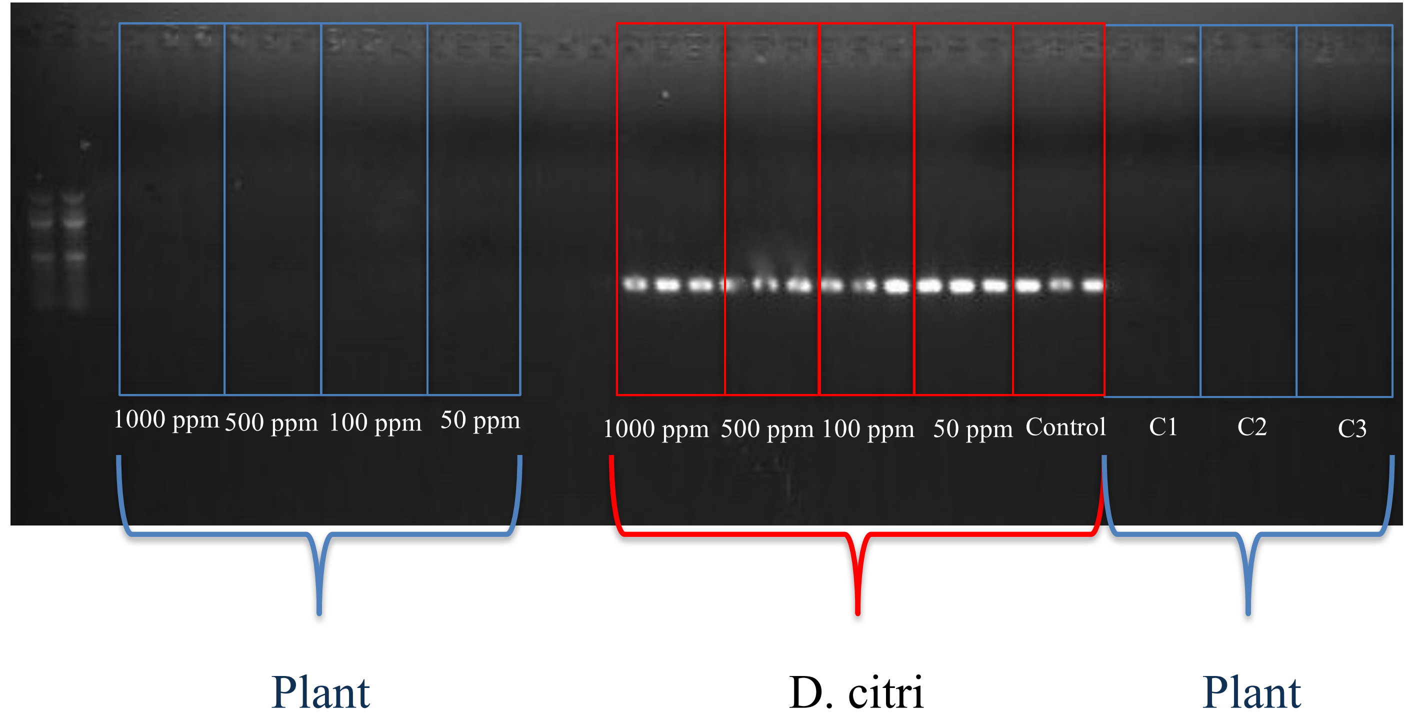

Figure 3.10 Detection of *C.* L. asiaticus. (1000 ppm (Blue)) *C. sinensis* with 1000 ppm flonicamid. (500 ppm (Blue)) *C. sinensis* with 500 ppm flonicamid. (100 ppm (Blue)) *C. sinensis* with 100 ppm flonicamid. (50 ppm (Blue)) *C. sinensis* with 50 ppm flonicamid. (C1,C2,C3) *C. sinensis* controls. (1000 ppm (Red)) *D. citri* exposed to 1000 ppm flonicamid (500 ppm (Red)) *D. citri* exposed to 500 ppm flonicamid. (100 ppm (Red)) *D. citri* exposed to 100 ppm flonicamid. (50 ppm (Red)) *D. citri* exposed to 50 ppm flonicamid. (Control (Red)) *D. citri* control.

3.4 Discussion

3.4.1 Systemic and foliar flonicamid behavioral assay

Both the systemic and foliar bioassays resulted in an altered feeding behavior in *D. citri*. *C. sinensis* leaves treated with the two highest concentrations (1000 ppm and 500 ppm) of flonicamid showed a significant alteration in feeding behavior ($P<0.001$) compared to the control groups. The higher concentrations of flonicamid could have caused a greater effect on the nerves involved in *D. citri* feeding. This reduction and/or alteration in feeding behavior decreases the likelihood that *C.* L. asiaticus will be transmitted to citrus. Further studies should be done to assess what factors are influencing the feeding alteration in *D. citri*. Both bioassays indicated that

under laboratory conditions systemic and foliar flonicamid yield similar results. Future studies should include a section regarding what factors could affect the absorption of flonicamid applied to the roots. The pairwise test confirmed the significant similarity between the two applications at the two highest flonicamid concentrations under laboratory conditions (1000 ppm: $P<0.024$; 500 ppm $P<0.002$). The systemic applications of flonicamid at 500 ppm resulted in fewer *D. citri* on the leaves than foliar applications at 500 ppm, which could indicate that less flonicamid could be used in systemic application compared to foliar applications. The potential use of systemically applied flonicamid makes this compound a extremely relevant because foliar applications can be more expensive than systemic applications (Black et al. 2007). These findings could result in substantial saving for citrus growers.

3.4.2 Fecal output assay

The fecal output of *D. citri* was significantly altered ($P<0.001$) on *C. sinensis* leaves treated with the two highest concentrations (1000 ppm and 500 ppm) of foliar flonicamid compared to the control groups. This is likely due to flonicamid's mode of action as a chordotonal organ modulator. The higher concentrations of flonicamid are presumed to have affected the nerve impulses involved in *D. citri* feeding, and thus affected the quantity of feces produced. These finding confirm the hypothesis that greater concentrations of flonicamid will cause an alteration in feeding behavior that will result in a reduction in fecal output. Future studies should focus on identifying the mechanism that is causing the alteration in feeding behavior.

3.4.3 *C.* L. asiaticus in *C. sinensis* treated with foliar or systemic flonicamid

Transmission of *C.* L. asiaticus transmission from *D. citri* to *C. sinensis* was not detectible in the *D. citri* mortality bioassay in a traditional real time PCR or a nested real time PCR. This could be due to the fact that *C.* L. asiaticus levels in the *C. sinensis* were not at detectable levels after only 4 days of exposure. It is also possible that the 50% positive *C.* L. asiaticus *D. citri* used in this study were not positive enough to inoculate the *C. sinensis. Diaphorina citri* are always a vector for *C.* L. asiaticus; however, the highest rates of *C.* L. asiaticus in the *D. citri* population occur in summer and coincide with the *C. sinensis* bloom.

References

Burckhardt, D., and Ouvrard, D. 2012. Article A revised classification of the jumping plant-lice (Hemiptera : Psylloidea). Entomology 34: 1–34.

Davidson, E. W., Segura, B. J. 1994. Microorganisms influence the composition of honeydew produced by the silverleaf whitefly, *Bemisia argentifolii*. Journal of Insect Physiology 40(12): 1069-1076.

Eyer, J., Crawford, R. 1933. Observations on the Feeding Habits of the Potato Psyllid (*Paratrioza cockerelli* Sulc.) and the Pathological History of the" Psyllid Yellows" Which It Produces. Journal of Economic Entomology 26(4): 846-850.

Goolsby, J., Adamczyk, J. 2007. Development of an IPM program for management of the potato psyllid to reduce incidence of zebra chip disorder in potatoes. Subtropical Plant Science 59: 85-94.

Halbert S. E. 2005. The discovery of huanglongbing in Florida, presentation H3. Proceedings of 2nd International Citrus Canker and Huanglongbing Research Workshop. 1:7-11.

Hall, D. G., Hentz M. G., Adair R. C. 2008. Population ecology and phenology of Diaphorina citri (Hemiptera: Psyllidae) in two Florida citrus groves. Environ Entomol. 37(4):914-24

Hall, D. G., Richardson, M. L., Ammar, E.-D., Halbert, S. E. 2013. Asian citrus psyllid, Diaphorina citri, vector of citrus huanglongbing disease. Entomol. Exp. Appl., 146(2): 207–223.

Hodges, A. W., and Spreen, T. H. 2012. Economic Impacts of Citrus Greening (HLB) in Florida. Entomology 3:3-6.

Hunter, W., Dowd, S. 2009. Psyllid biology: expressed genes in adult Asian citrus psyllids, *Diaphorina citri* Kuwayama. Open Entomology Journal 3: 18-29.

Jagoueix, S., Bove, J. M., Garnier, M. 1994. The Phloem-Limited Bacterium of Greening Disease of Citrus Is a Member of the α subdivision of the proteobacteria. Int. J. Syst. Bacteriol., 44(3): 379–386.

Kumagai L.B., LeVesque C. S., Blomquist C. L., Madishetty K., Guo Y., Woods P. W., Rooney-Latham S., Rascoe J., Gallindo T., Schnabel D., Polek M. 2013. First report of "Candidatus Liberibacter asiaticus" associated with citrus huanglongbing in California. Plant Dis 97:283. http:// dx.doi.org/10.1094/PDIS-09-12-0845-PDN.

Kuwayama, S. 1908. Die Psylliden Japans. I," Trans. Sapporo Nat. Hist. Soc., 2: 149–189.

León, J. H. De, Sétamou, M., Gastaminza, G. A., Buenahora, J., Cáceres, S., Yamamoto, P. T., Bouvet, J. P., Logarzo, G. A. 2011. Two Separate Introductions of Asian Citrus Psyllid Populations Found in the American Continents. Ann. Entomol. Soc. Am., 104(6): 1392–1398.

Li W., Hartung J. S., Levy L. 2006. Quantitative real-time PCR for detection and identification of Candidatus Liberibacter species associated with citrus huanglongbing. J Microbiol Methods 66:104 –115. http://dx.doi.org/ 10.1016/j.mimet.2005.10.018.

Manjunath, K., Halbert, S. 2008. Detection of '*Candidatus* Liberibacter asiaticus' in *Diaphorina citri* and Its Importance in the Management of Citrus Huanglongbing in Florida. Phytopathology 98(4):387-396.

Setamou, M., J. V. Da Graca. J. L. Sandoval. 2015. Siutability of native North American Rutaceae to serve as host plants for the Asian citrus psyllids. (Hemiptera: Liviidae). Journal of Applied Entomology. 10:4-9.

Stelinski, L.L., D.R. Boina, and W.L. Meyer. 2010. Oviposition marking behavior of *Diachasma alloeum*, (Hymenoptera: Braconidae), parasitizing *Rhagoletis pomonella*, (Diptera:

Tephritidae). Journal of Insect Behavior. 23: 419-430.

Rogers, M. E., Stansly, P. A., Stelinski L. L. 2014. Florida Citrus Pest Management Guide: Asian Citrus Psyllid and Citrus Leafminer. IFAS Extension 1-9.

Rosenthal, G., and McNally, A. 2010. USDA Announces Quarantine to Prevent Spread of Citrus Disease. Entomology 9:13-20.

Teixeira, D., Saillard C., Eveillard S., Danet J., Costa P., Ayres A., Bové J. 2005. The Spread of HLB. Int J Syst Evol Microbiol 55(5):1857-1862. doi:10.1099/ijs.0.63677-0.

Thao, M. L. L., Clark, M. A. 2001. Phylogenetic Analysis of Vertically Transmitted Psyllid Endosymbionts (*Candidatus* Carsonella ruddii) Based on atpAGD and rpoC: Comparisons with 16S–23S rDNA-Derived Phylogeny. Current Microbiology 42(6): 419-421.

Tiwari, S., Gondhalekar, A. D., Mann, R. S., Scharf, M. E., Stelinski, L. L. 2011. Characterization of five CYP4 genes from Asian citrus psyllid and their expression levels in Candidatus Liberibacter asiaticus-infected and uninfected psyllids. Insect Mol. Biol. 20(6): 733–44.

Yang, Y., Huang, M., Beattie, G. A. C., Xia, Y., Ouyang, G., Xiong, J. 2006. Distribution, biology, ecology and control of the psyllid Diaphorina citri Kuwayama, a major pest of citrus : A status report for China. Int. J. Pest Manag. 52(12): 343–352.

Chapter 4

Identification of an Alimentary Tract Associated Bacterium for Future Use in Biological Management Strategies for *D. citri*

4.1 Introduction

Diaphorina citri, the Asian citrus psyllid, is an invasive phloem feeding plant pest in the United States and a vector for the bacterium *Candidatus* Liberibacter asiaticus (Kuwayama, 1908). This bacterium is the primary causal agent of Huanglongbing, a major threat to the citrus industry in the United States. *Diaphorina citri* was first observed in Florida in 1998. Since its introduction, *D. citri* transmitting *C.* L. asiaticus has cost more than 8.92 billon dollars of revenue from the citrus industry in Florida alone (Hodges and Spreen, 2012). Traditional pest management protocols have not adequately managed the spread of the pathogen (Halbert, 2005). While many new chemical management strategies are under development, the problem of eventual chemical resistance remains; therefore, a biological management strategy would be desirable.

Adult female *D. citri* lay eggs on the newly emerged citrus leaves known as flush. When the *D. citri* nymphs emerge they begin to feed on the flush and excrete sugary fecal matter known a "psyllid sugar". The *D. citri* life cycle includes 5 instars before molting into their adult form (Liu and Tsai, 2000). It has been determined that *C.* L. asiaticus is not a necessary bacterium for *D. citri* survival (Chia-Ching et al., 2016). This means that removing *C.* L. asiaticus could have little effect on the *D. citri* population and they could return to their status as a minor plant pest.

Bacterial symbionts play a key role in the insects ability to adapt to new environments and food sources (Planck et al., 2015). It is recognized that mutualistic bacterial symbionts contribute

to the acquisition of nutrients vital to insect growth and reproduction. It is also possible that some bacterial symbionts may aid in the detoxification of secondary plant metabolites giving the insects access to otherwise unavailable nutrients (Planck et al., 2013). The three major bacterial endosymbionts associated with *D. citri* are *Wolbachia*, *Canadidatus* Carsonella ruddii and *Candidatus* Profftella armature (Chu et al., 2016). *Wolbachia* from the Alphaproteobacteria group of bacteria has been highly studied in insects and it has been confirmed that this bacterium is vertically transmitted in insects (Chu et al., 2016). *Wolbachia* can be found in large numbers in insect eggs, however it has never been found in sperm. This bacterium has been linked to cytoplasmic incompatibility, meaning that *Wolbachia* infected males will not successfully mate with uninfected females, or females infected with a different strain of *Wolbachia*. *Canadidatus* Carsonella ruddii is a species of unculturable Gammaproteobacterium housed in the bacteriome, of *D. citri,* where it is associated with nutritional acquisition in all species of phloem feeding insects (Nakabachi et al., 2006). *Candidatus* Profftella armature is a species of Betaproteobacterium that produces a defensive polyketide called disphorin (Chu et al., 2016). *Candidatus* Profftella armature is currently unculturable and has an extremely streamlined genome from 464,857 to 174,014 bps indicating that it is an obligate symbiont (Chu et al., 2016). Fifteen percent of the *Candidatus* Profftella armature genome is dedicated to producing diaphorin. Other *D. citri* associated bacterial symbionts are *Nitrosospira multiformis*, *Alkanindiges illinoisensis*, *Buchnera aphidicola* and *Enterobacter cloacae*. *Nitrosospira multiformis* is an ammonia oxidizing bacteria associated with soil. *Alkanindiges illinoisensis* is a anerobic obligate hydrocarbonoclastic bacterium. *Buchnera aphidicola* has been studied in phloem feeding insects and could be important in synthesizing riboflavin for *D. citri* growth. *Enterobacter cloacae* is a gram negative facultative anaerobic bacterium that has been fully

sequenced, as have its associated plasmids. *Enterobacter cloacae* can be easily cultured in a laboratory setting and its plasmids have been shown to be transformable (Watanabe et al., 2000). In 1998 Dr. Kenji Watanabe and Dr. M. Sato isolated 5 strains of *Enterobacter cloacae* from plant and insect sources and usefully grew the bacteria in the guts of silk worm larva (Watanabe and Sato, 1998).

The first biological management strategy attempted on the *D. citri* population was in 1998 with the introduction of *Tamarixia radiate*, a parasitic wasp (Hall et al., 2010). This wasp is native to Southeast Asia where it acts as a natural parasitoid of *D. citri* (Hall et al., 2010). Currently, studies are being performed to assess the effectiveness of this biological management strategy (Hall et al., 2010). However, even with the implementation of this strategy the *C*. L. asiaticus pathogen continues to be spread by *D. citri*. Other biological management strategies, like paratransgenesis, that do not introduce a new invasive species into the United States are gaining support. However, in order to implement a biological management strategy like paratransgenesis, a great deal of information needs to be gathered. One paratransgeneic model for *D. citri* would be to eliminate *C*. L. asiaticus from the *D. citri* population through the transformation of a bacterium naturally occurring in *D. citri*. The foundation of this model is an understanding of the microbiome of *D. citri* and work is being done to characterize the microbiome of *D. citri* (Chia-Ching et al., 2016).

In this study, an alimentary tract-associated bacterium was recovered from *C*. L. asiaticus positive and negative insects. Genome data of this bacterium and the associated plasmid was sequenced, assembled, and annotated. The information gained in this study provides greater insight into the microbiome of *D. citri*.

4.2 Materials and Methods

4.2.1 Psyllid acquisition from USDA-ARS

At Dr. Carol Lazon's lab, dead *D. citri* adults (N = 227) were shipped on ice, overnight from the USDA-ARS Horticultural Laboratory in Fort Pierce, Florida to California State University where they were processed the day of receipt, or kept at 0 °C for 24 h. The *D. citri* were surface sterilized using a three-step chemical soak (Lauzon et al., 2010). Whole abdomens, individual gut samples, fore-, mid-, and hindgut, were tested for bacterial inhabitants.

4.2.2 Bacterial Culture Techniques

Abdomens and individual gut samples were individually and aseptically placed into sterilized Reasoner's 2 broth (Difco Laboratories, Detroit, MI). The samples were then incubated at 24°C for 24 hours. Then, the cultures were streaked onto Reasoner's 2 agar and incubated overnight under air. The individual cultures were isolated and the pure colonies of aerobic bacteria were identified using standard biochemical and morphological tests (Bergey, 2002) with API analytical strips (BioMerieux, Durham, NC). In order to identify any anaerobic bacterial cultures, the other portion of the bacterial samples were streaked onto sterilized media (Hardy Diagnostics, Santa Maria, CA) and incubated in an anaerobic chamber at 24°–26° for 48 hours. These experiments were carried out in Dr. Carol Lazon's lab at UC Riverside.

4.2.3 Initial Identification of Isolated Bacterial Cultures

Bacterial DNA was isolated from 18 hour pure cultures from the abdomens and gut samples of dead adult *D. citri* using the DNeasy kit (Qiagen, Hilden, Germany). DNA was quantified using a UV spectrophotometer (Genesys 8, Thermo Scientific, Rochester, NY). Then, the DNA was amplified using polymerase chain reaction (PCR) under the following conditions:

Two sets of primers were used to amplify regions specific for almost all bacterial 16S sequences. A region of approximately 1,500 bp from the 16S rRNA gene was amplified with the primers B27F (5' AGAGTTTGATCMGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Lazon, 2010). For the reaction, each 25-µL PCR mixture contained 10 ng DNA, 2X PCR Master Mix (Promega, Madison,WI), with primers. The amplification cycle was constructed as initial denaturation step of 2 minutes at 95°C, and 35 cycles of 30 s at 95°C, 30 s at 55°C and 1 minute at 72°C, and a final extension step of 7 minutes at 72°C. Template DNA was omitted from the reaction mixture to serve as a negative control. Then, 5 µL of each suspension was electrophoresed on 1% agarose gels in 1X TBE buffer. Five µL of PCR products were subjected to an ExoSAP-IT kit (Affymetrix, Inc) at 37°C for 15 minutes followed by 80°C for 15 minutes, to cleave excess primers and inactivate free nucleotides. Next, the cleaned PCR products were used as the template for sequencing. DNA from all samples was subjected to a BigDye V3.1 Terminator Kit (Applied Biosystems, Foster City, CA), and the reactions were run in a PTC-200 Thermal Cycler (MJ Research, Waltham, MA). The conditions were initial denaturation at 96°C for 1 minute, followed by 25 cycles at 96°C for 10 s, 50°C for 5 s, 60°C for 4 minutes. X-Terminator Mix (Applied Biosystems, CA,USA) was added. DNA was sequenced using a 3130 Genetic Analyzer (Applied Biosystems, CA, USA). Peak Scanner™ Software v1.0 (Applied Biosystem, CA, USA) was used to view the sequence peaks. Each sequence was run in a BLAST search limited to a bacterial database (http://blast.ncbi.nlm.nih.gov/). Bacterial DNA from pure cultures was identified by examining the high scoring sequences from the BLAST search results. These techniques were completed at Dr. Carol Lazon's lab at UC Riverside.

4.2.4 Bacterial DNA Extraction for Genome Sequencing

The isolation protocol for gram-negative bacteria was used according to the manufacturer protocol (Qiagen Blood and and tissue Kit, Foster City, CA). Bacterial cells from a diluted overnight culture, were pelleted by centrifuging at 5000 x g for 10 minutes. Then, 180 ul of enzymatic lysis buffer was added and allowed to incubate for 30 minuets and 37°C. Buffer AL and proteinase K were added to the samples and incubated at 56°C for 30 minuets. The rest of the procedure followed the extraction of blood or cells protocol (Qiagen Blood and tissue Kit, Foster City, CA). The samples were stored at 20°C until they were sent on ice for Ilumina sequencing.

4.2.5 Plasmid Isolation for Sequencing

The bacterial plasmids were isolated in accordance with the Qiagen Plasmid Purification Mini Kit. The bacterial cultures were harvested from the starter cultures and centrifuged at 6000 x g for 15 minutes. The bacterial pellets were re-suspended in 0.3 ml of Buffer P1 (Qiagen Plasmid Purification Mini Kit). Buffer P2 was added and incubated at room temperature for 5 minutes. Then, 0.3 ml of cold P3 Buffer was added and incubated on ice for 5 minutes. The samples were centrifuged at 6000 x g for 10 minutes and the supernatant was removed into another micro centrifuge tube. The supernatant was then filtered, washed and eluted. The samples were precipitated with isopropanol and washed with 70% ethanol. The samples were allowed to air dry before being dissolved in water. Plasmid size was ascertained by running a standard gel electrophoresis and analyzing the gel using gel analyzer **(Figure 4.1)**. The samples were stored at 20°C until they were sent on ice for Illumina sequencing.

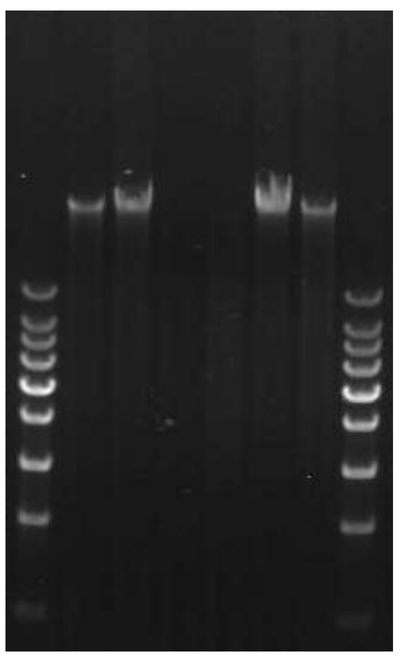

Figure 4.1 The plasmids isolated from a *D. citri* bacterial culture were run on a gel. A gel analyzer was used to calculate the relative size of the extracted plasmids.

4.2.6 Sequencing and Analysis of Bacterium and Plasmids

The bacterial DNA and plasmid preps were sent for individual analysis to the Research and Testing Laboratory (RTL, Lubbock, Texas) for MiSeq Illumina whole genome single end sequencing. The samples were sequenced using standard protocols. The genomic data for both the plasmid and the bacterium were quality filtered using standard protocols (Geneoius software version 8.05) A *de novo* assembly was completed for the bacterium and the plasmid using Geneoius (software version 8.05). The contigs from the bacterium were used to identify the bacterium by preforming an NCBI nucleotide BLAST. The sequences were then compared to the reference genome and annotated.

4.3 Results

4.3.1Bacterial identification using 16S

The identify of the isolated bacterium was ascertained using a nucleotide BLAST on the NCBI database. The nucleotide BLAST using the NCBI database returned a distribution of 200 blast hits with an alignment of >=200 on a scores **(Table 4.1)**. The alimentary tract-associated bacterium was identified as *Enterobacter cloacae*.

4.3.2 Bacterial and Plasmid Genome Sequencing

The bacterial DNA returned 1,477,787 nucleotide sequences with a minimum sequence length of 35bps and a maximum sequence length of 300 bps. The three assembled contigs from the bacterium were (1) 28624, (2) 28556 and (3) 27784 base pairs (bps). Contig 1 from the *de novo* assembly spans from 2383415 to 2412038 bps on the reference genome. This region contains genes that encode for phage-like proteins and an intergrase **(Figure 4.2)**. **Figure 4.3** shows the second contig which spans from 3763079 to 3791634 bps on the reference genome. This regions contains genes that encode for phage-like protein, tail fiber, tail shaft, coat, portal and terminase. The third contig spans from 3801810 to 3829593 bps on the reference genome. This region contains genes that encode for Intergrase, phage-like protein, attachment site, terminase and tail shaft **(Figure 4.4)**.

The plasmid prep retuned 971,141 nucleotide sequences with a minimum sequence length of 35 bps and a maximum sequence length of 300 bps. After identification of the bacterium as *Enterobacter cloacae,* all of the complete plasmids associated with *Enterobacter cloacae* were compared to the plasmid isolated from the *D. citri* bacterium. The isolated plasmid was compared to the pDNA reference and had 100% coverage (Geneious software version 8.05). The

de novo assembly of the plasmid yielded large contigs with good coverage and assembled to Enterobacter *cloacae* plasmid DNA (pDNA). The contig for the plasmid spans from from 2820556 to 2852732 bps on the reference plasmid of Enterobacter cloacae. This region of the plasmid contains genes that encode for Intergrase and phage-like protein **(Figure 4.5)**.

Table 4.1 The top five hits from the nucleotide BLAST using the NCBI database. The BLAST returned a distribution of 200 blast hits with an alignment of >=200 on a scores.

***Enterobacter cloacae* strain**	**Max Score**	**Total Score**	**Query Cover**	**Ident.**	**Accession**
ATCC 13047 16S ribosomal RNA gene, complete sequence	2856	2856	100%	100%	NR_102794.1
AZ-3 16S ribosomal RNA gene, complete sequence	2846	2846	99%	100%	KJ675625.1
GGT036, complete genome	2830	22580	100%	99%	CP009756.1
DSM 30054 16S ribosomal RNA gene, partial sequence	2808	22464	99%	99%	NR_117679.1
MR2 16S ribosomal RNA gene, partial sequence	2791	2791	99%	99%	KC999876.1

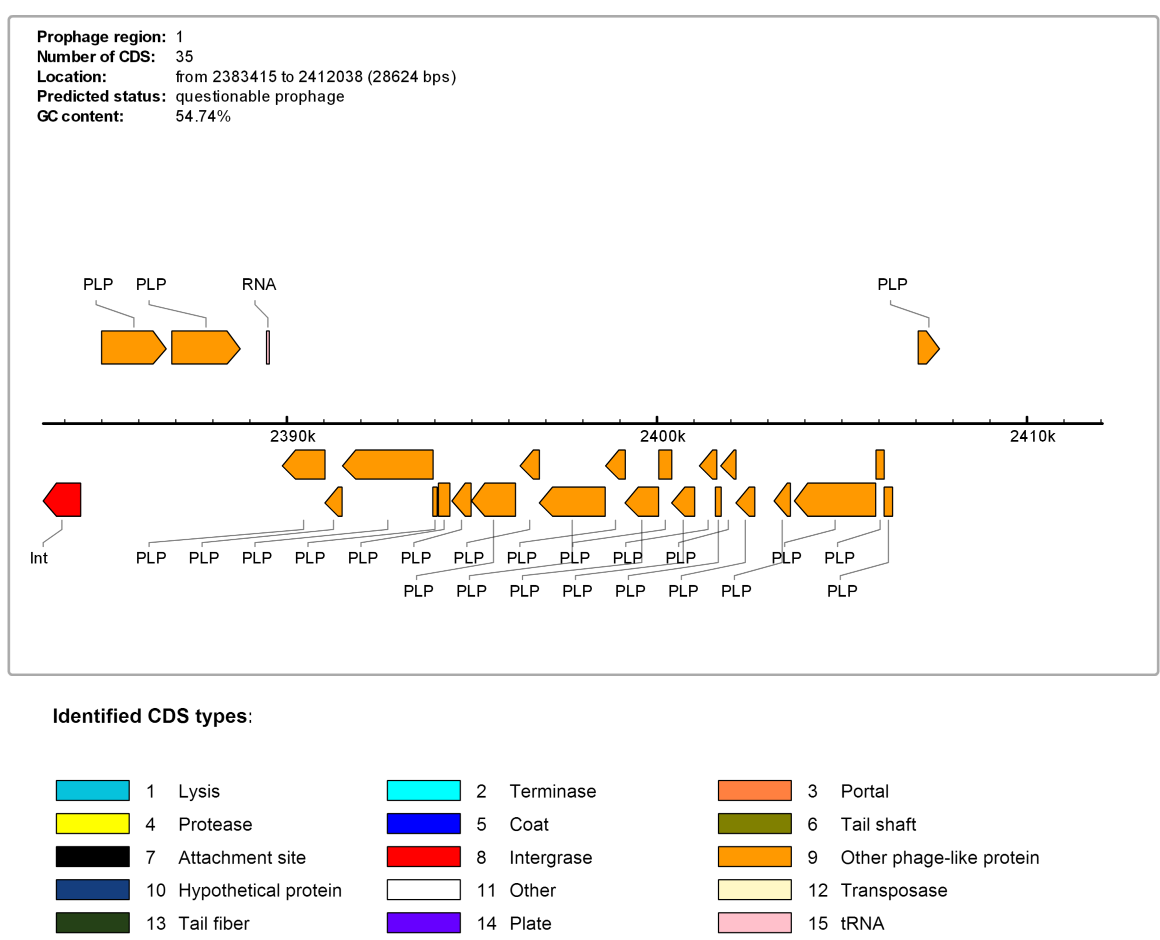

Figure 4.2. Contig 1 of the bacterium isolated from *D. citri* and identified as *Enterobacter cloacae.* This region is 28624 bps with 35 CDS and a GC content of 54.74%. The location of this region on the reference genome is from 2383415 to 2412038 bps. This region contains genes that encode for phage-like proteins and an intergrase.

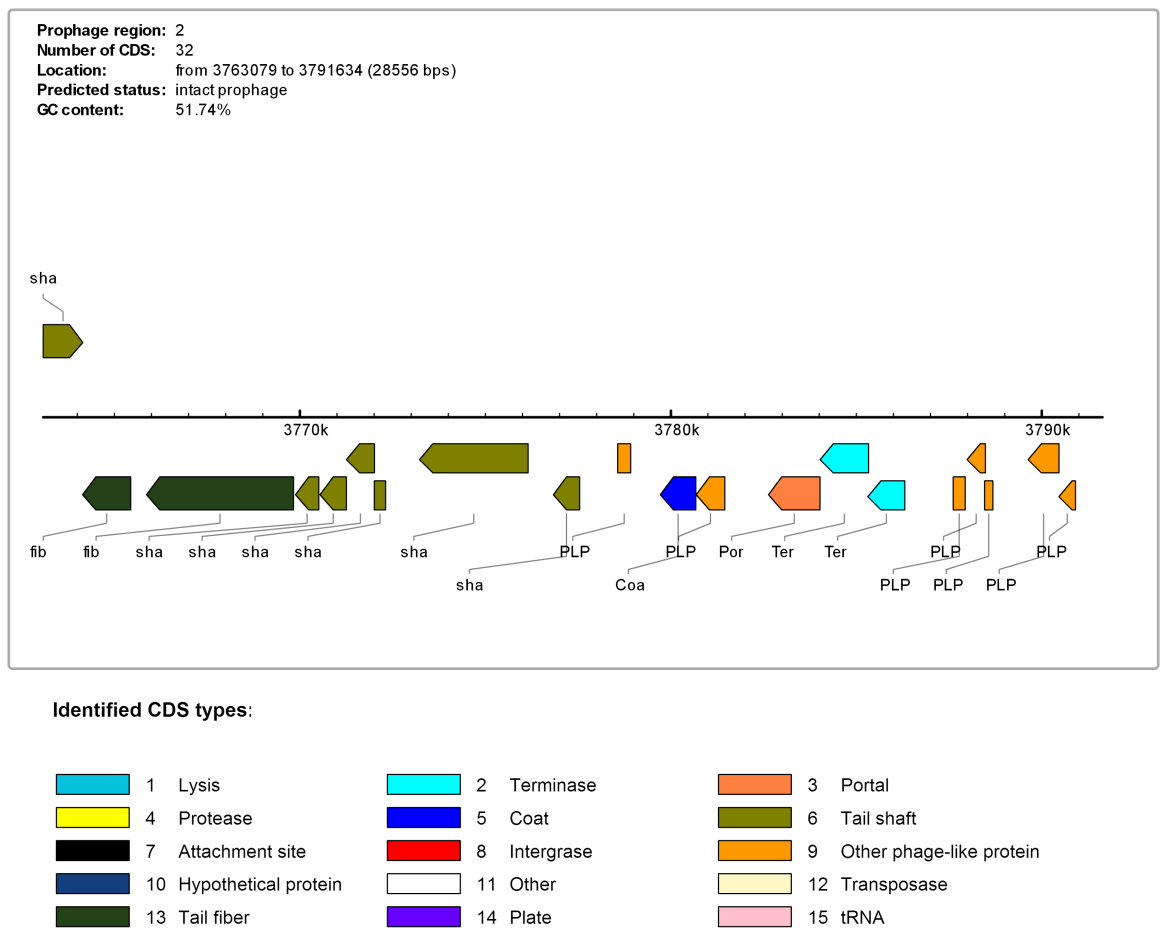

Figure 4.3. Contig 2 of the bacterium isolated from *D. citri* and identified as *Enterobacter cloacae.* This region is 28556 bps with 32 CDS and a GC content of 51.74%. The location of this region on the reference genome is from 3763079 to 3791634 bps. This regions contains genes that encode for phage-like protein, tail fiber, tail shaft, coat, portal and terminase .

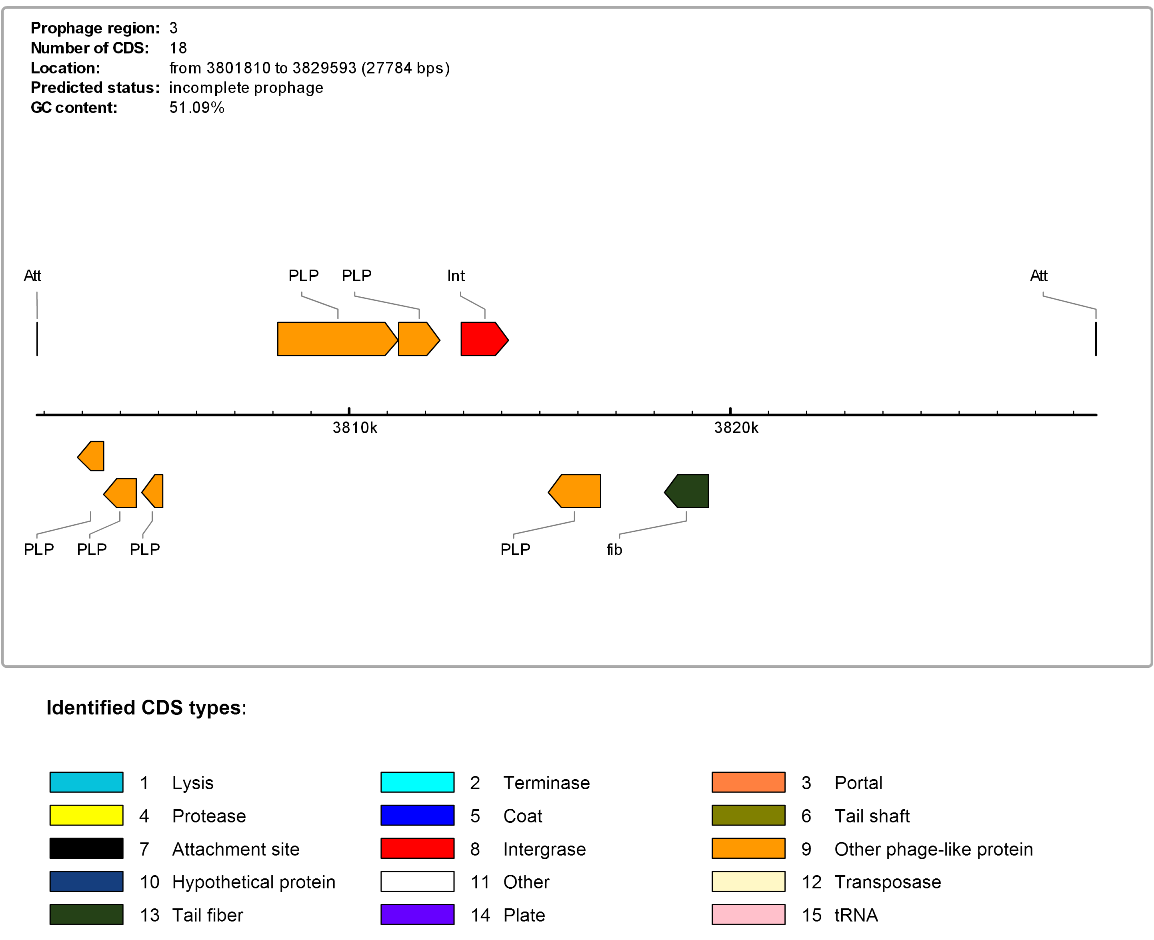

Figure 4.4. Contig 3 of the bacterium isolated from *D. citri* and identified as *Enterobacter cloacae.* This region is 27784 bps with 18 CDS and a GC content of 51.09%. The location of this region on the reference genome is from 3801810 to 3829593. This region contains genes that encode for Intergrase, phage-like protein, attachment site, terminase and tail shaft.

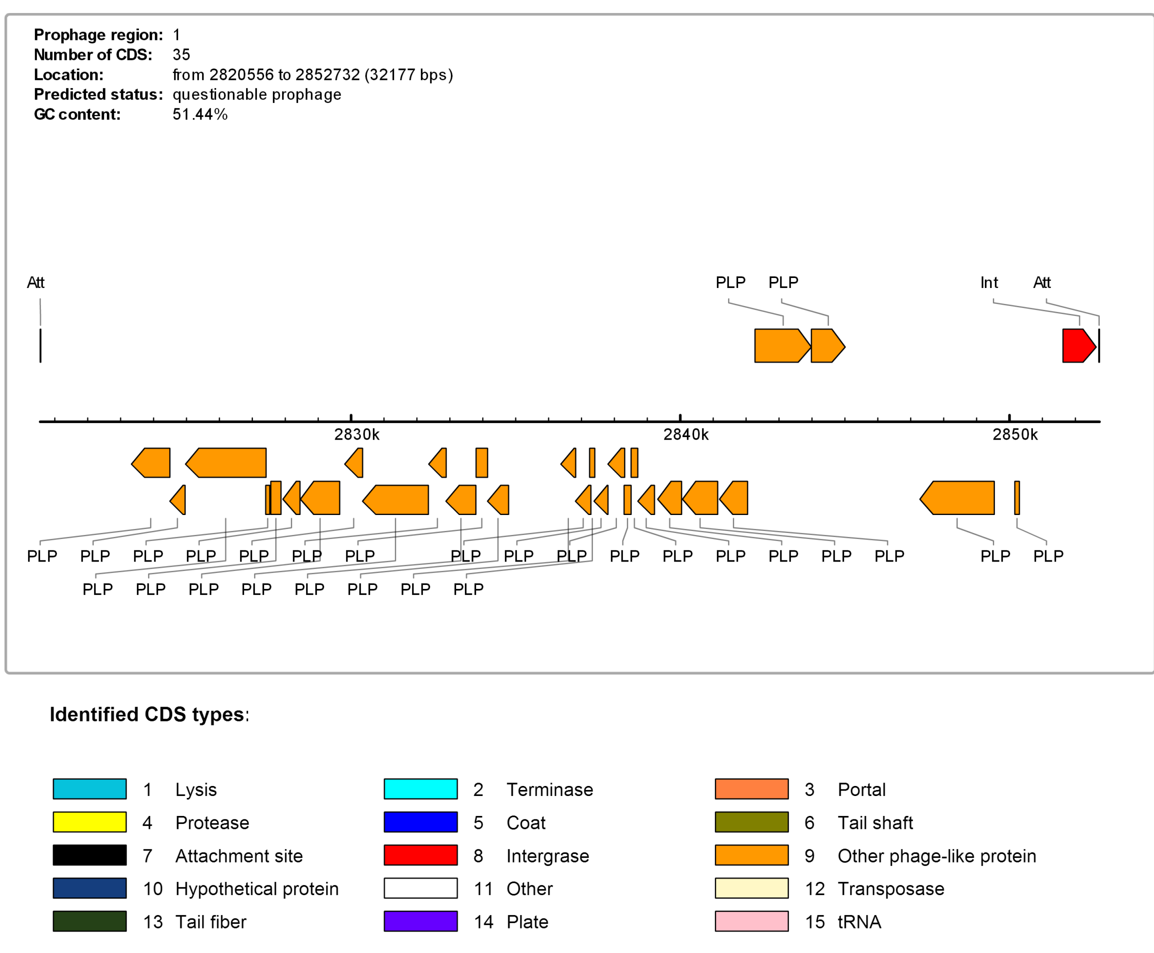

Figure 4.5. Contig 1 of the plasmid isolated from the bacterium isolated from *D. citri* and identified as *Enterobacter cloacae.* This region is 32177 bps with 35 CDS and a GC content of 51.44%. The location of this region on the reference genome is from 2820556 to 2852732 bps. This region contains genes that encode for Intergrase and phage-like protein.

4.4 Discussion

The two set of techniques used to isolate and identify bacteria in microbiology are culture dependent and culture independent techniques (Dickson et al., 2014). Culture dependent techniques rely on traditional culturing methods and may fail to recognize relevant unculturalable and fastidious bacteria (Takikawa et. al., 2002; Stefani et al., 2015). Culture independent methods, which gained recognition in the late 1990's, do not rely on bacterial cultivation and instead, use methods like denaturing gradient gel electrophoresis to identify unculturalable bacteria (Dickson et al., 2014). *Candidatus* Liberibacter asiaticus, the causal agent of HLB, is an extremely relevant unculturalable bacteria that was identified using culture independent techniques (Garnier et al., 2000; Planet et al., 1995; Crosslin et al., 2009). Many of the bacteria housed in the gut of *D. citri* are unculturalable and have recently been identified using culture independent techniques (Su et al., 2012). One downside to cultural independent methods is that they can lead to culture bias because culture independent methods identify bacterial DNA that may or may not be biologically active (Kisand et al., 2003). Culture dependent methods require that the bacteria is biologically active because if it was not, the bacteria would not grow on media (Hirsch et al., 2010). Bacteria that are able to be cultured are required for bacterial transformations, since it is not currently possible to make competent cells without a bacterial culture (Watanabe et al., 2000). This is one reason why the identification of *Enterobacter cloacae* as a *D. citri* associated bacterium is significant for use in a biological management strategy like paratransgenesis. *Enterobacter cloacae* is easily cultured, has a well studied genome, and contains a native plasmid. The native plasmid offers more stability in a transformation when compared with non-native plasmids which has been tested in termites (Watanabe and Sato, 2006). This bacterium and associated plasmid have been successfully

transformed in other insects including silkworms and termites (Watanabe and Sato, 2006). Many compounds have been confirmed to suppress *C.* L. asiaticus in citrus these include Ampicillin, Carbenicillin, Penicillin and Cafalexin (Zhang et al., 2014). It is possible that antibiotic genes could be incorporated into the bacterial plasmid in order to suppress *C.* L. asiaticus. Before that can happen, these compounds would need to be tested on *D. citri* in order to assess the impact they would have of the microbial community of *D. citri*. It is also important to note that strains of *Enterobacter cloacae* associated with insects have not been identified as human pathogens (Watanabe and Sato, 2006). This study recovered three bacterial contigs which represent a small fraction (1.58%) of the *Enterobacter cloacae* genome. The plasmid from the isolated bacterium has 100% coverage when compared to *Enterobacter cloacae* pDNA. The identification, sequencing, assembly and annotation of the bacterium and plasmid isolated from *D. citri* provides the scientific community working on ebbing the spread of *C.* L. asiaticus through biological management strategies like paratransgenesis, with another important piece of the *D. citri* puzzle. By confirming that *Enterobacter cloacae* can be found as a bacterial symbiont of *D. citri* and that it contains a transformable native plasmid, progress has been made toward a stable biological management strategy for *D. citri*.

References

Baldo, L., Hotopp-Dunning, J., Jolley, K., Bordenstein, S., Biber, S., Choudhury, R., Hayashi, C., Maiden, M., Tettelin, H., and Werren, J. 2006. Multilocus sequence typing system for the endosymbiont Wolbachia pipientis. Appl Env. Microbiol. 72(11):7098–110.

Baumann, P. 1995. Genetics, physiology, and evolutionary relationships of the genus *Buchnera*: intracellular symbionts of aphids. Annual Reviews in Microbiology 49(1): 55-94.

Boykin, L. M., De Barro, P., Hall, D. G., Hunter, W. B., McKenzie, C. L. Powell, C. A., Shatters, R. G. 2012. Overview of worldwide diversity of Diaphorina citri Kuwayama mitochondrial cytochrome oxidase 1haplotypes: two Old World lineages and a New World invasion. Bull. Entomol. Res. 102(05): 573–582.

Chia-Ching C. A., Torrence, M., Hoffmann, K S., Pelz-Stelinski K. S. 2016. Inter- Population Variability Endosymbiont Densities in the Asian Citrus Psyllid (Diaphorina citri Kuwayama). Invertebrate Microbiology 1-9.

Davidson, E. W., Segura, B. J. 1994. Microorganisms influence the composition of honeydew produced by the silverleaf whitefly, *Bemisia argentifolii*. Journal of Insect Physiology 40(12): 1069-1076.

Dickson, R. P., Erb-Downward, J. R., Prescott, H. C., Martinez, F. J., Curtis, J. L., Lama, V. N., Huffnagle, G. B. 2014. Analysis of Culture-Dependent versus Culture-Independent Techniques for Identification of Bacteria in Clinically Obtained Bronchoalveolar Lavage Fluid. Journal of Clinical Microbiology 52(10): 3605–3613. http://doi.org/10.1128/JCM.01028-14.

Duan, Y., Zhou, L., Hall, D. G., Li, W., Doddapaneni, H., Lin, H., Liu, L., Vahling, C. M., Gabriel, D. W., Williams, K. P., Dickerman, A., Sun, Y., Gottwald, T. 2009. Complete

Genome Sequence of Citrus Huanglongbing 38 Bacterium, 'Candidatus Liberibacter asiaticus' obtained through metagenomics. MPMI 22: 1011–1020

Gai, C. S., Lacava, P. T. 2009. Transmission of *Methylobacterium mesophilicum* by *Bucephalogonia xanthophis* for paratransgenic control strategy of Citrus Variegated Chlorosis. The Journal of Microbiology 47(4): 448-454.

Halbert, S. E. 2005. The discovery of huanglongbing in Florida, presentation H3. Proceedings of 2nd International Citrus Canker and Huanglongbing Research Workshop. 1: 7-11.

Hall, D. G., Hentz M. G., Adair R. C. 2008. Population ecology and phenology of Diaphorina citri (Hemiptera: Psyllidae) in two Florida citrus groves. Environ Entomol. 37(4):914-24.

Hall, D. G., Richardson, M. L., Ammar, E.-D., Halbert, S. E. 2013. Asian citrus psyllid, Diaphorina citri, vector of citrus huanglongbing disease. Entomol. Exp. Appl., 146(2):207–223.

Kumagai, L. B., LeVesque C. S., Blomquist C. L., Madishetty K., Guo Y., Woods P. W., Rooney-Latham S., Rascoe J., Gallindo T., Schnabel D., Polek M. 2013. First report of "Candidatus Liberibacter asiaticus" associated with citrus huanglongbing in California. Plant Dis 97:283-300. http:// dx.doi.org/10.1094/PDIS-09-12-0845-PDN.

Kuwayama, S. 1908. Die Psylliden Japans. I. Trans. Sapporo Nat. Hist. Soc. 2:149–189.

Nakabachi, A., Yamashita, A. 2006. The 160-kilobase genome of the bacterial endosymbiont *Carsonella*. Science 314(5797): 267.

Pearson, W. R. 1990. Rapid and Sensitive Sequence Comparison with FASTP and FASTA. Methods Enzymol. 183:63–98.

Yang, Y., Huang, M., Beattie, G. A. C., Xia, Y., Ouyang, G., Xiong, J. 2006. Distribution, biology, ecology and control of the psyllid Diaphorina citri Kuwayama, a major pest of citrus : A status report for China. Int. J. Pest Manag 52(12): 343–352.

Chapter 5

Conclusions and Future Research

Conclusion

As the primary vector of *C.* L. asiaticus, *D. citri* have contributed to billions of dollars of lost revenue for the citrus industry in the United States. Current chemical and biological management strategies have not been able to manage the spread of Huanglongbing effectively without consequences to non-target organism like important insect pollinators or vertebrates. The integration of both chemical and biological management strategies is central to dealing with the *D. citri* issue.

The development and testing of new insecticides like cyclaniliprole on *D. citri* has yielded significant mortality and could contribute to a new management strategy. Cyclaniliprole and like other diamides has been shown to have little to no effect on off target species like pollinators or vertebrates. This is due to the mode of action for diamides; they bind specifically to insect ryanodine receptors and initiate a constant stream of Ca^{2+} resulting in death. In this study the bioassays done with cyclaniliprole yielded significant *D. citri* mortality ($P<0.001$) after 4 days of exposure with concentrations at and above 500 ppm. The dose response curve completed at 84 hrs. indicated that the LC_{50} was 95 ppm. Future research should focus on concentrations of cyclanilipole between 50 ppm and 500 ppm in order to identify the LC_{50} at shorter lengths of exposure. Cyclaniliprole should be considered when creating a management strategy for *D. citri*.

Another important compound is the antifeedant flonicamid. This compound has been shown to have little impact on non-target organisms because of its novel mode of action. Flonicamid works on nerve action by modulating a chordotonal organ. This mode of action is

different from other compounds and shows no cross resistance with other compounds. *Diaphorina citri* have a similar response whether flonicamid is exhibiting systemic or translaminar action. The ability of citrus plants to take flonicamid up systemically could be advantageous, because this would allow treatments to be administered through irrigation lines and reduce the need for expensive and time consuming foliar applications. The similarities between the foliar and systemic application results found in this study warrant further research with flonicamid. This study provides justification for whole plant studies with flonicamid, this would allow the ability of the plant roots to systemically take up flonicamid to be analyzed. This could contribute to long term management of *D. citri*.

Development of a biological management for *D. citri* is has been sought for many years. The issue with the development of this type of strategy is a lack of knowledge of microbiome of *D. citri*. The characterization of the microbiome of *D. citri* has made the creation of a biological management strategy for *D. citri* a greater possibility. One current biological management strategy is paratransgenesis. This strategy would remove *C*. L. asiaticus from *D. citri* by transforming a bacterial symbiont of *D. citri*. This would affect the ability of *D. citri* to transmit *C*. L. asiaticus and help to ebb the spread of the pathogen. In order to make this strategy a possibility the isolation, identification and annotation of *D. citri* bacterial symbionts is necessary. In chapter three of this study, an alimentary bacterium and associated plasmid were isolated from *D. citri*. The bacterium and plasmid were then identified and annotated. This work on the microbiome of *D. citri* gives greater insight in to the symbiotic relationship *D. citri* has with its alimentary bacteria and plasmids and contributes to a greater understanding of *D. citri* and symbiont biology that could be used in a biological management strategy.

The research done in this study gives important insights into the chemical and biological management of *D. citri* that can be used currently and in future strategies. The two chemical compounds cyclaniliprole and flonicamid offer modes of action that effect the mortality of *D. citri* with limited non- target side effects. Flonicamid can also affect the feeding behavior of *D. citri*. In chapter four the identification and annotation of a *D. citri* alimentary track associated bacterium *Enterobacter cloacae* and plasmid could contribute future biological management strategies.