Fall 11-1-2015

RNA Interference: Potato/Tomato Psyllid, Bactericera Cockerelli, Oral Delivery of Double-Stranded RNAI Construct

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RNA INTERFERENCE: POTATO/TOMATO PSYLLID, BACTERICERA COCKERELLI, ORAL DELIVERY OF DOUBLE-STRANDED RNAI CONSTRUCT

by

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A thesis submitted in partial fulfillment
of the requirements for the degree of
Master's of Science
Department of Biology

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College of Arts and Sciences

The University of Texas at Tyler
November 2015

The University of Texas at Tyler
Tyler, Texas

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November 2, 2015
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DN: c=US, o=U.S. Government, ou=Department of Agriculture, cn=WAYNE HUNTER, 0.9.2342.19200300.100.1.1=12001000102348
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Acknowledgements

Firstly, I would like to express my sincere gratitude to my advisor Dr. Blake Bextine for the continuous support of my MS study and related research, you have been a tremendous mentor for me. I am always thankful for your priceless advice on both research as well as on my career.

My sincere thank also goes to my committee members, Dr. Ali Azghani, Dr. Jon Seal and Dr. Wayne Hunter for serving as my committee members and for their insightful comments and encouragements.

I am equally grateful to Dr. Srini Kambampathi, Department Chair, and all the faculty members in the Department of Biology at The University of Texas at Tyler, for their valuable guidance and supports.

My sincere thanks also goes to Mr. Brad Low, Miss Maequeta Humber and Miss Jessica Coleman, who provided their valuable suggestions and time.

I thank my fellow lab mates and all the members from Bextine's Research Laboratory for their help and support. My sincere gratitude also goes to Mr. Christopher Powell, Mr. Patrick Rydzak, Miss Gretta Marie Sharp and Miss Megan Rudolph for their valuable comments that greatly improved the manuscript.

I would like to thank Dr. Don Henne and Adrian Silva for providing potato psyllids. I would also like to thank Miss Hema Kothari for assisting me during fluorescence microscopy. I would like to express my warm thanks to Mr. Daymon Hail for providing me the primer for the dsRNA construct.

Lastly, I would like to express my sincere gratitude towards my family for supporting me spiritually throughout my study. I am equally thankful to my friend, Aman Gautam for his untiring support and help.

Thank You,

Bijaya Kumar Sharma

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Abstract

RNA INTERFERENCE: POTATO/TOMATO PSYLLID, BACTERICERA COCKERELLI, ORAL DELIVERY OF DOUBLE-STRANDED RNAI CONSTRUCT

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November, 2015

The potato/tomato psyllid, *Bactericera cockerelli* (Sulc.), is a serious and economically important pest of the potato, tomato and other solanaceous crops. This insect is the putative primary vector of the phytopathogenic bacterium *Candidatus* Liberibacter solanacearum which causes the Zebra Chip in potato. This disease has caused millions of dollars loss to the potato industry. While traditional management programs have minimized the negative impacts of this disease system, a sustainable alternative to chemical treatment is needed. Management of this pest by downregulation of endogenous mRNA using RNA interference (RNAi) technology is one of the best molecular method available; however, several technical challenges exist which must be overcome to demonstrate RNAi strategies in potato production. First, the oral delivery of double stranded RNA construct need to provide an effective and efficient method that can be used in the field. Second, potential genes to target and downregulate the endogenous mRNA level through RNAi technology needs to result in insect mortality or reduction in pathogen transmission. Therefore, dsRNA construct were synthesized *in vitro* and offered to adult potato psyllid, mortality was recorded overtime and significant downregulation of mRNA level for target gene was assessed using qPCR technology. Moreover, to study the synergism between RNAi and insecticide treatments, dsRNA against one of the xenobiotic metabolizing gene, CYP450 6BQ13 was orally delivered to the adult *B. cockerelli* along with the imidacloprid, a neonicotinoid insecticide, mortality caused due to the conjugal treatment was recorded overtime and qPCR was utilized to observe the possible downregulation of endogenous CYP450 6BQ13 mRNA level.

Chapter One

Literature review

Insecta

Insecta is a broad class of the phylum arthropoda and is divided into several subclasses and many more orders. Insects are classified according to their morphological structure and biological process of development. The two forms of insect metamorphosis are, hemimetabolism (incomplete metamorphosis) and holometabolism (complete metabolism) found in insects. Holometalous insects undergo complete metamorphosis and develop through four complete life stages – embryo or egg, larva, pupa and adult. Unlike holometabolists, hemimetabolous insects undergo incomplete metamorphosis and consists of only three life stages – egg, nymph and adult. (Borror et al., 1989).

The insects lying in the order Coleoptera, diptera, hymenoptera, lepidoptera and all other members of endopterygota exhibits complete metamorphosis while the major order for insects exhibiting incomplete metamorphosis includes, paraneoptera, orthoptera, blattaria, mantodea, isopteran, odonta and hemiptera (Borror et al.,1989). More than 80,000 species of hemiptera have been classified and most of them are economically important to agricultural crops, as they are associated with the direct damaging of the plant through feeding on the plant's sap and indirect damaging by transmitting plant pathogens (Dolling, 1991).

Psyllids

Psyllids (jumping plant lice) are small sap sucking insects, which belongs to the order hemiptera and superfamily psylloidea (Burckhardt, 2014; Lubanga et al., 2014). Almost forty species (19 genera) of order hemiptera have been discovered to be economically important (Percy, 2005). Like all hemipterans, psyllid life cycle are comprises of three distinct life stages,

an egg stage, five larval instars and a sexually reproductive adult stage (Lubanga et al., 2014). Temperature and precipitation are the two major determining factors for the development of psyllid eggs and nymphs (Lubanga et al., 2014). Psyllids are host specific and host plants are another factor in psyllid growth and development (Hodkinson, 2009). They feed on plants by penetrating with their needle-like stylets (Eyer and Crawford, 1933), this may be one of the reason this taxa is associated with the transmission of microbial pathogens. Psyllids receive their major nutrients by feeding on host plants. This causes the nutrient deficiency in the host plant and causes systemic phytotoxaemia (Hodkinson, 2009).

Psyllid mouth parts are modified for piercing and sucking, this is one of the primary factor accounting for the transmission of plant pathogens. These insects transmit more than 50% of vector-borne pathogenic viruses and bacteria in plants (Huot et al., 2013). During feeding on a host plant, psyllid nymphs release toxic secretions through saliva causing severe symptoms to the host plant. Psyllids also show a close association with pathogenic microbes, such associations are detrimental to the host plant's physiology by reducing the levels of defensive chemicals, there by weakening the plants immune response (Hodkinson, 2009).

While feeding on infected plants, psyllids ingest pathogenic viruses and bacteria (liberibacters and phytoplasmas) and later introduce them into other healthy host plants (Hodkinson, 2009). Both adults and nymphs are associated with the transmission of phytoplasmas. These bacteria have been found to be vertically transferred by female psyllids to the offsprings, e.g. *Cacopsylla pruni* (Hodkinson, 2009). Recently, four psyllid species (*Diaphorina citri*, *Trioza erytrea*, *Bactericera cockerelli* Sulc and *Trioza apicalis*) have become subject of intense research because these species are strongly associated with the transmission of various species of Liberibacter (Munyaneza, 2010).

***Bactericera cockerelli* (Sulc.)**

The Potato psyllids (*Bactericera cockerelli* Sulc.) are insects; which lay in the order Hemiptera sub-order Sternorrhyncha, and family Triozodae. They are hemimetabulous and undergo five nymphal instars. Adults are black to brown and have stripes of different colors along their abdomen. Both; male and female have clear wings and the length of their body can be between 1.3 and 1.9 mm (Lehman, 1930; Liu and Trumble, 2007). Female *B. cockerelli* lay eggs on the upper and lower surface of leaves of host plants (Knowlton and Janes, 1930). The eggs are yellow in color and oblong in shape, they are attached to the leaves through stalks (Pletsch, 1947) (Figure 1). Normally, eggs take 3-15 days to hatch and the sex ratio of offspring is around 1:1 (Pack, 1930; Knowlton and Janes, 1930). Adult males are smaller than female, as the females have higher metabolic rate than the male (Hodkinson, 2009). Nymphs feed on underside of leaves and rarely move (Lehman, 1930).

Figure 1.1. Life stages of *Bactericera cockerelli* (Rondon et al., 2012)

Potato psyllids are polyphagous insects and have wide host range of more than 20 plant families and more than 40 host species where they can oviposit and complete their life cycle (Knowlton and Thomas, 1934; Wallis, 1951). However, they prefer the plants in the family solanaceae (Wallis, 1955), hence the common names of the tomato or potato psyllid. They are serious pest of solanaceous crops such as potato, tomato (*Solanum lycopersicum* L.) peppers, and eggplant (*S. melongena* L.) (Crosslin et al., 2010).

Life history and development are highly dependent on temperature and other various conditions such as; host plants, sex and regional differences in haplotype (Yang et al., 2010). Optimal temperature for the growth and development of potato psyllid has been determined to be 26.7°C or 32.2°C begins to be detrimental and 38.8°C is lethal to eggs and nymphs and prevents mature females from oviposition (List, 1939). The underside of the larger plant canopy is cooler than the upper side of host leaves, *B. cockerelli* may use the shade of the leaves to stay in optimal temperature (Wallis, 1946).

B. cockerelli are endemic to North America and are regularly found in Oklahoma, Kansas, Nebraska, Arizona, Colorado, Idaho, California, Montana, Minnesota, Nevada, New Mexico, Utah, Oregon, Texas, North and South Dakota, Washington and Wyoming (Pletsh, 1947; Munyaneza et al., 2009; Cranshaw, 1994; Munyaneza et al., 2010). They follow optimal temperatures and undergo a seasonal range variation and/or migration. They have been observed in the upper provinces of Canada, Alberta, Saskatchewan, British Columbia, and Ontario (Pletsch, 1947; Wallis, 1995; Ferguson et al., 2002), as well as Mexico and Central America as far south as Guetemala, El Salvador, Hondurus and Nicaragua (Espinoza, 2010; Munyaneza, 2012, Powell et al., 2012; Bextine et al., 2013) and New Zealand (Butler and Trumble, 2012; Nachappa et al., 2012). In an aeriel insect survey, they have been collected at an altitude up to

1200 m and have also been reported from Sierra Nevada Mountains suggesting that potato psyllids migrate using air currents (Glick, 1939; Papp and Johnson, 1979).

All insects harbor microbial symbionts which help them in growth and development, symbionts also save them from pathogenic attack. Some microbial endosymbionts are heritable i.e., transfer vertically from parents to offspring (Oliver et al., 2014; Cayetano and Vorburger, 2015). Insects also acquire some microbial endosymbionts through food sources or diet (Oliver et al., 2014; Cayetano and Vorburger, 2015). *B. cockerelli* harbors *Candidatus* Carsonella ruddii as its primary endosymbiont, with *Wolbachia*, *Acinetobacter Methyllibium*, *Rhizobium*, *Gordonia*, *Mycobacterium*, and *Xanthomonas* as secondary endosymbionts (Nachappa et al., 2011; Hail et al., 2012). To supplement of diet of phloem deficient amino acids, phloem-feeding insects harbor the symbiotic microbial community in a specialized bundle of cells called bacteriomes (Hail et al., 2012). Though the purpose of having secondary endosymbionts is unknown, some might play great role in providing resistance towards the insecticides (Arp et al., 2014; Hail et al., 2012).

Disease associated with *Bactericera cockerelli*

B. cockerelli is a polyphagous phloem feeding insect (Alvarado et al., 2012; Nachappa et al., 2012). They penetrates their needle-like stylets through the plant tissues (Eyer and Crawford, 1933), to suck the plant sap this causes mechanical tissue damage towards the host plants. They acquire pathogenic bacteria while feeding on infected plants and transmit to the other host plants through direct feeding (Hodkinson, 2009; Rondon et al., 2012). While feeding on potato plants, psyllid nymphs inject a toxin that causes plants' yellowing and underdevelopment, this pathology is known as "psyllid yellows disease", which affects both tuber yield and quality (Munyaneza et al., 2007; Liefting et al., 2009). Direct feeding by this pest causes the tissue

damage and nutrients deficiency in plants. The indirect damages due to *B. cockerelli* are associated with the transmission of phytoplasma by adults and nymphs, which is the causal agent of the purple top potato disease, and the transmission of the bacterium *Candidatus* Liberibacter solanacearum, the causal agent of Zebra Chip disease in potato (Ramírez-Davila et al., 2012; Buchman et al., 2011; Liefting et al., 2009).

Zebra Chip Disease

In 1994 a new disease, commonly called as Zebra chip, was reported in potato fields in Saltilo, Mexico (Munyaneza et al., 2007). The disease was first documented in the United States from 2000 in the potato fields in Southern Texas (Munyaneza et al., 2007; Secor et al., 2009). Zebra chip disease is associated with the bacteria *Candidatus* Liberibacter solanacearum, which is transmitted by *B. cockerelli* (Munyaneza et al., 2007; Hansen et al., 2008). As of 2013, Zebra Chip has been reported through far northern state Washington and Idaho up to the Central America Nicaragua, and in 2008 was also reported in New Zealand (Munyaneza et al., 2009; Crosslin et al., 2012; Bextine et al., 2013; Liefting et al., 2008). The geographical map showing the presence of Zebra chip in North and Central America as of 2012 is shown in figure 2.

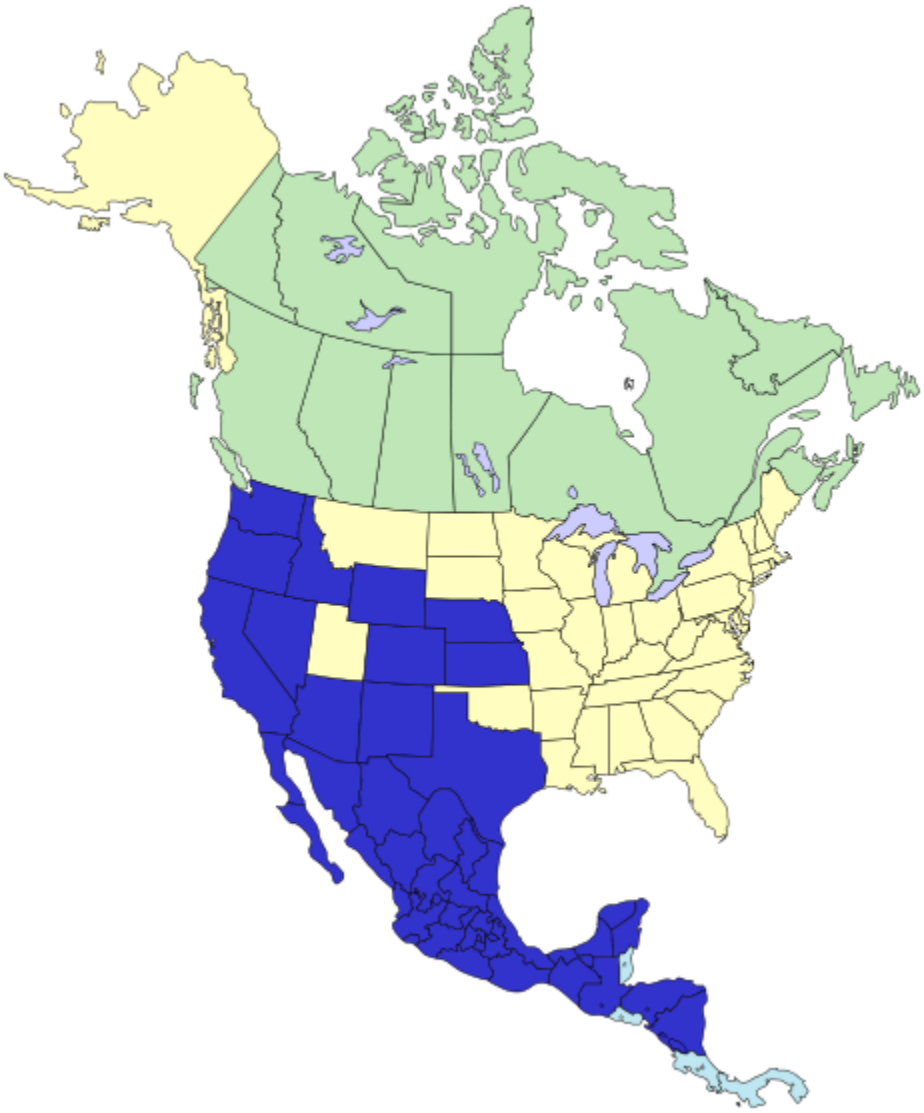

Figure 1.2. Map showing the presence of zebra chip in North and Central America (dark blue) as of 2012 (Munyaneza et al., 2012).

The foliar symptoms of Zebra Chip infected potatoes is similar to psyllid yellow disease, but the physiological changes in tubers are different (Arslan et al., 1985; Senogeda et al., 2010). Foliar symptoms of Zebra Chip is characterized by; stunting growth, leaf chlorosis and scorching, proliferation of axillary buds, aerial tubers, browning of vascular tissue and premature plant death (Munyaneza et al., 2007; Crosslin et al., 2010) (Figure 1.3). A freshly cut potato

tuber shows alternating light and dark bands (Figure 1.4), as the starches converted into soluble sugars in the medullary rays (Gao et al., 2009). These symptoms become more severe when frying the infected tubers, the fried tubers show very dark blotches and stripes (Figure 4) rendering them unsellable for chipping industries and other uses (Munyaneza et al., 2007). Navarre et al. (2009) discovered an increase in aromatic amino acids and phenolic compounds with an eight folds increase in tyrosine concentration in extract of ZC infected potato tubers compared to that of healthy potato. In a similar study, Wallis and Chen, (2011) also discovered high levels of peroxidases, polyphenol oxidases, chitinases, and β-1,3 glucanases. Such findings could be used to suggest that; the significant increase in amino acids and phenolic compounds may contribute to enzymatic browning of the infected potato.

Figure 1.3. Damaged associated with Zebra Chip disease. (A) Healthy plants (left) and Zebra Chip disease infected plants (right); (B) Aeriel tubers. (Butler and Trumble 2012).

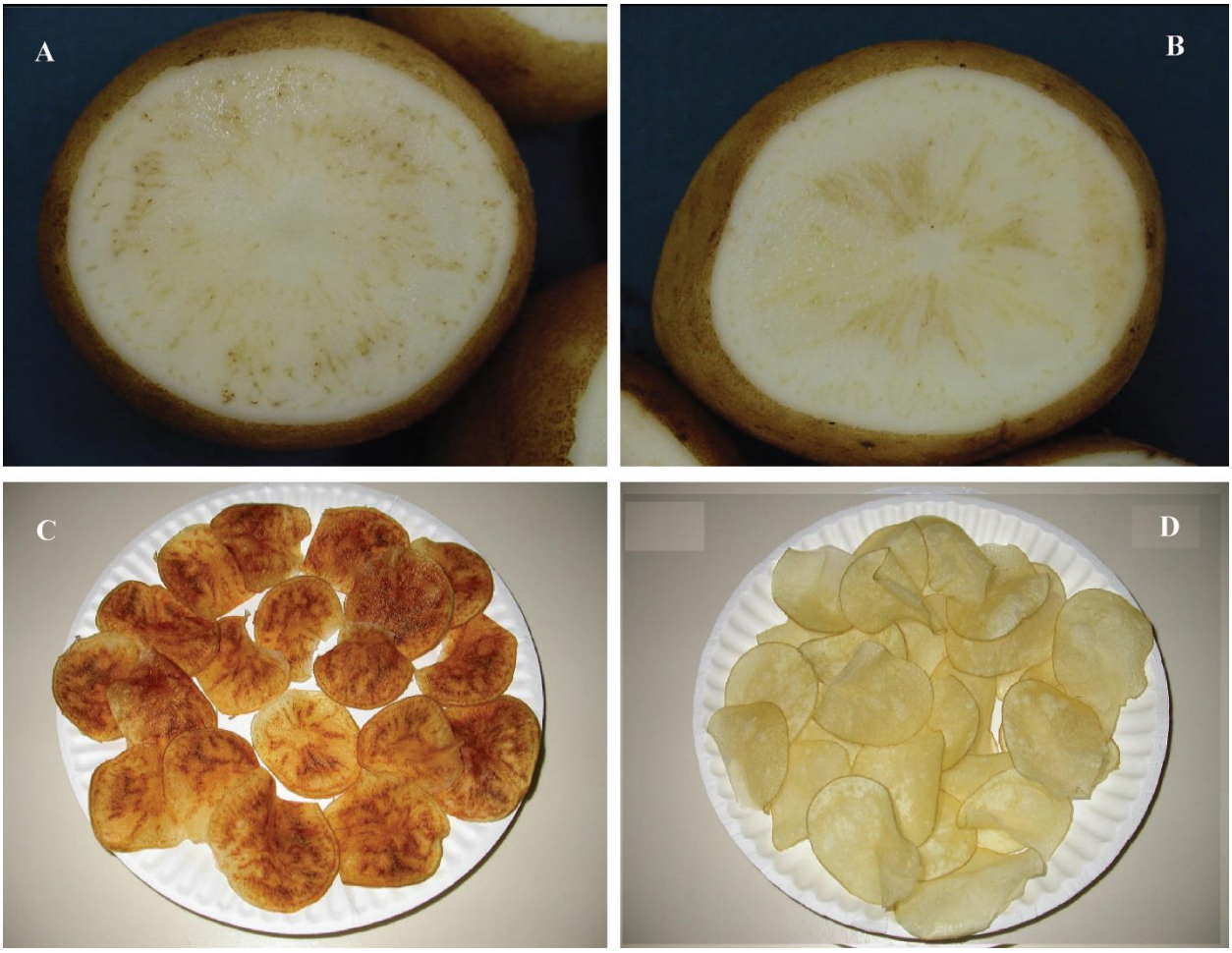

Figure 1.4. Zebra Chip infected potato tubers showing (A) Necrotic flecking; (B) Streaking of medullary ray tissue; (C) Fried chips obtained from infected potato tubers, and (D) chips from healthy potato. (Butler and Trumble 2012).

Candidatus Liberibacter solanacearum (Lso)

The liberibacters are bacteria that belongs to *Alphaproteobacteria* group and are phloem limited, Gram negative and unculturable (Jagoueix et al., 1994; Bove, 2006). Like other Liberibacters, Lso has a rod-shaped morphology (Liefting et al., 2009; Secor et al., 2009) and about 0.2 µm wide and 4 µm long (Liefting et al., 2009). Psyllids are the primary vectors of these bacterial plant pathogens (Bove, 2006). The putative cause of zebra chip disease was unknown until 2008. Liefting et al. (2009) implicated that *Candidatus* Liberibacter solanacearum as the

putative causal agent of ZC through transmission electron microscopy and 16S rDNA sequencing study.

Candidatus Liberibacter solanacearum (Lso) is closely related to liberibacters that are associated with the Huanglongbing, citrus greening disease (Hansen et al., 2008; Munyaneza, 2012). This pathogen has been observed in Western and Central region of USA, Mexico, Central America and New Zealand and has also been documented in Northern Europe and the Mediterranean region (Munyaneza, 2012). Lso has a wide host range, including: pepper (*Capsicum annum*), chilli pepper (*C. frutescens*), tomatillo (*Physalis peruviana*), tomato (*Lycopersicum esculentum*), potato (*Solanum tuberosum*), tamarillo (*S. betaceum*), tobacco (*Nicotiana tobacum*), eggplant (*S. melongena*) and several weeds in the solanaceae family (EPPO 2013; Janse, 2012; Munyaneza, 2012). Recently, Lso has been documented associated with several other non-solanaceous species, suggesting that this liberibacter species has multiple hosts and vectors (Munyaneza, 2012).

Mangement of Potato psyllid

Currently, *B. cockerelli* are attempted to be managed by chemical applications, and commonly used insecticides such as; acephate, metamidophos, thiacloprid, buprofezin, abamectin, cypermethrin, deltamethrin, lambda-cyhalothrin, esfenvalerate, spinosad, spirotetramat, imidacloprid, thiamethoxam, spiromesifen, dinotefuran, pyriproxyfen and pymetrozine (Goolsby et al., 2007; Berry et al., 2009; Gharalari et al., 2009; Butler and Trumble, 2012). Management of psyllids with insecticides is difficult because they are primarily found on the ventral surface of the leaves and total coverage by foliar insecticides is impossible (Nansen et al., 2010; Butler and Trumble, 2012). Various kinds of insecticides have been registered for use according to the life stage of the psyllids (Rondon et al., 2012). The insecticides that is effective

against adult stage might not have the same insecticidal activity against nymph stages or egg stage (Gharalari et al., 2009; Zens et al., 2010; Rondon et al., 2012). In addition, repeated use of insecticides may lead to resistance to pests (McMullen and Jong, 1971; Rondon et al., 2012). Liu and Trumble (2007) found the populations of psyllids from California were resistance to imidacloprid and spinosad compared to the psyllids from Central USA. Treatments of psyllids ideally should begin immediately after the first detection of the insect in the field, within hours these insects can transmit *Candidatus* Liberibacter solanacearum, the causal agent of Zebra Chip disease, to healthy plants (Buchman et al., 2011; 2012). The total costs associated with Zebra Chip control in Texas in 2010 and 2011 range from $-170 to $-590 per acre for the application of pesticides (Guenthner and Greenway, 2010; Guenthner et al., 2011).

Cultural control of *B. cockerelli* refers to the alteration and administration of the cropping environment to reduce the risk of pest population and damage associated with infestation (Pedigo and Rice, 2006). By shifting the planting time, the damage associated with *B. cockerelli* can be minimized. Eyer and Enzie, (1939) observed that early-planted potato and tomato crops developed psyllid yellows as severely as those planted later in the season. Wallis (1948) showed significantly higher numbers of *B. cockerelli* in early planted potato field in Wyoming and Nebraska compared to middle or late season planting. Other solanaceous crops can also be used as an alternate trap crop to protect the main crop from severe damage. Pepper plants were used as a secondary crop or more likely as an alternate trap crop to attract *B. cockerelli* from potatoes in Colorado (Cranshaw, 1994). Researchers have also focused on finding *B. cockerelli* resistant host plants. Of the thirty nine potato varieties screened, none were found to tolerate psyllid yellows disease (Babb and Kraus, 1937). All commercial potato varieties tested were found to be succeptible to *B.cockerelli* (Linford, 1928; Starr, 1939).

Research on biological control of this pest is still ongoing. The use of natural enemies of this pest is one of the environmental managing tactic. There are no practical non-chemical control strategies for potato psyllids that have been developed yet (Rondon et al., 2012). Therefore, it is important to develop a targeted, long-term management control treatment against *B. cockerelli*. Ribo-Nucleic Acid interference, RNAi, technology is a potential strategy of managing insect pests and plant pathogens and is therefore gaining the attention of the scientific community (Price and Gatehouse, 2008).

RNA interference

RNA interference (RNAi) is a technique that uses the introduction of novel, targeted double stranded RNA (dsRNA) sequences into organisms to degrade messenger RNA (mRNA) complementary to the sequences in the dsRNA, inhibiting gene expression (Mello and Conte, 2004). A summary of RNA silencing can be seen in figure 5 below. Sequence specific gene silencing in some insects can be achieved by feeding double stranded RNA through RNA interference (RNAi) technology (Baum et al., 2007). RNAi technology is a potential strategy of managing insect pests and plant pathogens therefore gaining attention of the scientific community (Price and Gatehouse, 2008). In a 2010 survey of adult and late instar potato psyllid transcriptomes, Hail et al. (2010) utilized 454 pyrosequencing (Roche) to identify several potential targets for RNAi.

The efficiency of dsRNA uptake depends on various factors such as, suitable delivery system, delivery efficiency, length and stability of dsRNA (Yu et al., 2013). Double stranded RNA can be deliver directly to the insect body through microinjection (Fire et al., 1998), ingestion (Turner et al., 2006; Baum et al., 2007), soaking (Tabara et al., 1998; Maeda et al., 2001) and transfection (Johnson et al., 2010). Microinjection is used to deliver the accurate

amounts of dsRNA to the specific target organ and is highly efficient way to deliver the dsRNA (Yu et al., 2013). Oral delivery of dsRNA construct is a less-invasive and high throughput method for delivering dsRNA into the insects (Scott et al., 2013). Wuriyanghan et al. (2011) successfully delivered gene specific dsRNAs targeting actin towards *B. cockerelli*, that led to increased mortality and injection of dsRNA corresponding to ATPase reduced 30% endogenous ATPase mRNA level.

Gene silencing by RNAi is a biological process, in which, dsRNA is cleaved by a conserved RNase III protein (dicer) into a short RNA duplex of 21 to 23 nucleotide called as short interfering RNAs (siRNAs). The siRNA combines with other cellular proteins to form RNA induced silencing complex (RISC). The RISC unwinds the duplex of siRNA into two single strands; a guide strand and a passenger strand. The guide strand, complimentary to the target mRNA sequence, binds to the mRNA halting translation while the passenger strand is degraded (Stevenson, 2003).

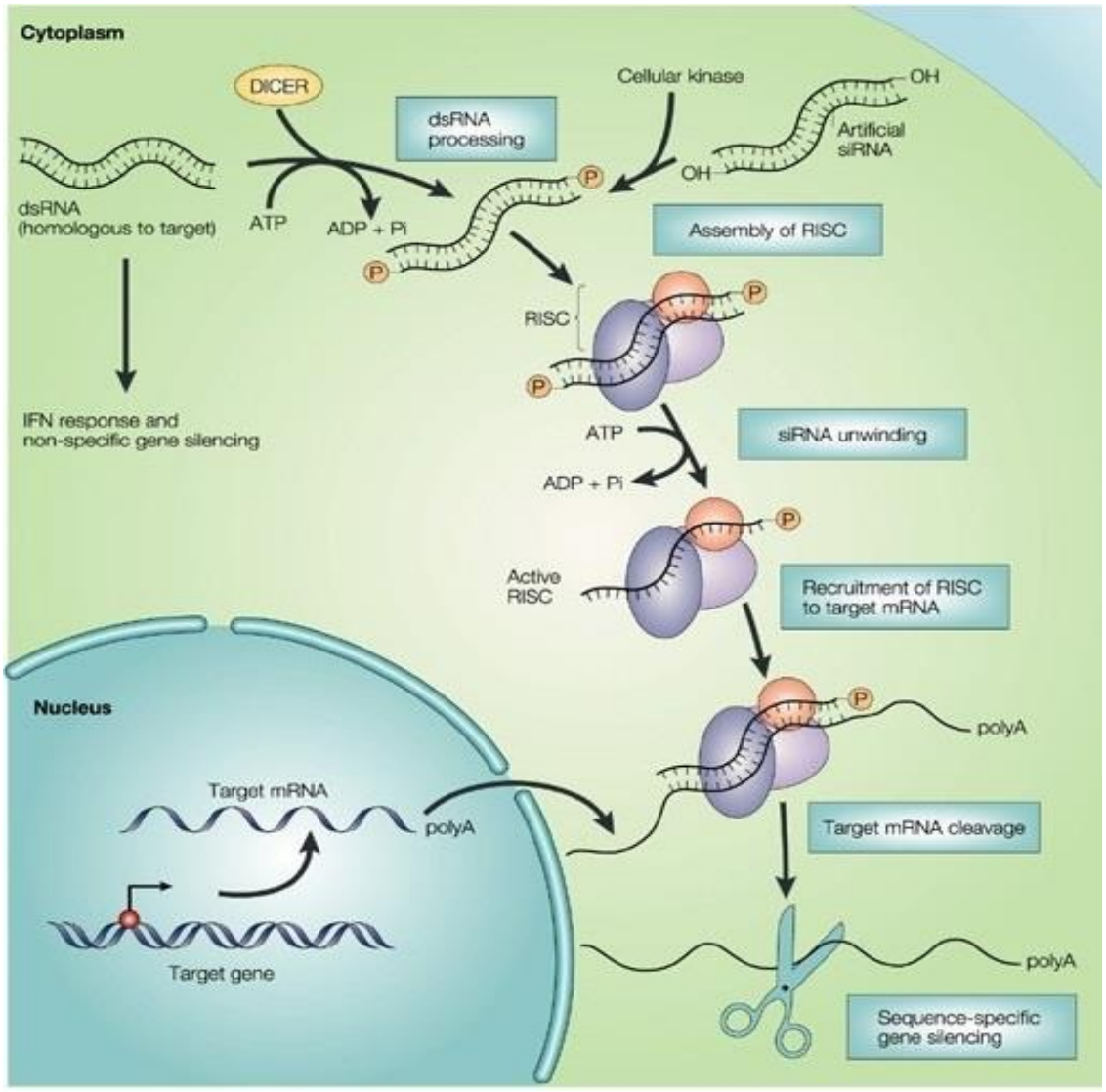

Figure 1.5. Gene silencing by RNA interference. (Stevenson, 2003)

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Chapter Two

The systems for the delivery of dsRNA

1. Introduction

The potato/tomato psyllid, *Bactericera cockerelli* (Sulc.), is a phytophagous insect and a serious pest of solanaceous crops such as potato (*Solanum tuberosum*), tomato (*S. lycopersicum* L.), eggplant (*S. melongena* L.) and peppers (*Capsicum annum*) (Crosslin et al., 2010). *B. cockerelli* is an economical pest associated with the transmission of the bacterial pathogen *Candidatus* Liberibacter solanacearum (Lso) which is the putative causal agent of Zebra Chip disease in potato (Liefting et al., 2009; Hansen et al., 2008; Munyaneza et al., 2007). The foliar symptoms of Lso infection is characterized by; stunting growth, leaf chlorosis and scorching, proliferation of axillary buds, aerial tubers, browning of vascular tissue and premature plant death (Crosslin et al., 2010; Munyaneza et al., 2007). When frying the infected tubers, the fried tubers show very dark blotches and stripes rendering them unsellable for chipping industries and other uses (Munyaneza et al., 2007). Since the first incidence of Zebra Chip in the 1990s this disease has caused millions of dollars in losses for the potato industry in the United States, Mexico, Central America and New Zealand (Munyaneza, 2012; Nachappa et al., 2012; Munyaneza, 2007; Secor and Rivera-Varas, 2004).

Currently, potato psyllids management is attempted through chemical application (Butler and Trumble, 2012; Berry et al., 2009; Gharalari et al., 2009; Goolsby et al., 2007). Management of potato psyllids with insecticides is difficult because psyllids are found on the ventral surface of the leaves and total coverage by foliar insecticides is impossible, therefore systemic insecticides are most effective (Nansen et al., 2010; Butler and Trumble, 2012). Treatment of psyllid infestations must begin immediately after first detection of the insect in the field, within a few hours these insects can transmit *Candidatus* Liberibacter solanacearum to healthy plants

(Buchman et al., 2011; 2012). Use or overuse of insecticide may lead to resistance in pests (McMullen and Jong, 1971; Rondon et al., 2012). Liu and Trumble (2007) found the populations of psyllids from California have developed resistance to imidacloprid and spinosad, when compared to the psyllids from central USA.

Management of *B. cockerelli* by down-regulation of endogenous mRNA using RNA interference (RNAi) technology is possible and these strategies are less prone to resistance development in their targets. Wuriyanghan et al. (2011) utilized RNAi technology and recorded significant mortality of *B. cockerelli* through oral delivery of gene specific double stranded RNA (dsRNA) targeting to Actin. RNA interference (RNAi) is a technique that uses the introduction of novel, targeted double stranded RNA (dsRNA) sequences into pest organisms to degrade messenger RNA (mRNA) complementary to the sequences of the dsRNA, and finally inhibits the gene expression (Mello and Conte, 2004). Sequence specific gene silencing in some insects can be achieved by feeding double stranded RNA through RNAi technology (Baum et al., 2007). RNAi is a potential technology of managing insect pest which is gaining attention from the agricultural community (Price and Gatehouse, 2008).

The efficiency of dsRNA uptake depends on various factors such as suitable delivery system, delivery efficiency, length and stability of dsRNA (Yu et al., 2013). Double stranded RNA can be deliver directly to the insect body through microinjection (Fire et al., 1998), ingestion (Turner et al., 2006; Baum et al., 2007), soaking (Tabara et al., 1998; Maeda et al., 2001) and transfection (Johnson et al., 2010). Microinjection is used to deliver the accurate amounts of dsRNA to the specific target organ and is high efficient way to deliver the dsRNA (Yu et al., 2013). Oral delivery of dsRNA construct is a less-invasive and high throughput method for delivering dsRNA into the insects (Scott et al., 2013). Wuriyanghan et al. (2011)

successfully injected the dsRNA corresponding to ATPase that reduced ~30% endogenous ATPase mRNA level of *B. cockerelli*.

In this study, five different delivery systems were developed and tested for the oral delivery of dsRNA (RNAi construct). For the oral delivery of dsRNA/RNAi construct, an artificial feeding system, commonly called as sachet has been designed that consists of artificial diets [15% sucrose solutions (W: V)] and three different fluorescent compounds (curcumin, quinone and riboflavin), contained between two thin layers of stretched parafilm. The purpose of using fluorescent compounds as a feeding supplement is to track those compounds making their way to gut through oral feeding. Wuriyanghan et al. (2011) utilized Cy^{TM} 3-labelled green fluorescent protein dsRNA in feeding solution and observed fluorescence around the gut area. Another possible feeding system for delivering dsRNA constructs to adult potato psyllids has been designed using plants with multiple methods for the administration of the feeding supplements. qPCR can be used to confirm the presence of dsRNA in plants used in delivery systems. The objective of this study is to determine whether the delivery systems tested can be used further for the delivery of dsRNA construct.

2. Materials and Methods

2.1 Insect colonies

B. cockerelli were initially provided by Dr. Don Henne (Texas A&M University). The psyllid colonies were maintained in separate insect cages (36in. x 24in. x 18in.) in the insectarium at temperature of 26°C and 70% humidity under 14:10 (light: dark) photoperiod. Three different solaneceous crops, tomato plants, potato plants and pepper plants were used to maintain the potato psyllid population. Plants were grown in 6-in. pots with autoclaved soil and fertilized once a month with the label rate of Miracle-Gro water soluble tomato plant food

(Scotts Company, OH) and irrigated with nanopure water. Mixed male/female adult potato psyllids were used in this study.

2.2 dsRNA synthesis

For the feeding assay, one target site for alpha tubulin dsRNA was selected using cDNA library data of *B. cockerelli* (Hail, unpublished). The primer sets were designed to amplify desired length of amplicon for respective target gene using Geneious version 7.1.7 (Biomatters Limited 2014). For each primer sets, temperature gradient PCR was conducted to determine the optimum annealing temperature of the primers in the temperature range 50-60°C using the Amplitaq Gold 360 PCR kit as per manufacturer protocol (Applied Biosystems, Foster City, CA). Each 25 µl reaction mixture included 12.5 µl AmpliTaq Gold 360 Master Mix (Life Technologies, Austin, TX), 1 µl GC enhancer (Life Technologies, Austin, TX), 2.5 µl of each forward and reverse primer, 1 µl of total genomic DNA (~100 ng) and 6.5 µl of nuclease free water (ThermoFisher Scientific, Grand Island, NY). The thermal profile was run as follows: cycle 1, 95°C for 10 min (1x), cycle 2, 95°C for 30s, 50-60°C for 30s, 72°C for 60s (45x), and cycle 3, 72°C for 7 min (1x). Amplicons were visualized on a 2% agarose gel stained with ethidium bromide. If a single band of correct size was visualized, it was excised and the DNA was recovered using QIAquick Gel Extraction kit (QIAGEN, Valencia, CA) following the manufacturer protocol. A second PCR was done using the same primer amended with T7 promoter sequence (5'-TAATACGACTCACTATAGGG-3') at 5' end using identical PCR protocol. The amplicons visualization and purification was done according to the previous described method. The purified product was further used for dsRNA preparation using Hiscribe T7 in vitro transcription kit (New England Biolabs, Ipswich, MA) according to the manufacturer protocol (Figure 2.1).

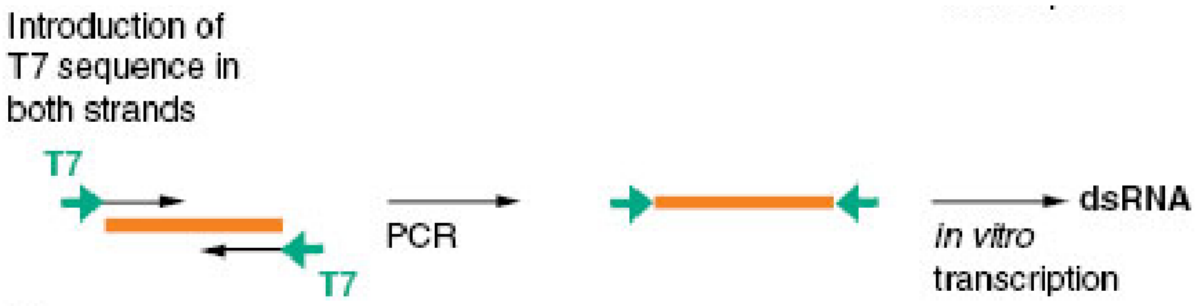

Figure 2.1. The *in vitro* transcription of dsRNA using T7 promoter amended amplicon (Hiscribe T7 *In Vitro* Transcription Kit, New England BioLabs, Ipswich,MA).

2.3. dsRNA/RNAi construct delivery using sachet feeding system

An artificial sachet feeding system (Figure 2.2) was developed for the oral delivery of dsRNA (RNAi construct). Four different fluorescent chemicals (Curcumin, Riboflavin, Uranine and Quinone) and green food coloring of different concentration were used in feeding assay. In this feeding system, the food supplement was a 15% (W: V) sucrose solution and the concentrations for all fluorescent compounds were arbitrarily chosen. Adult potato psyllids were grouped into one of eleven treatment groups, with ten adult psyllids in each treatment group. The treatment group were as follows: (1) psyllids that received 1: 10 (V: V) green food coloring, (2) psyllids that received 1: 5 (V: V) green food coloring, (3) psyllids that received 0.1 M curcumin, (4) psyllids that received 0.5 M curcumin, (5) psyllids that received 1: 10 (V: V) quinone, (6) psyllids that received 1: 5 (V: V) quinone, (7) psyllids that received 0.1 M riboflavin, (8) psyllids that received 0.5 M riboflavin, (9) psyllids that received only artificial feeding solution 15% (W: V) sucrose solution, (10) psyllids that received autoclaved tap water and (11) psyllids that received nothing. Except the treatment groups 10 and 11, all other treatment groups were mixed in 15% (W: V) sucrose solution. In each treatment group, 200 µl treatment solution was placed in the lid of 1.5 ml microfuge tube partitioned by thinly stretched parafilm and inverted through the

hole made in a 15 ml centrifuge tube (Figure 2.2). Ten adult psyllids were housed and offered sachet that contained artificial diets amended with fluorescent compounds (above described) and monitored for ten days. Each feeding assay was replicated three times. A fluorescence microscope (NIKON ECLIPSE Ti series) was used to visualize the psyllids after feeding with fluorescent compounds. The exposure time for all images was, auto exposure (AE) 6S (+1.0 EV). Blue, red and green filters were used for acquiring images. Psyllid mortality data were analyzed by using Graph PadPrism (version 5.0b).

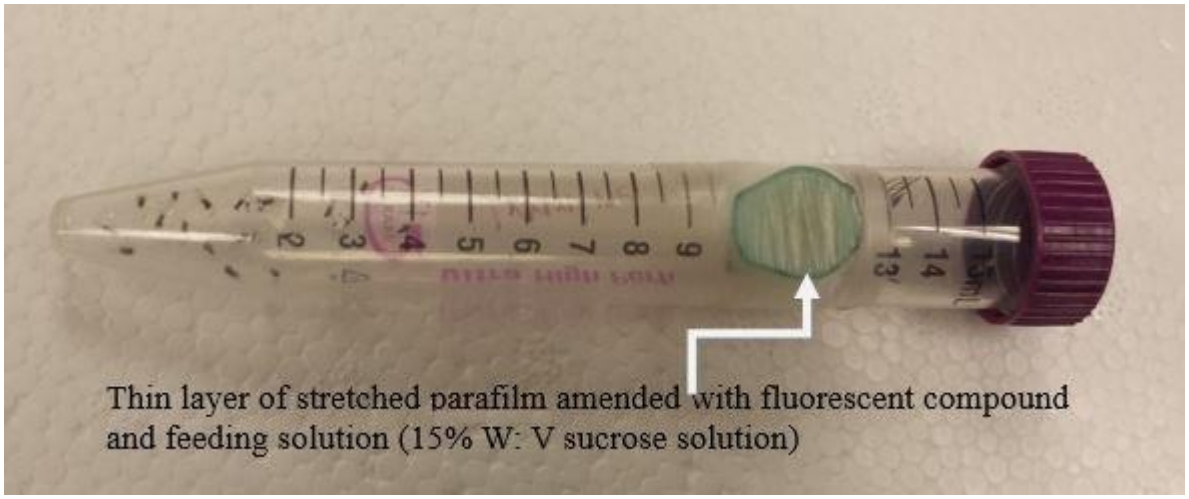

Figure 2.2. Sachet apparatus used for oral delivery of dsRNA. In each treatments, ten adult psyllids were housed and offered various concentration of fluorescent compounds mixed with feeding solutions (15% sucrose solution W: V) and monitored for ten days. Each treatment was replicated three times.

2.4. Sucrose feeding assay

Sucrose solutions of different concentrations; 3% (W: V), 5% (W: V), 7.5% (W: V), 10% (W: V), 15% (W: V) and 20% (W: V) were prepared and offered to potato psyllids for feeding using sachet feeding system. Each concentrations of sucrose were offered in different treatment and replicated three times. Ten adult psyllids were used in each treatment. Psyllid mortality was recorded for 10 days. Autoclaved tap water was used as a positive control and empty sachet was

used as a negative control. Mortality data was analyzed using Graph PadPrism (version 5.0b) and significance was assessed using repeated measures ANOVA.

2.5. DsRNA/RNAi construct delivery through root soaking method

Four weeks old seedlings of pepper plant (*Capsicum annuum*) were taken and roots were cleaned using sterile water. The roots were allowed to absorb the dsRNA construct, anti-alpha tubulin, to determine the ability of *C. annuum* to deliver the dsRNA construct through vascular tissues, xylem and phloem, to the various plant parts. A total volume of 300 µl of dsRNA construct (400µg) was put in the 1.5 ml microfuge tube. Pepper plant was allowed to absorb the dsRNA construct for 6 hours, and the nanopure water was refilled twice for 48 hours. The whole plant was then subjected to total RNA extraction using TRIzol (Life Technologies, Carlsbad, CA) reagent as per the manufacture's protocol. The total RNA was reverse transcribed to produce cDNA using M-MuLV Reverse Transcriptase (New England BioLabs) following manufacturer protocol. To identify dsRNA construct in plant system, qPCR was performed on the resulting cDNA using a specifically-designed qPCR primer set: sense primer (5'-GTGGATTCTGGGGTAGGGC) and anti-sense primer (5'- CTTGGACATCGAACGCCCC) to amplify a 151-bp product. Each 25 µl reaction mixture included 12.5 µl Amplitaq Gold 360 Master Mix (Life Technologies, Austin, TX), 1 µl GC enhancer (Life Technologies, Austin, TX), 1 µl of each forward and reverse primer, 1.25 µl of Evagreen 20 in water (Biotum, Hayward, CA), 2 µl of cDNA (~90 ng) and 5.5 µl of nuclease free water (ThermoFisher Scientific, Grand Island, NY). qPCR was performed with the following thermal profile: 50°C for 2min, 95°C for 2min, denatured at 95°C for 15sec, annealed at 60°C for 30sec, a hold at 72°C for 30sec, repeated over 40 cycles, followed by a melt curve obtained by ramping from 50°C to 90°C by adding 1°C each step for 90s, with five seconds between each step, followed by a hold at 4°C.

Cycle Threshold (CT) values obtained after qPCR were compared for the different treatment groups.

2.6. dsRNA/RNAi construct delivery using leaf and stem soaking method

Besides root soaking methods, two other plant based delivery systems were also tested for the delivery of dsRNA construct. A freshly cut petiolated leaf and stem were allowed to absorb 300µl of dsRNA construct, anti-alpha tubulin (120 µg), amended with 50µl red food coloring (McCormick and Co., Inc., Hunt Valley, MD) in 1.5 ml microfuge tube for 6 hours. The purpose of adding red food coloring in the dsRNA construct was to confirm the absorption by examining the color of the leaf as the leaf color turns red after absorption. The experiment was run in full light condition to increase the transpiration rate and absorption. The leaf and stem were then subjected to total RNA extraction using TRIzol (Life Technologies, Carlsbad, CA) reagent as per the manufacture's protocol. Similar protocol described in section 2.4.1 was followed to produce cDNA and to run qPCR. The obtained CT values were compared across the different treatment groups (Figure 2.3).

Figure 2.3: Leaf and stem were allowed to absorb 300 µl of dsRNA/RNAi construct (120 µg) amended with 50 µl of red food coloring for 6 hrs and nanopure water was refilled twice for 48 hrs. This experiment was set up in full light condition.

2.7. Delivery of treatment solutions through syringe amended with red food coloring

In this delivery system, 1000 µl of red food coloring (McCormick and Co., Inc., Hunt Valley, MD) was drawn into a 3 ml syringe and attached to the stem and petiolated leaf separately and sealed with the parafilm then a gentle pressure was applied to the syringe which replaced the xylem fluid with the red food coloring and forced out of the terminal end of the plant stem and leaf. When the terminal end was dabbed on the white paper towel, the red color could easily be observed (Figure 2.4).

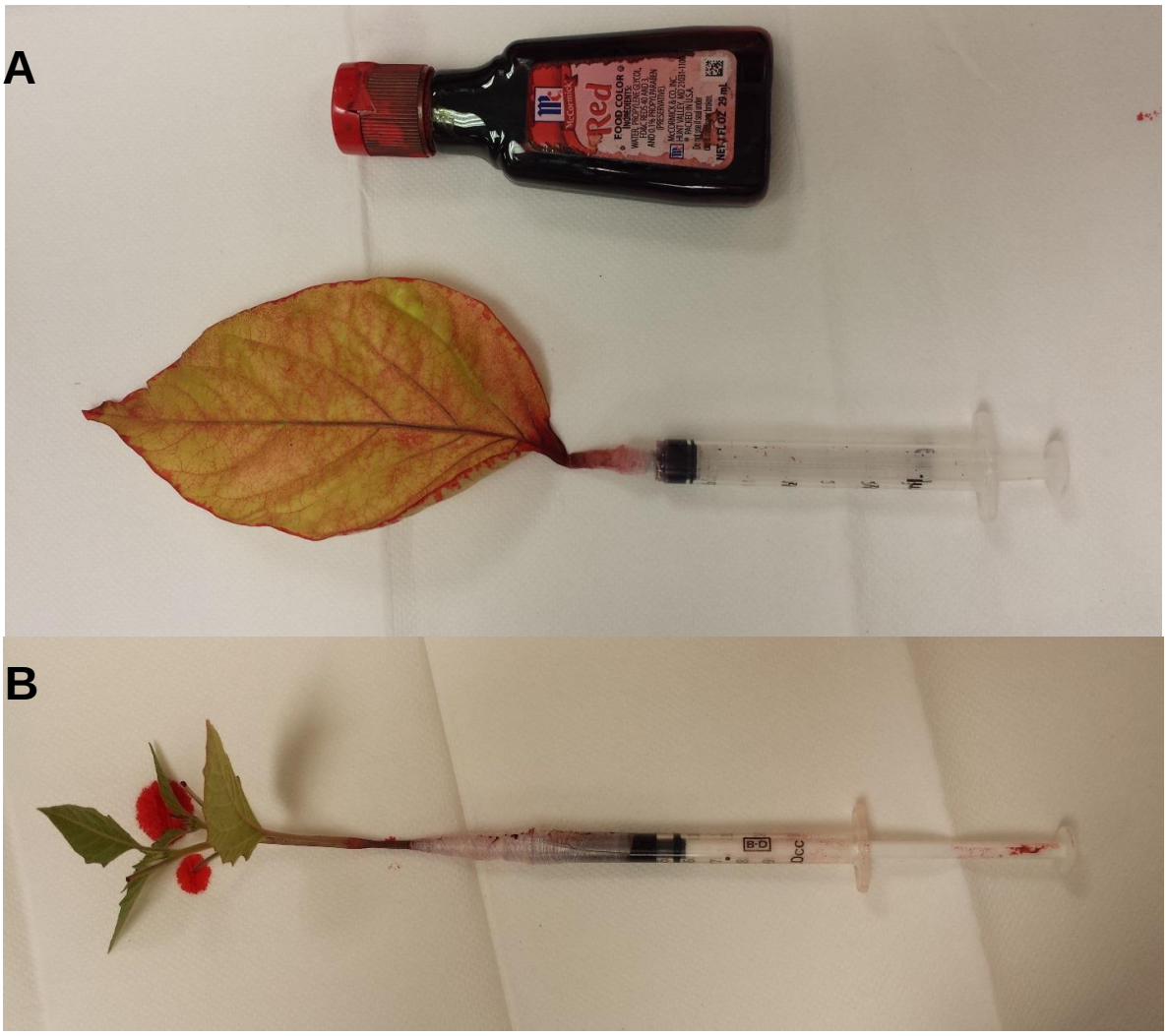

Figure 2.4. Syringe amended with red food coloring used in delivery system through leaf (A) and stem (B). 1000 µl of red food coloring was drawn in a syringe and attached to the leaf (A) and stem (B) of juvenile pepper plant and sealed with parafilm. Gentle pressure was applied on the syringe and observed the red color coming out of the leaf (A) and stem (B).

3. Results

3.1. Efficiency of sachet feeding method

The mortality of adult psyllids after feeding on highly concentrated green food coloring [1: 5 (V: V)] (96.67%±3.33% SEM) was higher than the low concentrated green food coloring [1: 10 (V: V)] (43.33%±24.04% SEM) (Figure 2.5A). Similarly, other fluorescent compounds of higher concentrations also caused higher mortality when compared with the low concentration

fluorescent compounds (Figure 2.5). Almost all the fluorescent compounds used in the feeding assay caused higher mortality of adult psyllid when compared with psyllids fed with artificial feeding diet (15% W: V sucrose solution).

After feeding with fluorescent compounds, imaging of the psyllids was done using fluorescence microscope (NIKON ECLIPSE Ti series). Psyllids fed on fluorescent compounds showed their gut fluorescing but psyllids fed on non-fluorescent compounds, water, sucrose and green food coloring did not show any fluorescence (Figure 2.6). On feeding with green food coloring, psyllids showed green coloration along the abdominal area.

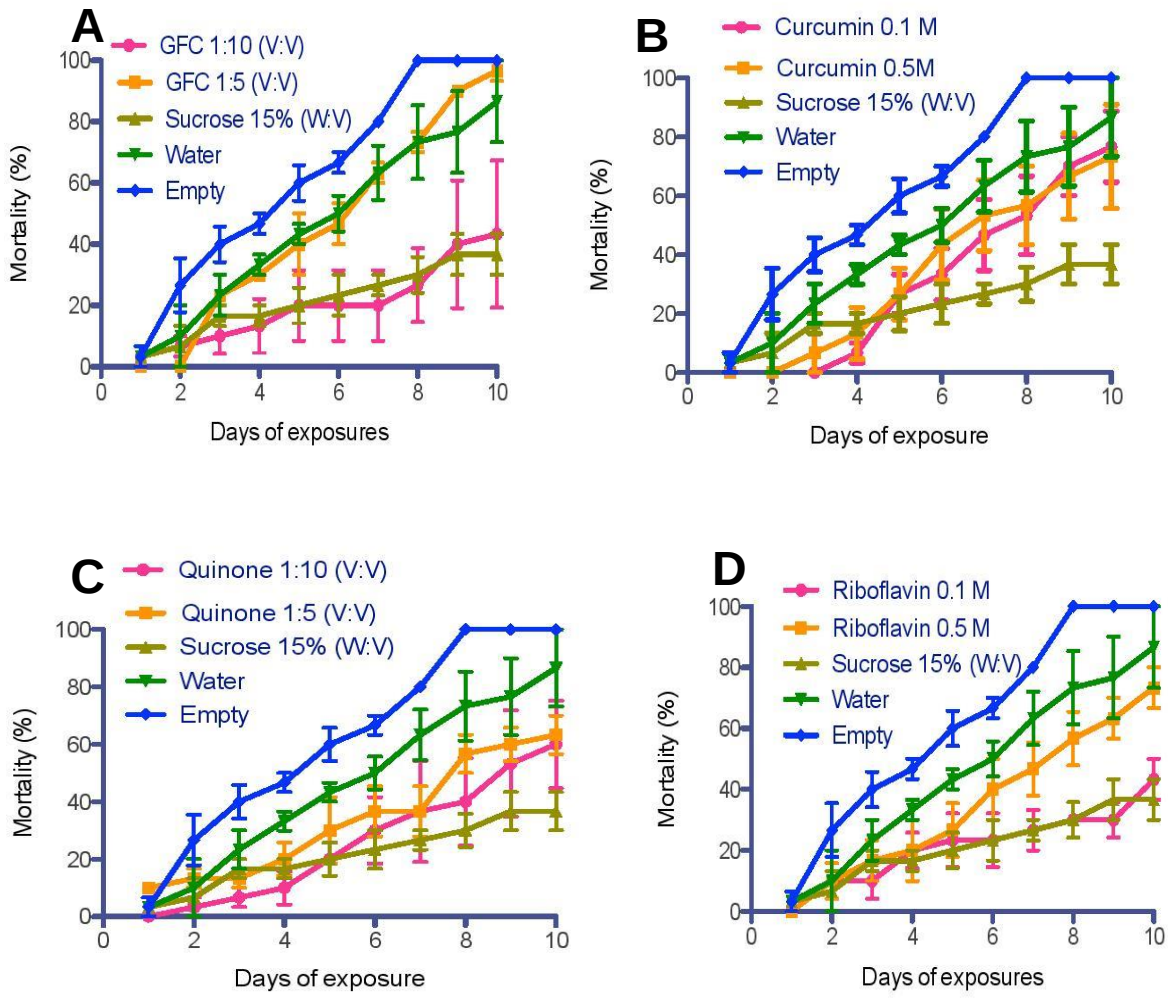

Figure 2.5: Psyllid mortality percentage and time response after feeding with different compounds. (A) Green food coloring (GFC). (B) Curcumin. (C) QUINONE and (D) Riboflavin. Ten adult potato psyllids were offered sachet amended with two different concentration of fluorescent compounds in artificial feeding diet (15% sucrose solution W: V) and monitored for ten days. Psyllids were offered with artificial feeding diet (15% sucrose solution W: V) as a positive control and sterile tap water and nothing as a no application control. Each treatment was replicated three times. The mortality data was analyzed using Graph PadPrism (version 5.0b). Standard error is represented for each day.

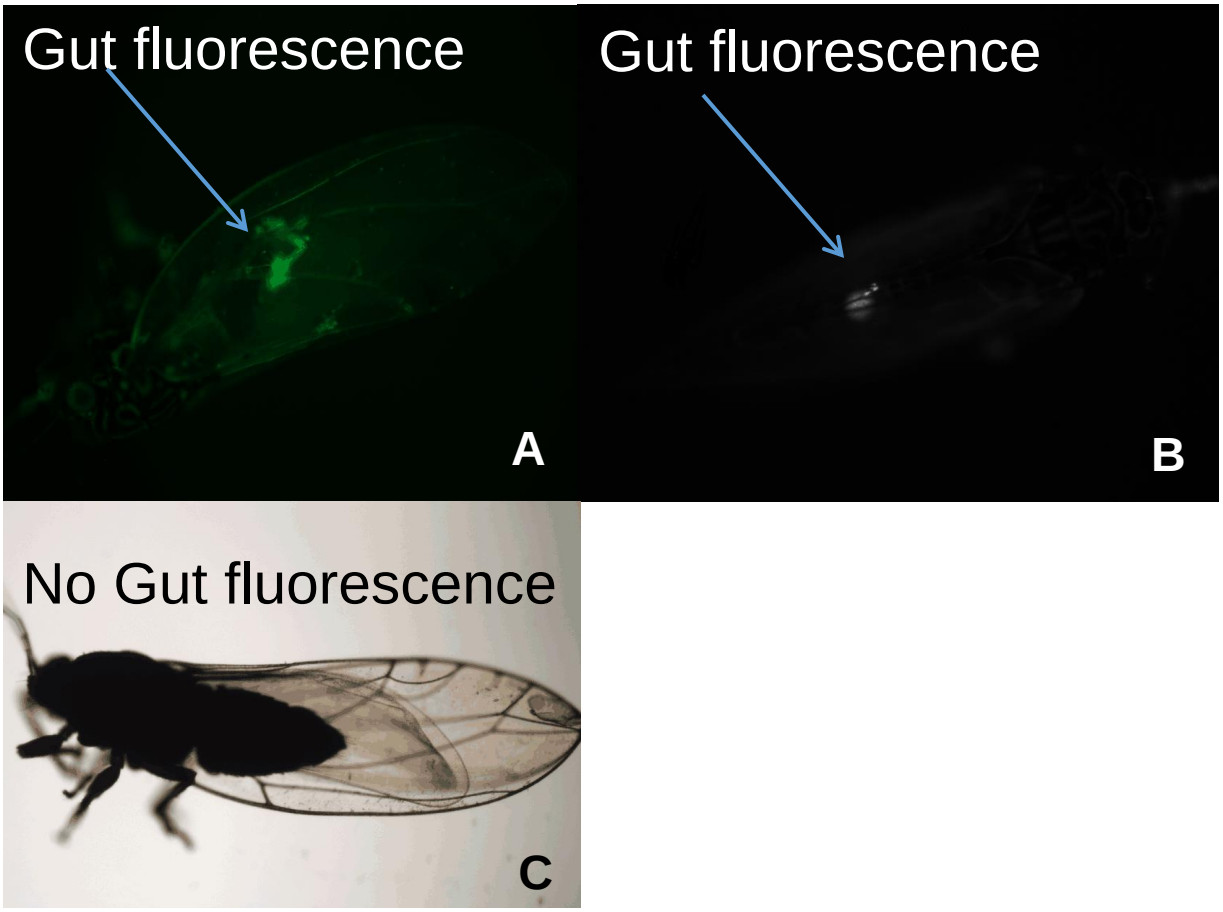

Figure 2.6. Psyllid feds with fluorescent compounds (A) curcumin and (B) quinone shows gut fluorescence whereas (C) psyllid fed with only sucrose shows no fluorescence at all. After feeding with fluorescent compounds, psyllids were imaged through fluorescence microscope (NIKON ECLIPSE Ti series). The exposure time for all images was, auto exposure (AE) 6S (+1.0 EV). Blue, red and green filters were used for acquiring images.

3.2. Sucrose feeding assay

Psyllids fed with low concentration sucrose solutions, 3% W: V sucrose (87%±8.82% SEM) and 5% W: V sucrose (93.33%±3.33% SEM) caused higher mortality when compared with psyllids fed with 7.5% W: V sucrose (70%±5.77% SEM) and 10% W: V sucrose (67%±18.26% SEM) (Figure 3.6A, 3.6B, 3.6C and 3.6D). Similarly, psyllids fed with high concentrated sucrose solutions, 15% W: V sucrose (90%±5.77% SEM) and 20% W: V sucrose (73.33%±17.63% SEM) mortality which is again higher than the psyllids fed with 7.5% W: V

sucrose (70%±5.77% SEM) and 10% W: V sucrose (67%±18.26% SEM) (Figure, 3.6C, 3.6D, 3.6E and 3.6F). Adult potato psyllids that received autoclaved tap water stayed alive for 6 days (100%) whereas psyllids that received nothing stayed alive for only 4 days (Figure 2.7).

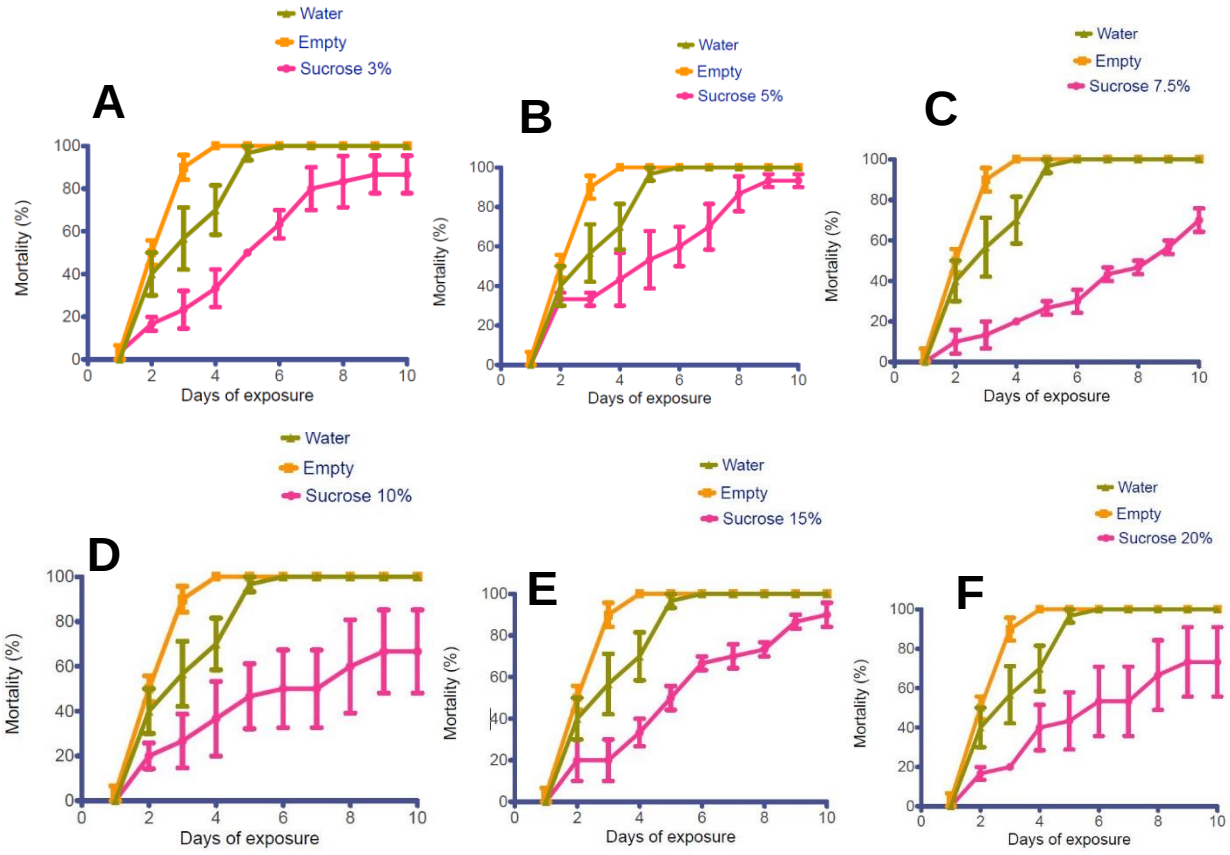

Figure 2.7. Potato psyllid mortality overtime by oral feeding of (A) 3% sucrose solution W: V, (B) 5% sucrose solution W: V, (C) 7.5% sucrose solution W: V, (D) 10% sucrose solution W: V, (E) 15% sucrose solution W: V, (F) sucrose solution 20% W: V. Ten adult potato psyllids were offered various concentration of sucrose solution through sachet feeding system and monitored for ten days. Each treatments were replicated for three times. Graph PadPrism version 5.0b was used for statistical analysis. Standard error is represented for each day.

3.3. Detection of DsRNA/RNAi construct in plant tissue in plant delivery system

The cDNAs obtained from the total extracted RNA from every plants used in delivery method were subjected individually for the qPCR using specific qPCR primer designed for the detection of alpha-tubulin dsRNA construct. The average CT values obtained from qPCR for the

root soaking treatment (8.39±0.13 SEM) and leaf soaking (7.57±0.31 SEM) treatment were significantly lower than the CT value obtained for no template control (34.68±1.60 SEM) (P<0.0001) (Figure 2.7). Whereas, no significant difference in the CT values for positive control (5.875±0.34 SEM) were noted when compared with the CT values for the root soaking treatment (8.39±0.13 SEM) and leaf soaking (7.57±0.31 SEM) treatment (P<0.0122) (Figure 2.8).

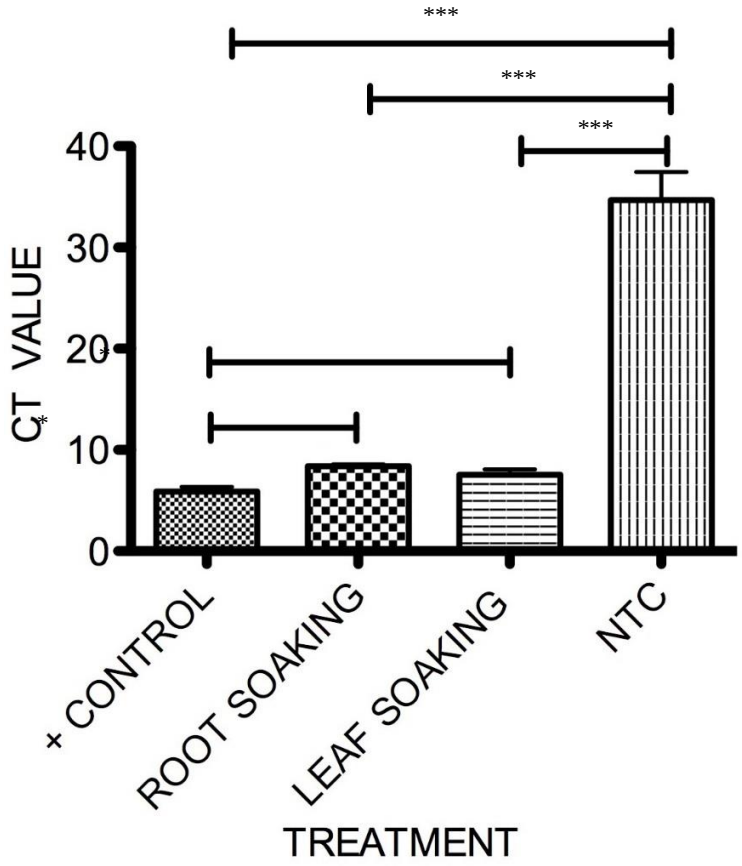

Figure 2.8. Average CT values for the quantification of dsRNA in plant based delivery system. Here the template cDNA, represented as positive control (5.875±0.13 SEM) was obtained from the reverse transcription of alpha-tubulin dsRNA using the M-MuLV Reverse Transcriptase (New England BioLabs) following manufacturer protocol. Root soaking treatment (8.390±0.13 SEM) represents the cDNA obtained from the reverse transcription of total RNA obtained from the plant sample used in root soaking delivery system. Likewise, leaf soaking treatment (7.567±0.31 SEM) represents the cDNA obtained from the reverse transcription of total RNA obtained from leaf samples used in leaf soaking delivery system. Finally, NTC (no template control) (34.68±1.60 SEM) represents sample without any cDNAs, instead, using nuclease free water in place of template. Standard error of the mean is represented in error bars and significance was determined using ANOVA. (* indicates P<0.05 and *** indicates P<0.001).

3.3. Syringe amended with red food coloring

In order to assess the validity of syringe amended delivery system, we chose the visualization methods to identify the movement of treatment solutions along the plant tissues. To deliver the treatment solution, red food coloring was drawn into the syringe and applied towards the plant leaf and stem separately. The syringe and plant junction was sealed using parafilm then a gentle pressure was applied on the syringe. The red colors forming in the edge of the leaf and stem acted as a visual confirmation that fluid from the vascular tissues was being replaced by the treatments (Figure 2.4A and 2.4B).

4. Discussion

In this study, the oral delivery of treatment solutions, amended with the fluorescent compounds through sachet feeding system has been successfully achieved. The fluorescence was observed around the gut/abdominal area of the psyllids after fed with fluorescent compounds compared with sucrose fed psyllids that displayed no florescence. After feeding psyllids with various concentrations of fluorescent compounds, mortality was shown to be significantly higher than sucrose fed psyllids. The purpose of this study was to determine the efficiency of sachet feeding method and to identify whether this is an effective way to orally deliver dsRNA constructs. Almost all fluorescent chemicals at higher concentration killed more psyllids, more quickly with respect to the lower concentration (Figure 2.5). Feeding with the treatment solutions causes ingestion of fluorescent chemicals, this might be one of the reasons contributing to the high mortality with respect to the artificial feeding diet (15% W: V sucrose solution). Using sachet feeding system, we offered sucrose solutions of various concentration to the adult psyllids to find the optimum concentration of sucrose solution. Our study suggest that, sucrose solution of 7.5% W: V is the optimum concentration for adult potato psyllid. These data showed that

psyllids are actively feeding on the provided treatments. Hence, in the next phase of the work, we used the sachet feeding method to deliver different dsRNA/RNAi construct designed to produce a mortality and downregulation of targeted mRNA sequences.

All the plant feeding systems studied here support the delivery of dsRNA/RNAi construct and treatment solutions. Whenever the root, leaf or stem are allowed to soak the treatment solutions, the existing liquid in vascular tissues is replaced by the treatment solutions amended with dsRNA/RNAi construct. Delivery of dsRNA/RNAi construct to the target insect is one of the crucial steps of applying RNA interference as a tool to manage pest insect species. There are various ways to deliver the dsRNA to the particular insect. Microinjection is one of the possible way to deliver the exact amount of dsRNA to the particular tissue (Yu et. al, 2013). However, microinjection is not a good way of delivering dsRNA in a potential pest management in a field experiment (Gu and Knipple, 2012). In addition, microinjection has various limitation including non–specific mechanical damage of tissues whereas oral delivery is a less-invasive and high throughput method for delivering dsRNA into the insects (Scott et al., 2013). Oral feeding of treatment liquids amended with dsRNA construct is one of the practical methods to deliver dsRNA constructs towards the target insect (Yu et al., 2013).

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Chapter Three

RNA Interference: Potato/Tomato Psyllid, *Bactericera cockerelli*

1. Introduction

The potato/tomato psyllid, *Bactericera cockerelli*, is phytophagous and exclusively feed on phloem (Knowlton and Thomas, 1934; Wallis, 1951). *B. cockerelli* is an economically important pest of crop plants, preferring solanaceous crops for survival and development (Munyaneza et al., 2007; Wallis, 1975). Potato psyllids are serious pests of solanaceous crops like, potato and tomato and are associated with psyllid yellows and Zebra Chip disease of potatoes (Hansen et al., 2008; Munyaneza et al., 2007). *B. cockerelli* can cause direct damage to the plants by feeding, nymphs have been reported to inject a toxin, causing psyllid yellows disease in potato which effects tuber yield and quality (Munyaneza et al., 2007; Ramírez-Davila et al., 2012; Liefting et al., 2009). Direct feeding by this pest causes the tissue damage and a deficit of certain nutrients in plants. The indirect damage due to *B. cockerelli* are associated with the transmission of the bacterium, *Candidatus* Liberibacter solanacearum (Lso), the causal agent of Zebra Chip disease in potato (Ramírez-Davila et al., 2012; Buchman et al., 2011; Liefting et al., 2009).

Zebra Chip is a disease which first arose in potato fields in Saltillo, Mexico in 1994 (Munyaneza et al., 2007). Now Zebra Chip disease has been reported in as far north as Washington Oregon and Idaho, other states such as, California, Nevada, Wyoming, Colorado, Nebraska, Kansas, Texas, New Mexico, Arizona and also has been reported into Central America as far as Nicaragua, and recently in New Zealand (Secor and Rivera-Varas, 2004; Munyaneza et al., 2007; Munyaneza et al., 2012; Secor et al., 2009; Liefting et al., 2009; Bextine et al., 2013). This disease causes serious economic issues for the potato industries, because it reduces the commercialization of the fresh potatoes for chips as the infected potato tubers show

very dark blotches upon frying (Lin et al., 2009; Munyaneza et al., 2007). This disease is responsible for millions of dollar in loses for the potato industries in various locations in Unites States (Secor and Rivera-Varas, 2004). The management strategies for Zebra Chip disease and psyllid yellows disease are targeted against the potato psyllid (Butler and Trumble, 2012).

Agricultural pests, such as *B. cockerelli* have been attempted to manage through application of chemical insecticides (Goolsby et al., 2007; Berry et al., 2009; Gharalari et al., 2009; Butler and Trumble, 2012). However, overtime, the use and/or overuse of chemical pesticides has promoted the development of resistance against the pesticides through adaptation (Li et al., 2013; Rondon et al., 2012). Other risks associated with pesticides includes, killing beneficial or non-target insects. Chemical application is not a healthy way to manage agricultural pests and has various limitations. There are no practical non-chemical control strategies for potato psyllids have been developed (Rondon et al., 2012). RNA interference (RNAi) technology is a potential strategy of managing insect pests and plant pathogens, and is therefore gaining the attention of the scientific community (Price and Gatehouse, 2008).

RNAi is a gene silencing process which is induced by the introduction of exogenous double-stranded RNA which inhibits the translation of complimentary endogenous messenger RNA (Zamore, 2001; xu et al., 1998; Meister and Tuschl, 2004). At the cellular level, the long dsRNA is processed by an RNAse III enzyme called Dicer, which cleaved long dsRNA into short (21 – 25 nucleotide), small interfering RNAs (siRNAs) (Elbashir et al., 2001). The siRNA combines with other cellular protein to form an RNA induced silencing complex (RISC). The RISC unwinds the duplex of siRNA into two single strands; a guide strand and a passenger strand. The guide strand is complementary to the mRNA and binds to it and halting further translation, whereas the passenger strand is degraded (Stevenson, 2003; Hammond et al., 2001).

RNAi is the potential tool for the management of insect pests, and can be triggered by the introduction of double stranded RNA via microinjection or from feeding on an artificial diet (Whyard et al., 2009; Ghanima et al., 2007; Turner et al., 2006). Microinjection is not a practical way to deliver dsRNA as pest management in a field experiment (Gu and Knipple, 2012). The efficiency of RNAi depends on, suitable delivery of dsRNA and the genes being targeted (Scott et al., 2013). By selecting and suppressing promising genes through RNAi a reduction in fitness and increase in mortality could be achieve (Scott et al., 2013). In this study, the primary target is the gene encoding for an enzyme, arginine kinase. Arginine kinase is an enzyme which catalyzes a reversible transfer of phosphate group from MgATP to arginine to produce phosphoarginine (Newsholme et al., 1978). Arginine kinase plays unique role in balancing the cellular energy homeostasis in invertebrates, so, it becomes one of the prime target in the study of pest management (Newsholme et al., 1978; Brown and Grossman, 2004; Wu et al., 2007).

The most common delivery systems for RNAi in insects include, delivery of dsRNA to the hemolymph through microinjection and oral feeding. Microinjection has various limitation including non–specific mechanical damage of tissues whereas oral delivery is a less-invasive and high throughput method for delivering dsRNA to insects (Scott et al., 2013).

To show the proof of concept, arginine kinase gene was selected as a primary target and various other genes were identified, *in vitro* dsRNA constructs targeting those genes were synthesized and orally delivered through a sachet feeding system and a plant delivery system. The mortality was recorded overtime and downregulation of target mRNA was assessed using qPCR.

2. Materials and Methods

2.1. Insect colonies

The potato psyllids were initially obtained from Dr. Don Henne (Texas A &M University). The colony was maintained on pepper plants (*Capsicum annum*) in a bug dorm (36in. x 24in x 18in.) within insectarium at 26^0C, 70% humidity and a 14:10 (day: night) cycle. Mixed male/female adult potato psyllids were used in this study.

2.2. Double stranded RNA/RNAi construct selection and primer designing

To identify the two targets for the gene arginine kinase (Table 3.1), the *B. cockerelli* transcriptome database (Fisher et al., 2014) was examined and primers for double stranded RNA constructs against those two genes were designed using NCBI primer blast tool. The primer designing settings in NCBI primer blast tool included a PCR product size of 200 to 500 base pairs length, primer melting temperature in a range of 50 to 60 °C, and specificity was checked against the order hemiptera.

Table 3.1: List of two targets for gene arginine kinase, their respective primer sequence and expected amplicon size (in base pairs).

Genes	Forward primer (5'-3')	Reverse primer (5'-3')	size
Arginine Kinase –I	GGGAGCTCAAGGGTCAGTTC	GCTTGGGCAGTTTGATGTGG	387
Arginine Kinase -II	GCAATGCCGTCGTTCATCTC	GGGCCTCAGTCCACATCAAA	202

One dsRNA construct targeted for *Diaphorina citri* arginine kinase was obtained from Dr. Wayne Hunter (USDA – ARS, Florida).

Using the nucleotide database of *B. cockerelli* available in NCBI and the expressed sequence tag (EST) sequence information from another hemipteran, *D. citri* available in NCBI were utilized to find the potential target for dsRNA/RNAi constructs (Table 3.2).

Table 3.2. Target genes for the construction of dsRNA/RNAi construct, their respective primer sequence and NCBI accession numbers.

Gene Name	NCBI accession No.	Forward primer (5' to 3')	Reverse primer (5' to 3')
Gyrase	XM_008483112	TGGTGATACTTCTGATGGAACCG	CGGTTTTTCTGCGGCAGTTT
Topoisomerase 2	XM_008489264	GGCGAGATCGACTACTGTGG	TCTTCACCTTCAGCTGCCTG
Wing disc protein	KJ363929	GGCTTCAAGTTGGTGGGACT	CCTCTTACGGTTCCTGGAGC
Cytochrome c oxidase subunit I	EF372597	CGTGCCTATTTCACTTCCGC	ACACCTGTTAAACCTCCCAGTG
18S rRNA	U20416	CGATGGTAGGTTATGCGCCT	ACCAGACTTGCCCTCCAATG
Ecdysone like receptor	XM_008485557	GAGCTGGAGGCTGAACGTAA	CTTCTCCACTTTCCACGCCT
Zinc finger protein on ecdysone puffs	XM_008477852	ACATTTCCTGCACCCCAAGT	ACTTTCACCGGAGCTACGTC
Ecdysone 20-monooxygenase	XM_008489199	GTTGACTAGCGGCAGGATCA	AGCTTCAAATAGGCAGGCGT
Ecdysone induced protein 78C	XM_008483528	TGACACCAAACAGCTGCTCA	CTCGTTTGGCGAATTCCACT
Hexokinase type 2-like	XM_008474120	GGACGTCCAAATCGAGGTGT	GCAGCGTCCACCTCTCTATC
Glucose-6-P 1-dehydrogenase	XM_008480359	CTACATTTGCTTCGGCCGTG	CGCCATGCCTCTGATAGCTC
Pyruvate dehydrogenase	XM_008488213	CATAACCGCTACAGGACGCA	TCTGTGAGTCGCTTGGCAAT
Glycerol-3-P dehydrogenase	XM_008479272	TCTGCGACGTTCTTCGAGTC	TGTTGGGGTTTCATCTCACCA
HIF 1b	AF154417	ATGGCACTTACAAGCCCTCC	TGCGACAGATGAAACCACG
Glyceraldehyde-3-P dehydrogenase 2	XM_008481620	ACGACTCCACTCATGGCAAG	GTCGTAGGCGTCCAAGTTGA
Cytochrome oxidase subunit I	KC008074	CAAGGTGTAGGGACAGGTTGA	AATAGGGTCTCCTCCTCCGG
Hormone receptor 4-like	XM_008483528	AACCACCGCAGCAACATTTC	AAGGTGAGGACGCTGTTGAG
Actin-X2	XM_008470468	TTGTCCCGAAGCTCTGTTCC	GCCGGACTCGTCGTATTCTT

NCBI primer blast tool was used to design the primer for all these construct. PCR product size of 200-500 base pair length, melting temperature of range 50 °C – 60 °C were selected to narrow down to primer designing. Hemiptera was selected as an organism for primer pair specificity checking parameters.

Complimentary DNA (cDNA) library data of *B. cockerelli* (Hail, unpublished) and expressed sequence tag (EST) sequence of *D. citri* were utilized to identify certain potential target dsRNA constructs (Table 3.3). Geneious version 7.1.7 (Biomatters Limited 2014) was used to design primer pairs to target these genes.

Table 3.3. Target genes for dsRNA/RNAi construct, their amplicon sizes, and forward and reverse primer sets. (NA =Never Amplified)

Gene Name	Forward primer (5'-3')	Reverse primer (5'-3')	size (bp)
Yellow B	GGGGAACACCAGCGTCTCGC	CCTGGTGGACACCACCCCCA	550
Enolase	TCGACTCCCGTGGCAACCCA	CGCTCAGACCGACATGGGGC	1000
Alpha tubulin F1/R1	AGTGCACCGGACTGCAAGGC	GGCTCGAAGCAGGCGTTGGT	450
Alpha tubulin F1/R2	AGTGCACCGGACTGCAAGGC	CGTGTCGGGGGTCGCACTTC	500
Alpha tubulin F1/R3	AGTGCACCGGACTGCAAGGC	CTTGGCGAGGTCACCGCCTG	650
Alpha tubulin F2/R1	GCCATCTACCCCGCCCCTCA	GGCTCGAAGCAGGCGTTGGT	350
Alpha tubulin F2/R2	GCCATCTACCCCGCCCCTCA	CGTGTCGGGGGTCGCACTTC	400
Alpha tubulin F2/R3	GCCATCTACCCCGCCCCTCA	CTTGGCGAGGTCACCGCCTG	500
Alpha tubulin F3/R1	GCCATCTACCCCGCCCCTCA	GGCTCGAAGCAGGCGTTGGT	350
Alpha tubulin F3/R2	GCCATCTACCCCGCCCCTCA	CGTGTCGGGGGTCGCACTTC	375
Alpha tubulin F3/R3	GCCATCTACCCCGCCCCTCA	CTTGGCGAGGTCACCGCCTG	500
Integrin	ATCAGATCGTCCGCCCCCGT	GGGGCCCCCGGGAAGTTTCT	325
Gelsolin	AATGGTGGCGCGTCCGTTGT	GTCAACGCCGGCAGAGTCCC	650
Beta tubulin I	AAGGGGAGCAAAGCCAGGC	AAGGGGAGCAAAGCCAGGCA	NA
Beta tubulin II	GGGTGGGATGTCACACACGG	AAGGGGAGCAAAGCCAGGCA	NA
Beta tubulin III	TTCCCGGGGCAACTCAACGC	CCCCGCCTCCTGGTACTGCT	500
Inhibitor of apoptosis	AACGTCGAGCGCTGGGCAAA	TGGCCGGTCGTTGCGATTCC	NA
Actin I	GGGCGGCGCGATGATCTTGA	CGACGAGGCCCAGAGCAAGC	950
Actin II	GGCGATGCCGGGGTACATGG	CGACGAGGCCCAGAGCAAGC	800
Disulphide isomerase	GAAGACCGGCCCTCCCGCTA	GCATGCTCGGCACCTCCTCC	290
Proteasome β4 I	TCCACAGTGGGTCAAACTGG	ACAGGAACCTCCGTGTTGGGG	300
Proteasome β4 II	TCCACAGTGGGTCAAACTGG	CAGAATGGCCCAACTCTTGGC	420
Ribosomal protein S7e	TCACGGACCAGACGAGGTTG	CAAGAAGGGTGGGGAGCCCG	220
RP L18A	TGGACGCAAACTGCCCACTG	CGCTTGGGCAGAGGGAATCG	430
Ribosomal protein L7A	CCGCAGACTTGGAGCCGAGC	CCAGCCCTGTGCCGCAAGAT	220
Ribosomal protein S8	GCCATCCCCATCGGACGCAG	GGCGCGGTTGGAATGGCTCA	300
Ribosomal protein L7	AAAGCAGCAGCCGCTGGCAA	TGCGCCATCCACCAGTTGGG	650
Ribosomal protein L3	AACGGTGGCTCTGTGGCCGA	CCAGCTTGTCAGCCGGGGTC	600
Ribosomal protein SA	TCTGCGCTGACAGGGGTGGT	TGCTGGGTGCCCAAACCCAC	860
Ribosomal protein L34	TGTGGCACAGGACACCTCCA	ACCGAAGACTGACAAGCAGT	280
RP L27Ae	GCTGAGAGGTCACGTGAGTC	GGTTGCTTTGGGATGCGCCC	330
ATP carrier protein	GCCCATGGCCCCCGCATTAG	TCCCGACCAGCACCAGCCTT	500

2.3. DNA extraction and Double stranded RNA/RNAi construct synthesis

Total *B. cockerelli* genomic DNA was extracted using the cetyltriethylammonium bromide (CTAB) buffer protocol (Zhang et al., 1998). The concentration of extracted DNA was measured using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA).

For all designed primer sets, the temperature gradient PCR was conducted to determine the optimum annealing temperature of the primers in the temperature range 50-60°C using the Amplitaq Gold 360 PCR kit as per the manufacturer protocol (Applied Biosystems, Foster City, CA). The thermal profile was run as follows: Cycle 1= 95°C for 10min (1x), Cycle 2= 95°C for 30s, 50°C to 60°C for 30s, 72°C for 60s (35x), Cycle 3= 72°C for 7 minutes (1x), and Cycle 4= 4°C for 10 minutes (1x). Amplicons were visualized on a 2% agarose gel stained with ethidium bromide. Only the prominent and single band size were excised and recovered the DNA through QIAquick Gel Extraction kit following manufacturer protocol (QIAGEN, Valencia, CA). The second PCR was done using the same primer amended with T7 promoter sequence (TAATACGACTCACTATAGGG) at 5' end using the excised amplicon as a template DNA. The amplicons visualization and purification was done same as the previous described method. The purified product was then used for dsRNA preparation using Hiscribe T7 in vitro transcription kit (New England Biolabs, Ipswich, MA) according to the manufacturer protocol.

2.4. Oral delivery of dsRNA construct using sachet feeding system

To deliver dsRNA/RNAi construct, fifteen adult psyllids were offered a sachet (Figure 3.1) that contained artificial diet (15% W: V sucrose solution) amended with dsRNAs construct [75 µg] and monitored for five days. The mortality data were analyzed using Graph PadPrism (Version 5.0b). The dsRNA constructs selected to feed in this trial are listed in table 3.4.

Table 3.4. Lists of dsRNA/RNAi construct used in bioassay

Gene Name	Forward primer (5'-3')	Reverse primer (5'-3')	size (bp)
Yellow B	GGGGAACACCAGCGTCTCGC	CCTGGTGGACACCACCCCCA	550
Alpha tubulin F2/R1	GCCATCTACCCCGCCCCTCA	GGCTCGAAGCAGGCGTTGGT	350
Alpha tubulin F3/R1	GCCATCTACCCCGCCCCTCA	GGCTCGAAGCAGGCGTTGGT	350
Alpha tubulin F3/R2	GCCATCTACCCCGCCCCTCA	CGTGTCGGGGGTCGCACTTC	375
Integrin	ATCAGATCGTCCGCCCCCGT	GGGGCCCCCGGGAAGTTTCT	325
RP L7A	CCGCAGACTTGGAGCCGAGC	CCAGCCCTGTGCCGCAAGAT	220
Ribosomal protein S8	GCCATCCCCATCGGACGCAG	GGCGCGGTTGGAATGGCTCA	300
Proteasome β4 I	TCCACAGTGGGTCAAACTGG	ACAGGAACCTCCGTGTTGGGGA	300
Proteasome β4 II	TCCACAGTGGGTCAAACTGG	CAGAATGGCCCAACTCTTGGCA	420

Nine dsRNA constructs (Table 3.4) were selected and offered individually. For negative application control group, dsRNA construct [75 µg] targeting the viral capsid of *Solenopsis invicta* virus was used, for no treatment control only artificial diets (15% W: V sucrose solution) was used. Each treatment group was replicated three times. The psyllid mortality significance was assessed using repeated measures ANOVA.

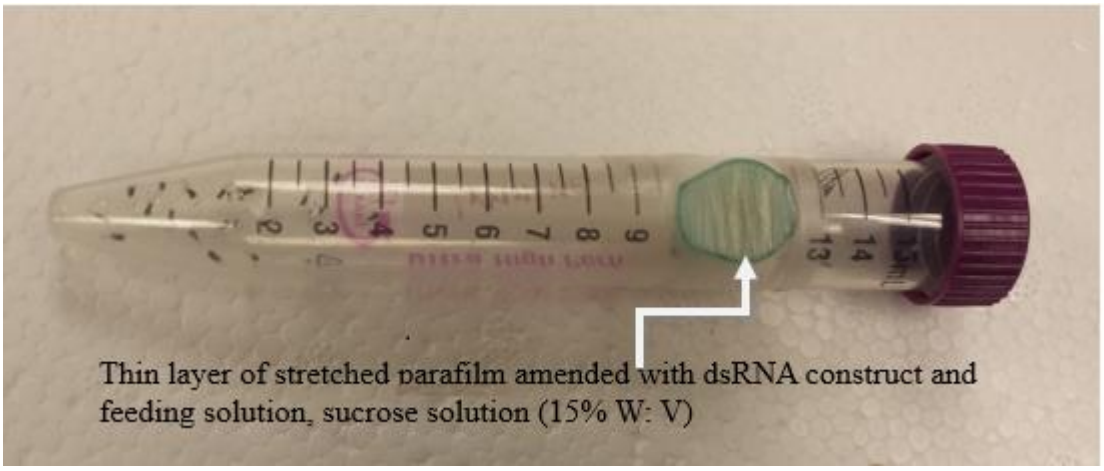

Figure 3.1: Sachet feeding system used to deliver dsRNA constructs. Adult psyllids were placed inside the 15 ml centrifuge tube. Double stranded RNA constructs [75 µg] described in table 4.4 were mixed individually with feeding solution, 150 µL of 15% sucrose solution (W: V), and partitioned by thin stretched parafilm and offered to adult psyllids and monitored for five days. The whole bioassay were maintained in room temperature in the dark.

2.5. dsRNA delivery using plant feeding system

The dsRNA construct for anti-arginine kinase, for *D. citri*, obtained from Dr. Wayne Hunter (USDA, ARS, Fort Peirce, Florida) was diluted to different concentrations (as 25 µg, 50 µg, 75 µg, 100 µg and 200 µg) in 300 uL of nanopure water, placed in 0.5 mL microfuge tube and freshly cut petiolated leaf was allowed to absorb the solution for 6 hours then nanopure water was added when the water level was low. The whole leaf was placed inside the pertri dish and 30 adult psyllids were housed and offered (Figure 3.2). Mortality was recorded in every 24 hours for 6 days. Each concentration was considered as a single treatments. For the no treatment control only nanopure water was applied to the leaf, for negative application control, dsRNA targeting the viral capsid protein of *Solenopsis invicta* virus (SINV) was used. Each treatment group was replicated for three times, mortality data was analyzed using Graph PadPrism (version 5.0b) and significance was assessed by using repeated measures ANOVA.

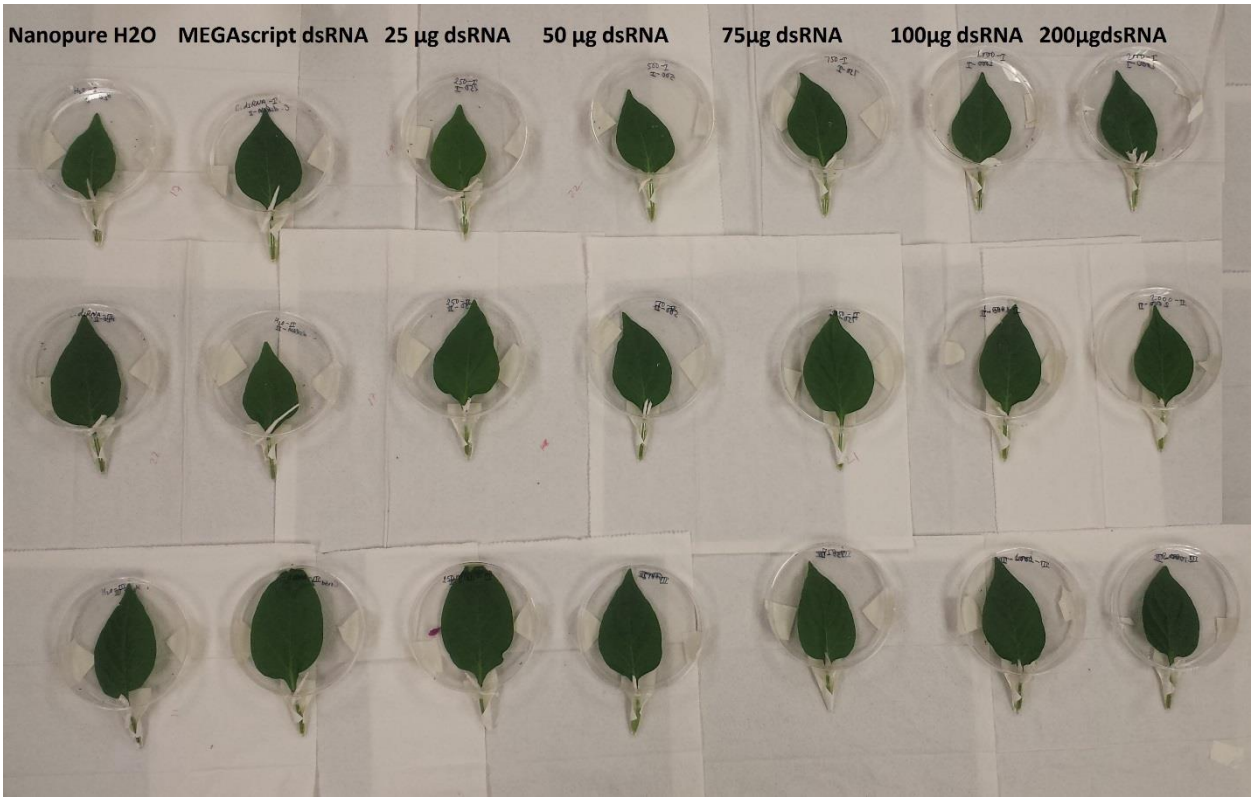

Figure 3.2. Experimental set up for the feeding assay for the oral acquisition of various concentration of dsRNA constructs synthesized against *D. citri* arginine kinase gene. Thirty adult psyllids were housed in petri plate and offered various concentrations of dsRNA constructs and monitored for six days. Nanopure water was used as no treatment control and dsRNA targeting for viral capsid protein for SINV was used as a no application control.

Two dsRNA anti-arginine kinase constructs (Table 3.1) were mixed in a single treatment and systemically delivered to the *B. cockerelli* through plant feeding system. Groups of fifteen psyllids were offered 400 µg of dsRNA constructs (200 µg of Arginine kinase I dsRNA + 200 µg of Arginine kinase – II dsRNA) and monitored for five days. For negative application control, MEGAscript positive control dsRNA was used and for no treatment control, nanopure water was used. Five psyllids were removed from each treatment after 24 hours to assess downregulation of targeted genes. Each treatment was replicated for three times, mortality data was analyzed using Graph PadPrism (version 5.0b) and significance was assessed by using repeated measures ANOVA.

2.6. Psyllid RNA isolation, cDNA synthesis and quantitative real time PCR (qRT-PCR)

Among all of the described treatments downregulation was assessed for the target genes; alpha tubulin F2/R1, alpha tubulin F3/R1 and the arginine kinase genes described in Table 3.1. Psyllids fed with alpha tubulin F2/R1 and alpha tubulin F3/R1 were subjected to total RNA extraction using TRIzol (Life Technologies, Carlsbad, CA) reagent as per the manufacturer protocol. The total RNA was reverse transcribed to produce complimentary DNA (cDNA) using M-MuLV Reverse Transcriptase (New England BioLabs) following manufacturer Protocol. To assess downregulation of target genes, quantitative real time PCR (qPCR) was performed on the total cDNA. Each 25 µl reaction mixture included 12.5 µl Amplitaq Gold 360 Master Mix (Life Technologies, Austin, TX), 1 µl GC enhancer (Life Technologies, Austin, TX), 1 µl of each forward and reverse primer, 1.25 µl of Evagreen 20 in water (Biotum, Hayward, CA), 2 µl of cDNA (~90 ng) and 5.5 µl of nuclease free water (ThermoFisher Scientific, Grand Island, NY). The thermal profile was run as follows: 94°C for 30s, 60°C for 30s, 72°C for 6s, 60°C for 5 s, repeated 40 times, followed by a melt curve obtained by ramping from 50°C to 90°C by adding 1°C each step for 90s, with five seconds between each step, followed by a hold at 4°C. qPCR was performed using a specially-designed qPCR primer sets (Table 3.5). For the no template control (NTEC), nuclease free water was used instead of cDNA as a template DNA. A similar protocol was followed for quantification of downregulation of two different arginine kinase genes. 18S ribosomal RNA was used as an endogenous control. Quantitative PCR normalization was performed by dividing CT values obtained from each treatment with the respective CT values obtained from the 18S rRNA amplification done in the same run.

Table 3.5. A list of Primer sets used for the qPCR

Gene Name	Forward primer (5'-3')	Reverse primer (5'-3')	Size
Alpha tubulin F2/R1	GTGGATTCTGGGGTAGGGC	CTTGGACATCGAACGCCCC	151
Alpha tubulin F3/R1	CCACCCTGGAACACTCTGAC	CGTTCGATGTCCAAGTTGCG	85
Arginine kinase – I	TGGTGCAACGAAGAAGACCA	ATCGTGCGAGAAGGGGATTC	126
Arginine Kinase - II	GGGCTCTTGGTTGTGTCAGA	AGAGTTCGATGCCGTCAAGG	100
18S rRNA	AAGCAGGCAAGCAAAGTGTG	CAAATTAAGCCGCAGGCTCC	

3. Results

3.1. dsRNA/RNAi construct synthesis

None of the primer sets mentioned in Table 3.2 provided a single prominent band, they gave either multiple bands or no amplification, so they were excluded from the further investigation.

The amplicons, mentioned in Table 3.3, displayed a band size above 400 base pairs failed to produce dsRNA construct except Yellow B (550 bp), and excluded from further investigation.

3.2. Oral delivery of dsRNA construct using sachet feeding system and mortality recording

Of nine treatments all three targeted at alpha tubulin (alpha tubulin F2/R1, alpha tubulin F3R1 and alpha tubulin F3/R2) caused 91.11% ± 5.88% SEM, 82.22% ± 2.22% SEM and 71.11% + 2.22% SEM of mortality respectively at day 5, which is greater than the mortality caused due to no treatment control (15% W: V sucrose solution) 62.22% ± 13.51% SEM and control dsRNA treatment 37.78% ± 8.01% SEM. (Figure 3.3A, 3.3B, and 3.3C). A P-value of $P < 0.0001$ was obtained from the repeated measures ANOVA suggested the significance of the mortality was due to the three alpha tubulin treatments. The ribosomal protein S8 construct caused 28.89% ± 9.69% SEM mortality which is less than both control dsRNA treatment 37.78% ± 8.01% SEM and no treatment control 62.22% ± 13.51% SEM (Figure 3.3G and 3.3H). This treatment did not cause significant mortality with respect to the control treatments ($P > 0.05$).

Four treatments, yellow B, integrin, proteasome B4 F1/R1 and proteasome B4 F1/R2 caused 46.67% ± 7.70% SEM, 37.78% ± 8.01% SEM, 46.67% ± 3.85% SEM and 51.11% ± 5.88% SEM of mortality respectively. These treatments induced a response greater than control dsRNA treatment 37.78% ± 8.01%SEM but less than the no treatment control 62.22% ± 13.11% SEM (Figure 3.3F, 3.3E, 3.3H and 3.3I). These four treatments also did not cause the significant mortality of psyllids (P>0.05) when compared with control treatments.

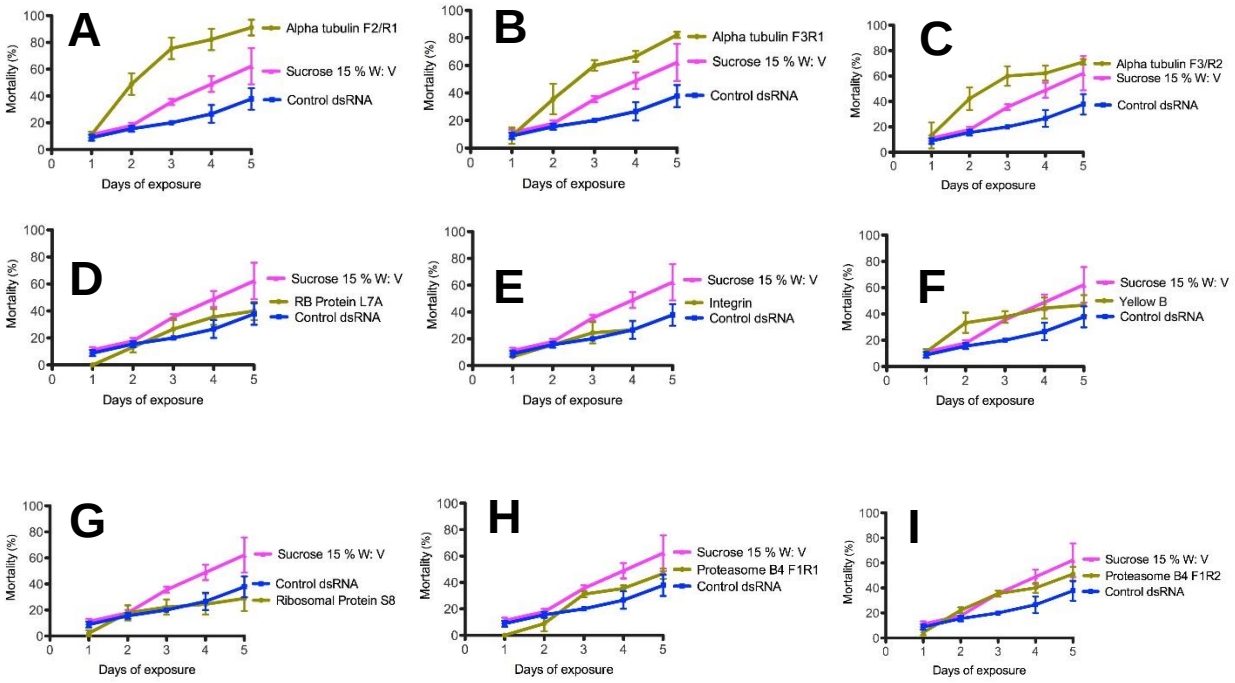

Figure 3.3. Mortality recording of psyllids overtime by oral feeding of dsRNA constructs (A) alpha tubulin F2/R1, (B) alpha tubulin F3/R1, (C) alpha tubulin F3/R2, (D) ribosomal protein L7A, (E) Integrin, (F) Yellow B, (G) ribosomal protein S8, (H) proteasome B4 F1/R1 and (I) proteasome B4 F1/R2. Groups of fifteen adult psyllids were used for each treatment. All the experiments were repeated three times. 15% W: V sucrose solution was used as a no treatment control and dsRNA targeted for viral capsid protein was used as a dsRNA control. Graph PadPrism version 5.0b was used for statistical data analysis. Standard error is represented for each day and the psyllid mortality significance was assessed using repeated measures ANOVA.

3.3. Oral delivery of dsRNA construct, arginine kinase targeted for *D. citri*, using plant feeding system and mortality recording

The average mortality was not significantly higher in the psyllids treated with 75 µg dsRNA than the psyllids treated with no treatment control and positive dsRNA control (P = 0.9471) (Figure 3.4). All other treatments also did not cause significant mortality of psyllids with respect to the positive control treatment and no treatment control (P < 0.05). Psyllids fed with 75 µg of dsRNA construct caused only 15.55% ± 5.88% SEM average mortality whereas psyllids fed with 150 µg, 225 µg, 300 µg and 600 µg dsRNA construct caused average mortality of 35% ± 12.37% SEM, 31.11% ± 4.45% SEM, 42.22% ± 12.37% SEM and 26.66% ± 3.84% SEM respectively, which is higher than the average mortality caused due to the positive control treatment (20% ± 0% SEM) and no treatment control (24.44% ± 4.44% SEM) (Figure 3.4B, 3.4C, 3.4D and 3.4E).

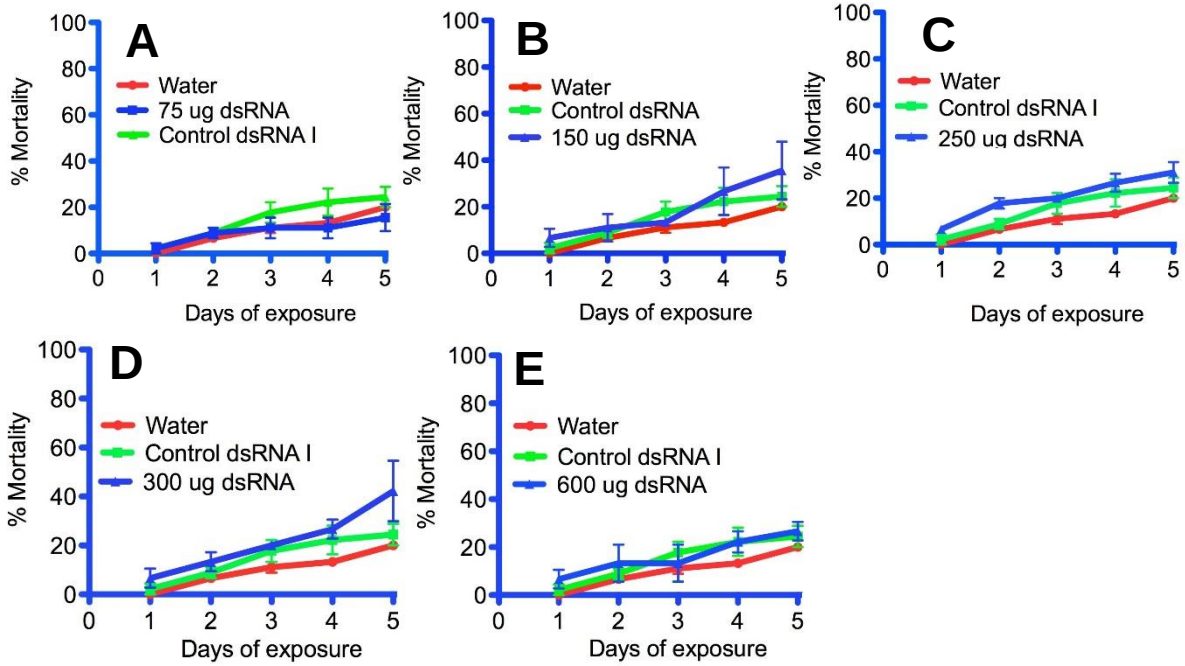

Figure 3.4. Average mortality of psyllids in overtime after acquisition of different concentration of arginine kinase dsRNA construct (A) 75 µg, (B) 150 µg, (C) 250 µg, (D) 300 µg and (E) 600 µg through systemic plant delivery system. Fifteen adult psyllids were used for each treatment

and each treatment was replicated three times. Nanopure water was used as a no treatment control and dsRNA construct targeted for viral capsid protein was used as a positive dsRNA control. All the data were analyzed using Graph PadPrism (version 5.0b). Standard error is represented for each day and the significance of mortality was assessed by using repetitive measures ANOVA.

3.4. Systemic delivery of dsRNA construct, arginine kinase targeted for *D. citri*, using plant feeding system

Overall mortality caused due to the oral delivery of mixtures of two arginine kinase dsRNA (arginine kinase-I and arginine kinase-II) 57%±3.33% SEM caused significantly higher mortality than the psyllids fed with no application control (MEGAscript positive control dsRNA) (30%±5.77% SEM) and nanopure water 33.33%±3.33% SEM ($P<0.05$) (Figure 3.5).

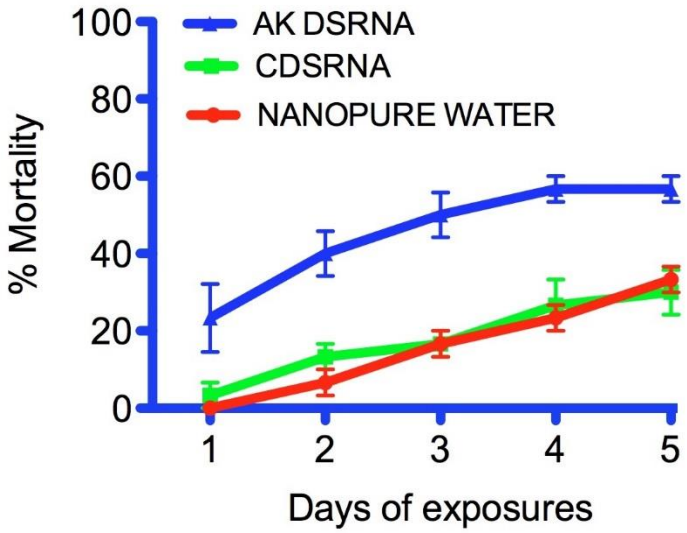

Figure. 3.5. Mortality of adult potato psyllids overtime after systemic delivery of argininine kinase dsRNA (AKDSRNA), MEGAscript positive control dsRNA (CDSRNA) and nanopure water through plant feeding system. Fifteen psyllids were housed in the petri-dish and allowed to feed on pepper systemically treatment with the solutions. Each treatments were replicated three times. The mortality of adult psyllids were recorded in every 24 hours. Graph PadPrism (version 5.0b) was used for statistical analysis. Standard error has been presented for each day and the psyllid mortality has been assessed using repeated measures ANOVA.

3.5. Down regulation of alpha tubulin mRNA level due to oral feeding of alpha tubulin dsRNA construct using sachet feeding system.

To assess the down-regulation of endogenous mRNA levels in potato psyllids treated with alpha tubulin F2/R1 and alpha tubulin F3/R1 in 24 hours, qPCR was used to monitor gene expression of reverse transcribed target mRNAs. The CT values obtained from the qPCR, were compared and analyzed to identify possible down-regulation over time. There is a significant difference in mean CT values among the treatments ($P<0.05$) this significant increase suggest the down-regulation of endogenous mRNA levels over time (Figure 3.6).

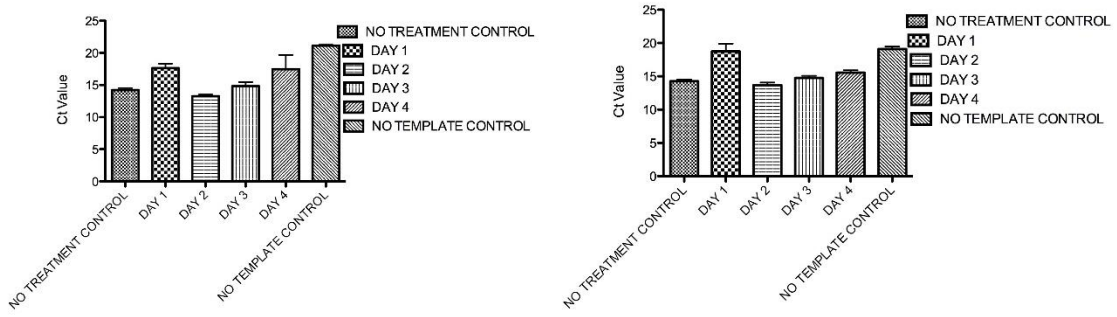

Figure 3.6. Average CT values obtained from qPCR done using total cDNA obtained from total *B. cockerelli* RNA treated with dsRNA construct (A) alpha tubulin F2/R1 and alpha tubulin F3/R1. Fifteen potato psyllids were orally fed with 15% W: V sucrose solution with dsRNA construct and mortality was recorded in every 24 hours. Each treatment was replicated three times. The total RNA was extracted from each replication and cDNA was prepared and used to run qPCR. CDNA obtained from the psyllids fed with only 15% W: V sucrose solution was consider as a no treatment control and the use of nuclease free water instead of template cDNA was consider as no template control. All the data were analysed using Graph PadPrism (version 5.0b). Standard error is represented for each day and the significance of the average CT values was assessed by using ANOVA.

3.6. Downregulation of arginine kinase mRNA levels due to oral acquisition of dsRNA constructs against arginine kinase I and arginine kinase II using systemic plant delivery system.

Psyllids fed with the anti-arginine kinase I dsRNA construct (Table 3.1) did not show significant downregulation of endogenous arginine kinase I gene, as indicated by their normalized index value ($P>0.005$) (Figure 3.7A) (Table 3.6). Similarly, no significant

downregulation of endogenous arginine kinase II gene was observed when analyzed with the normalized index value (P>0.005) (Figure 3.7B) (Table 3.6).

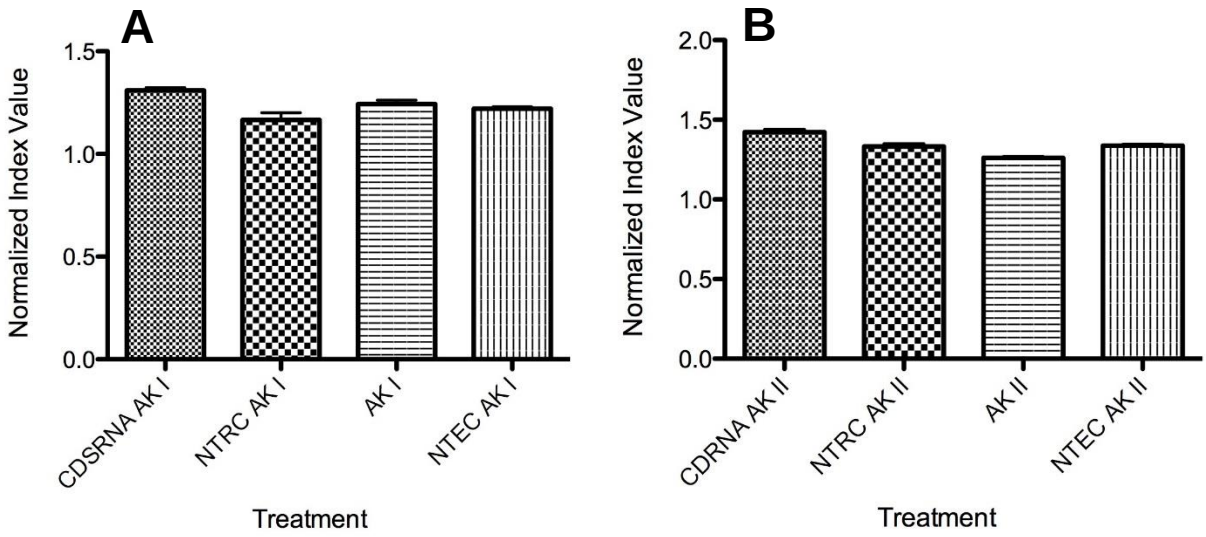

Figure 3.7. 18S gene expression normalization index of Arginine kinase-I (A) and Arginine kinase-II (B) gene downregulation in different treatment groups. In figure (A) CDSRNA AK I (1.31 ± 0.013 SEM) = psyllids that received MEGAscript control dsRNA, NTRC AK I (1.17±0.034 SEM) = psyllids that received nanopure water, AK I (1.24±0.017 SEM) = psyllids that received 200 µg arginine kinase I dsRNA and NTEC AK I (1.22±0.005 SEM) = no template control. In figure (B), CDRNA AK II (1.42±0.005 SEM) = psyllids that received MEGAscript control dsRNA, NTRC AK II (1.33±0.005 SEM) = psyllids that received nanopure water, AK II (1.26±0.005 SEM) = psyllids that received 200 µg arginine kinase II dsRNA and NTEC AK II (1.34±0.005 SEM) = no template control. Standard error is represented for each treatment and significance was determined using one-way ANOVA.

Table 3.6. A list of CT values, replicated CT values and calculated standard deviation for replicated CT values obtained by running qPCR on CDNAs obtained from psyllids systemically fed with MEGAscript control dsRNA, nanopure water, a total 400 µg dsRNA (200 µg arginine kinase I + 200 µg arginine kinase II dsRNA).

No.	Name	Ct	Rep. Ct	Std. Dev.
1	AK I	17.99	17.44	0.48
2	AK I	17.11		
3	AK I	17.24		
4	AK I	16.86	16.79	0.24
5	AK I	16.52		
6	AK I	16.99		
7	AK I	17.11	17.54	0.42

8	AK I	17.95		
9	AK I	17.55		
10	CDSRNA AK I	17.14	17.5	0.32
11	CDSRNA AK I	17.61		
12	CDSRNA AK I	17.74		
13	NTRC AK I	17.37	16.5	0.85
14	NTRC AK I	16.45		
15	NTRC AK I	15.68		
16	NTEC AK I	16.8	16.6	0.2
17	NTEC AK I	16.4		
18	NTEC AK I	16.59		
19	AK II	17.54	17.47	0.06
20	AK II	17.47		
21	AK II	17.42		
22	AK II	17.52	17.75	0.2
23	AK II	17.83		
24	AK II	17.9		
25	AK II	17.05	17.88	0.73
26	AK II	18.17		
27	AK II	18.42		
28	CDSRNA AK II	18.66	19	0.39
29	CDSRNA AK II	18.92		
30	CDSRNA AK II	19.43		
31	NTRC AK II	19.27	18.85	0.41
32	NTRC AK II	18.46		
33	NTRC AK II	18.83		
34	NTEC AK II	18.02	18.18	0.2
35	NTEC AK II	18.4		
36	NTEC AK II	18.11		
37	AK 18S	14.77	14.36	0.45
38	AK 18S	14.44		
39	AK 18S	13.87		
40	AK 18S	14.24	14.19	0.11
41	AK 18S	14.07		
42	AK 18S	14.27		
43	AK 18S	13.52	13.56	0.04
44	AK 18S	13.59		
45	AK 18S	13.57		
46	CDSRNA 18S	13.25	13.36	0.1
47	CDSRNA 18S	13.44		
48	CDSRNA 18S	13.38		
49	NTRC 18S	14.32	14.15	0.16
50	NTRC 18S	14.13		
51	NTRC 18S	14.01		
52	NTEC 18S	13.97	13.59	0.41
53	NTEC 18S	13.15		
54	NTEC 18S	13.64		

Discussion

In this study, significant mortality of adult potato psyllids has been demonstrated after feeding of anti-alpha tubulin dsRNA. The results obtained from qPCR show the downregulation of endogenous alpha tubulin mRNA. This suggests that introduction of exogenous dsRNA complimentary to certain genes can cause the silencing of target endogenous mRNAs.

All of the dsRNA construct did not cause significant mortality of adult potato psyllids. Some other dsRNA constructs caused a slightly greater percentage of mortality compared with negative application control, but did not cause significant mortality. By systemic delivery of two dsRNA constructs (mixed together) against the target gene (arginine kinase), the treatment caused significant mortality of individuals when compared with treatment group that received nanopure water and the treatment group MEGAscript positive control dsRNA. By mixing two different dsRNA constructs and allowing the insects to feed on such a treatment is a possible way to cause the significant mortality of potato psyllids. Significant downregulation of endogenous mRNA levels for two arginine kinase genes could not achieved, but comparing the normalized index value with other control treatments indicates a slight downregulation of endogenous arginine kinases mRNA levels (Figure 3.6). RNAi is a promising gene silencing technology and is a novel management tool for insect pests (Gu and Knipple, 2013). One of the limiting factor of RNAi is the delivery of dsRNA constructs (Scott et al., 2013). The dsRNA delivery systems proposed in this study are more efficient and economically valid.

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Chapter–Four

Oral delivery of insecticide, Imadicloprid, and dsRNA/RNAi construct, CYP450 6BQ13, towards potato/tomato psyllid, *Bactericera cockerelli*

1. Introduction

The potato/tomato psyllids, *Bactericera cockerelli* (Sulc.), (Order: Hemiptera) are hemimetabolous and undergo five nymphal instars (Lehman, 1930; Liu and Trumble, 2007). Potato psyllids are phytophagous insects and exclusively feed on phloem. They have been observed feeding and residing on more than 20 families of plant but have only been seen complete their development on 40 plant species (Knowlton and Thomas, 1934; Wallis, 1951). Potato psyllids prefer the plants from solanaceae family for survival and development (Wallis 1975). Although they will feed on plants besides solaneceous like, bindweed (*Convolvulus* spp) and sweet potato (*Ipomoea batatas*) (Munyaneza, 2012). *B. cockerelli* can cause damage to crop plants by direct feeding; hence, they have been a serious and economic pest of solanaceous crops (Casey and Trumble, 2011).

B. cockerelli is a serious pest of solanaceous crops like potato and tomato and is associated with psyllid yellows disease of potatoes (Munyaneza et al., 2007). *B. cockerelli* is associated with the transmission of the bacterial pathogen, *Candidatus* Liberibacter solanacearum, which is the causal agent of Zebra Chip disease in potato (Munyaneza et al., 2007; Hansen et al., 2008; Liefting et al., 2009). This disease had led to for millions of dollars in losses in potato industries in various locations in Unites States, Mexico, Central America and New Zealand (Munyaneza, 2012; Nachappa et al., 2012; Munyaneza, 2007; Secor and Rivera-Varas, 2004). Management strategies for Zebra Chip disease and psyllid yellows disease are targeted against the potato psyllid employing chemical and cultural strategies (Butler and Trumble, 2012).

Currently, the greatest focus in potato psyllids management is on chemical applications (Goolsby et al., 2007; Berry et al., 2009; Gharalari et al., 2009; Butler and Trumble, 2012). Various classes of insecticides have been registered for use according to the life stage of the psyllid (Rondon et al., 2012). The management of potato psyllid with insecticide application is difficult; in some cases, insecticides application should be specific. The insecticides that is effective against adult stage might not have the same insecticidal activity against nymph stages or egg stage (Gharalari et al., 2009; Zens et al., Rondon et al., 2012). There is always a risk of insects developing resistance towards chemical pesticides, such risk factors can be mitigate with suitable planning; to avoid development of resistance chemical pesticide users should follow insecticide resistance management plans (Rondon et al., 2012). Some populations of potato psyllids demonstrate resistance to field rates of imidacloprid (Rondon et al., 2012). Psyllids from California were found to be resistant to imidacloprid and spinosad compared to psyllids from Central USA (Liu and Trumble, 2007). Another risk of use of chemical pesticides is associated with killing other beneficial and non-target insects disrupting the polluting the surrounding ecosystem. There are no practical non-chemical control strategies for potato psyllids that have been made available to growers (Rondon et al., 2012).

Cultural control of *B. cockerelli* refers to the alteration and administration of the cropping environment to reduce the risk of pest population and damage associated with infestation (Pedigo and Rice, 2006). By shifting the planting time, the damage associated with *B. cockerelli* can be minimized. Eyer and Enzie., (1939) observed that early-planted potato and tomato crops developed psyllid yellows as severely as those planted later in the season. Wallis, (1948) showed significantly higher numbers of *B. cockerelli* in early planted potato field in Wyoming and Nebraska compared to middle or late season planting. Other solanaceous crops can also be used

as an alternate trap crop to protect the main crop from severe damage. Pepper plants were used as a secondary crop or more likely as an alternate trap crop to attract *B. cockerelli* from potatoes in Colorado (Cranshaw, 1994). Researchers have also focused on finding some *B. cockerelli* resistant host plants. Of the thirty nine potato varieties screened, none were found to tolerate psyllid yellows disease (Babb and Kraus, 1937). All commercial potato varieties tested were found to be susceptible to *B.cockerelli* (Linford, 1928; Starr, 1939). The research on biological management of this pest is still ongoing. The use of natural enemies of this pest is one of the environmental managing tactic (Butler and Trumble, 2012). All the tactics, chemical or cultural, should be used to mitigate the possible losses associated with *B. cockerelli*. RNAi technology is a potential strategy of managing insect pests and plant pathogens and is therefore gaining the attention of the scientific community (Price and Gatehouse, 2008).

RNAi is a technique that uses the introduction of novel, targeted double stranded RNA (dsRNA) sequences into pest organisms to degrade mRNA complimentary to the sequences in the dsRNA, and finally inhibits gene expression (Mello and Conte, 2004). Sequence specific gene silencing can be achieved via RNAi in insects by feeding dsRNA (Baum et al., 2007). All management strategies currently used to manage *B. cockerelli* are relatively expensive and pesticide intensive, further study towards integrated pest management (IPM) of this pest is required for sustainable pest control. In some cases, the administration of two or more than two different compounds/treatments together or sequentially may act synergistically, i.e., might cause much more toxicity than the compounds given alone or may act antagonistically, i.e., the effects due to the combination of two or more than two treatments might lower the individual toxicity effects (Hodgson, 2004). In this study, potato psyllids has been administered with CYP450

6BQ13 dsRNA and two different concentration of imidacloprid solutions and monitored for five days to identify the synergism between these two different treatments.

Insecticides have proven the only effective management tool for the management of potato psyllid infestations (Goolsby et al., 2007; Gharalari et al., 2009). Imidacloprid is one of the pesticides which has been used extensively to manage the potato psyllid (Goolsby et al., 2007). This compound is a systemic neonicotinoid insecticide and acts as an agonist of acetylcholine binding to post–synaptic nicotinic acetylcholine receptors, disrupting feeding behaviors and causing tremors, convulsions and finally death of insects (Butler et al., 2012; Nauen, 1995; Oliveira et al., 2011). The imidacloprid disrupts feeding behavior of potato psyllids reducing the transmission of *Candidatus* liberibacter solanaceraum (Lso) (Butler et al., 2011). Boina et al. (2009) observed negative effects on development, reproduction, survival and longevity of Asian citrus psyllid, *Diaphorina citri*, when exposed to sub lethal concentration of imidacloprid (0.1 µg/ml).

This study aims to advance the integrated pest management technique against the *B. cockerelli.* The *B. cockerelli* transcriptome has been examined to identify cytochrome p450s family genes involved in insecticide resistance and designed a dsRNA construct against one of the cyp450s gene. Anti-CYP450 6BQ13 dsRNA has been synthesized *in vitro* and offered to potato psyllids along with commonly used insecticide, imidacloprid, through artificial systemic plant feeding system. The mortality was recorded every 24 hours for five days and possible downregulation of cytochrome P450 6BQ13 gene was assessed using qRT-PCR.

2. Materials and methods

2.1. Insect colonies

Potato psyllids used in this experiment were obtained from Adrian Silva (Zebra Chip monitoring Program, Texas A&M Agrilife Research, Weslaco, Texas) and were maintained on pepper plants (*Capsicum annum*) in a bug dorm (36in x 24in x 18in) within insectarium at 26°C, 70% humidity and a 14:10 (day: night) cycle. The colony was maintained without any exposure to pesticides. Mixed male/female adult potato psyllids were used in this experiment.

2.2. Imidacloprid

The insecticide formulation, Tree & Shrub Protect & Feed (Bayer Advanced, Kansas City, MO 64120) was used as an imidacloprid source. The insecticide solution used in this experiment was provided by Mr. Irfan Vafaie (Texas A&M Agrilife Research & Extension Center at Overton). According to the manufacturer's protocol, the dosage for containerized plants (one teaspoon of insecticide solution in one gallon of water) was chosen. Converting this dosage amount into standard milliliter form, we calculated 4.928 ml (one teaspoon) of insecticides in 3785.41 ml (one gallon) of water. This concentration is equivalent to 1.3 uL of insecticide solution in 1 ml of water. In this experiment, we have considered this concentration as 1X imidacloprid solution and 10 fold dilution of the 1X imidacloprid solution has been named as 0.1X, 10 fold increase in concentration of the 1X imidacloprid solution has been named as 10X and 100 fold increase in concentration of the 1X imidacloprid solution has been named as 100X. All the solutions were made in nanopure water.

2.3. Double stranded RNA/RNAi construct selection, primer designing and synthesis

The *B. cockerelli* transcriptome database (Fisher et al., 2014) was examined to find the CYP450 6BQ13 gene, this gene is known to be involved in insecticide resistance. The NCBI

primer blast tool was used to design primers for CYP4506BQ13 gene. The settings in NCBI primer blast tool included a PCR product size of 200 to 500 bp length, primer melting temperature in a range of 50^0C to 60^0 C, and specificity was checked against the order hemiptera.

Cetyltriethylammonium bromide (CTAB) buffer protocol (Zhang et al., 1998) was used to extract total *B. cockerelli* genomic DNA. The quality of the extracted DNA was assessed using NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA) and visualized on a 2% agarose gel stained with ethidium bromide. The temperature gradient PCR was conducted using the Amplitaq Gold 360 PCR kit as per the manufacturer protocol (Applied Biosystems, Foster City, CA). The thermal profile was run as follows: cycle 1, 95°C for 10 minutes (1x), cycle 2, 95°C for 30s, 50-60°C for 30s, 72°C for 60s (45x), and cycle 3, 72^0 C for 7 min (1x). Amplicons were visualized on a 2% agarose gel stained with ethidim bromide. The prominent and single band size was excised and recovered DNA through QIAquick Gel Extraction kit following manufacturer protocol (QIAGEN, Valencia, CA). A second PCR was done using the same primer amended with T7 promoter sequence (TAATACGACTCACTATAGGG) at the 5'end. The excised amplicon visualization and purification was done the same as the previously described method. The purified product was analyzed using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA) and used for dsRNA synthesis via the MEGAscript RNA kit (Ambion, Austin, TX, USA) as per the manufacturer protocol.

2.4. Mortality bioassay of potato psyllids fed with various amount of imidacloprid solutions

For the preliminary study, potato psyllids were grouped into four treatment groups of ten psyllids. Each treatment group was replicated three times. The treatment groups were as follows: (1) psyllids that received 0.1X imidacloprid solution, (2) psyllids that systemically received 1X imidacloprid solution, (3) psyllids that received 10X imidacloprid solution and (4) psyllids that

systemically received nanopure water as no treatment conrol. To deliver the treatment solutions orally, the freshly cut petiolate pepper leaf was allowed to absorb the treatment solution put in 1.7 mL microfuge tube for six hours then refilled with nanopure water when the water level was low. The whole leaf was placed inside the petri dish and ten psyllids were housed. Mortality was recorded every 24 hours for five days. The data were analysed using Graph PadPrism (version 5.0b). The psyllid mortality significance was assessed by using repeated measures ANOVA.

2.5. Mortality bioassay of potato psyllids fed with Imidacloprid in conjunction with dsRNA targeted for CYP 450 6BQ13 gene

Potato psyllids were grouped into six treatment groups with fifteen psyllids in each groups. The treatment groups were as follows: (1) psyllids that systemically received 10X imidacloprid, (2) psyllids that systemically received 10X imidacloprid and 400 microgram CYP450 6BQ13 dsRNA (500 uL of 800 ng/uL), (3) psyllids that systemically received 100X imidaclorpid solutions, (4) psyllids that systemically received 100X imidacloprid solution and 400 microgram CYP450 6BQ13 dsRNA, (5) psyllids that received only nanopure water and (6) psyllids that systemically received 400 µg of MEGAscript positive control dsRNA (Figure 4.1). The oral delivery for this bioassay was similar as above described systemic method (2.4). To assess the particular downregulation of CYP450 6BQ13 gene, five psyllids from each treatments were removed and mortality was recorded for rest of the psyllids. The mortality was recorded every 24 hours for five days. The mortality data was analyzed using Graph PadPrism Version 5.0b. The psyllid mortality significance was assessed using repeated measures ANOVA.

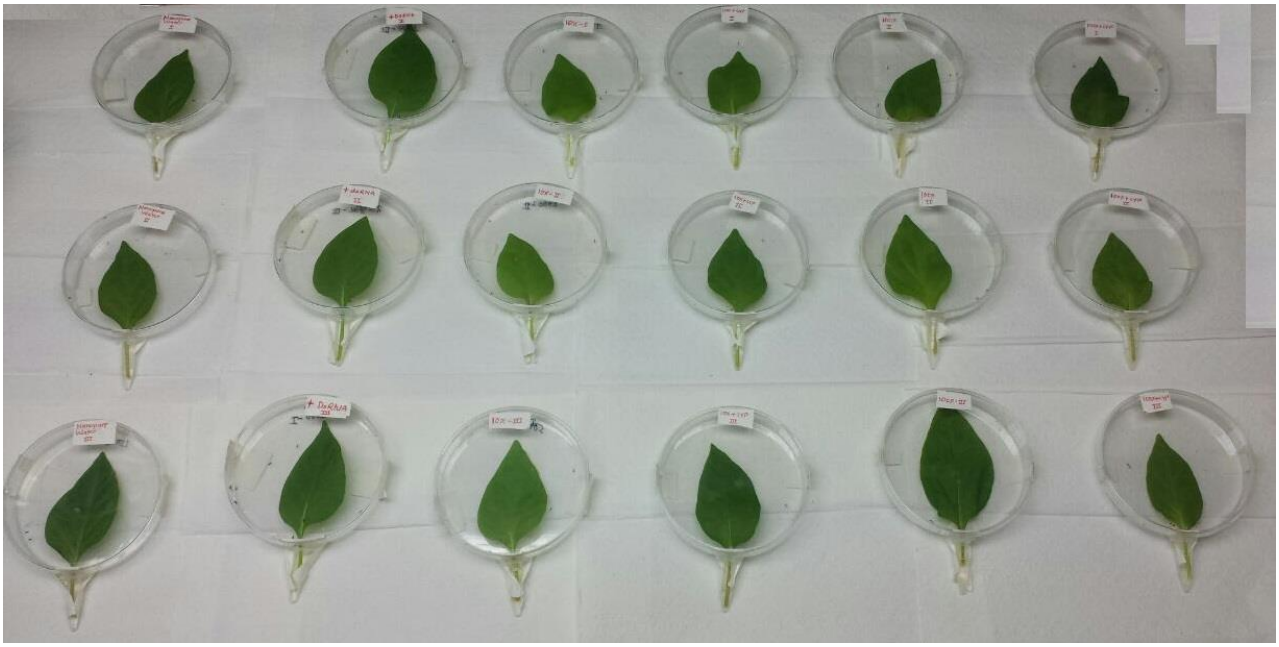

Figure 4.1. Experimental set up for the mortality study of *B. cockerelli* through systemic delivery of six different treatments, nanopure water, 10X imidacloprid solution, 10X imidacloprid solution + CYP450 6BQ13 dsRNA, 100X imidacloprid solution, 100X imidacloprid solutions + CYP450 6BQ13 dsRNA and MEGAscript positive control dsRNA. Each treatment was replicated three times. 10 psyllids were housed in each treatment. The mortality data was analysed using Graph PadPrism (version 5.1b).

2.6. Psyllid RNA isolation, cDNA synthesis and quantitative real time PCR (qRT-PCR)

The five psyllids removed from each treatment were pooled and subjected to total RNA extraction using TRIzol (Life Technologies, Carlsbad, CA) reagent as per the manufacturer protocol. The total RNA was reverse transcribed to produce complimentary DNA (cDNA) using M-MuLV Reverse Transcriptase (New England BioLabs) following manufacturer protocol. To assess the possible downregulation of endogenous CYP450 6BQ13 mRNA level, quantitative real time polymerase chain reaction (qRT-PCR) was performed on the total cDNA. The reaction mixture was 12.5 µL Amplitaq Gold 360 Master mix (Life Technologies, Austin, TX), 1 µL GC enhancer (Life Technologies, Austin, TX), 1 µL of each forward and reverse primer, 1.25 µL of Evagreen 20 in water (Biotium, Hayward, CA), 2 µL of extracted cDNA as a template DNA

(~90 ng) and 5.5 µL of nuclease free water (ThermoFisher Scientific, Grand Island, NY). The total volume was 25 µL per reaction. The thermal profile was run as follows: 94°C for 30s, 60°C for 30s, 72°C for 6s, 60°C for 5 s, repeated 40 times, followed by a melt curve obtained by ramping from 50°C to 90°C by adding 1°C each step for 90s, with five seconds between each step, followed by a hold at 4°C. qPCR was performed using a specially-designed qPCR primer set: sense primer (5'-CCCTCAATTGTGTGGGACGA-3') and reverse primer (5'–TGACGGGGACTTCAAAGGTG-3') to amplify a 126-bp product. For the no template control (NTEC), nuclease free water was used instead of cDNA as a template DNA. 18S ribosomal RNA was used as an endogenous control. CT values obtained from each treatments were compared and normalized to the 18S rRNA.

3. Results

3.1. Potato psyllids mortality fed with imidacloprid solutions

Psyllids fed with high concentrated imidacloprid solution (10X) caused comparatively high mortality (73% ± 16.68% SEM) than psyllids fed with lower concentrated imidacloprid solutions; 1X imidacloprid solution caused only 37% ± 8.83% SEM and 1X imidacloprid solution caused 37% ± 17.66% SEM mortality and nanopure water fed psyllids caused mortality of 40% ± 5.78% SEM. (Figure 4.2). The two lower concentration imidacloprid solutions, 1X and 0.1X did not cause significant mortality with respect to psyllids fed with nanopure water ($P>0.05$) (Figure 4.2A and 4.2B).

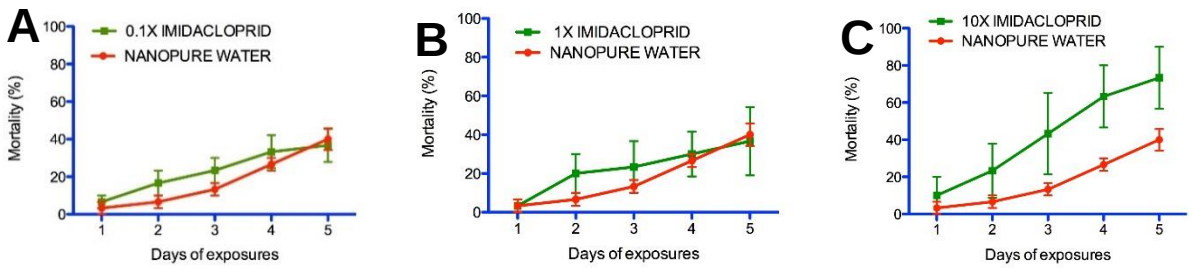

Figure 4.2. Potato psyllid mortality overtime by oral feeding of (A) 0.1X imidaclorid solution, (B) 1X imidacloprid solution and (C) 10X imidacloprid solution. Groups of ten adult psyllids were used for each treatment. Each treatment was replicated three times. Only nanopure water was used as a no treatment control. Graph PadPrism version 5.0b was used for statistical data analysis. Standard error is represented for each day and the psyllid mortality significance was assessed using repeated measures ANOVA.

3.2. Mortality bioassay of potato psyllids fed with Imidacloprid solution and CYP 450 6BQ13 dsRNA.

Cumulative mortality was significantly higher in the potato psyllids fed with both, 10X imidacloprid solution (93% ± 3.41% SEM) and 10X imidacloprid solution + CYP450 6BQ13 dsRNA treatments (100% ± 0% SEM) than psyllids fed with control dsRNA (23% ± 3.41% SEM) and nanopure water (17% ± 6.68% SEM) ($P<0.001$) (Figure 4.3). Psyllids treated with 10X imidacloprid solution + CYP450 6BQ13 dsRNA caused 100% ±0% SEM mortality but could not able to cause significant mortality than 10X imidacloprid solution (93% ± 3.41% SEM) ($P>0.005$) (Figure 4.3A).

Psyllids fed with 100X imidacloprid solution (97% ± 3.41% SEM) and 100X imidacloprid + CYP450 6BQ13 dsRNA (100% ± 0% SEM) caused significantly higher mortality of psyllids when compared to psyllids fed with control dsRNA (Mortality = 23% ± 3.41% SEM) and nanopure water (17% ± 6.68% SEM) ($P<0.0001$) (Figure 3 B). After 24 hours, 100X imidacloprid solution + CYP450 6BQ13 dsRNA treatment caused 33% ± 8.83% SEM mortality of psyllids whereas on the same day 100X imidacloprid solution caused only 13% ±3.34% SEM

of psyllid mortality but for remainder of the assay, except on day 5, when the 100X imidacloprid solution caused slightly higher mortality than 100X imidacloprid + CYP450 6BQ13 dsRNA treatment (Figure 4.3B).

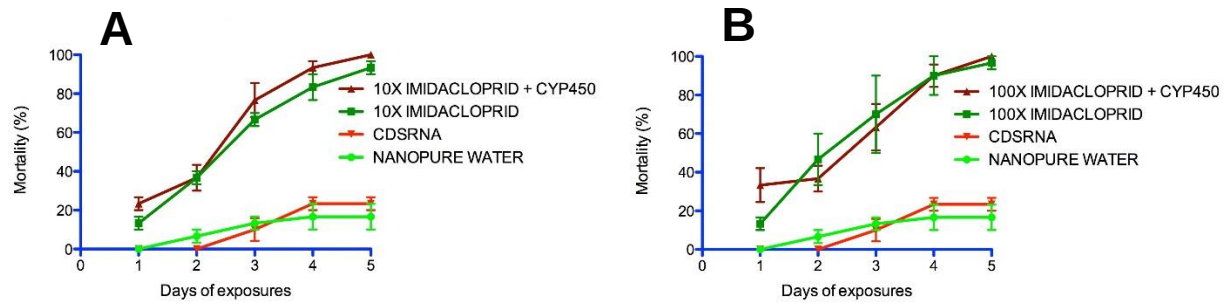

Figure 4.3. Mortality of potato psyllids overtime by oral feeding of (A) 10X imidacloprid solution and CYP450 6BQ13 and (B) 100X imidacloprid solution and CYP450 6BQ13. Groups of fifteen psyllids were used for each treatment. Each treatment was replicated for three times. Psyllids fed with only nanopure water was used as no treatment control. Psyllids fed with MEGAscript control dsRNA was used as a positive control dsRNA. Graph PadPrism version 5.0b was used for statistical data analysis. Standard error is represented for each day and the psyllid mortality significance was assessed using repeated measures ANOVA.

3.3. CYP450 6BQ13 gene downregulation analysis

Overall, there was no significant difference between CYP450 6BQ13 gene downregulation in all treatment groups as indicated by their normalized index value ($P>0.005$) (Figure 4) (Table 4.1). Psyllids that received 10X imidacloprid solution + 400 µg CYP450 6BQ13 dsRNA and Psyllids that received 100X imidacloprid solution + 400 µg CYP450 6BQ13 dsRNA did not show any endogenous CYP450 6BQ13 genes downregulation when compared with psyllids that received only control dsRNA and nanopure water ($P>0.05$) (Figure 4.4).

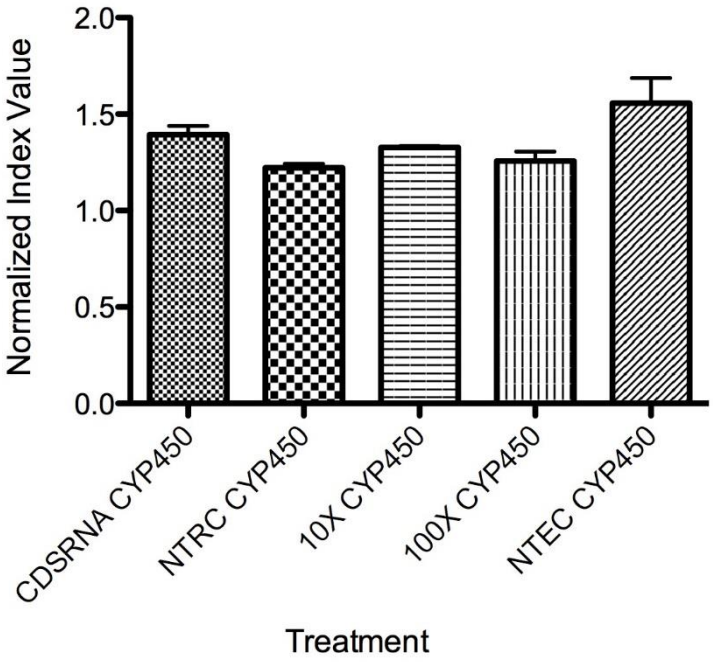

Figure 4.4. 18S gene expression normalization index of CYP450 6BQ13 gene downregulation in different treatment groups, where CDSRNA CYP450 = psyllids that received MEGAscript dsRNA, NTRC CYP450 = psyllids fed with only nanopure water, 10X CYP450 = psyllids fed with 10X imidacloprid solution and 400 milligram CYP450 6BQ13 dsRNA, 100X CYP450 6BQ13 = psyllids fed with 100X imidacloprid solution and 400 milligram CYP450 6BQ13 dsRNA, and NTEC CYP450 = no template control, instead of cDNA, nuclease free water was used. Standard error is represented for each day and significance was determined using one-way ANOVA. Index: CDSRNA CYP450 = 1.394 ± 0.045 SEM, NTRC CYP450 =1.222 ± 0.02 SEM, 10X CYP450 = 1.327 ± 0.009 SEM, 100X CYP450 = 1.257 ± 0.028 SEM and NTEC CYP450 = 1.557 ± 0.13 SEM.

Table 4.1. A list of CT values, replicate CT values, and calculated standard deviation obtained by running qPCR on cDNAs obtained from psyllids systemically fed with control dsRNA, nanopure water, 10X imidacloprid solution + 400 µg CYP450 6BQ13 dsRNA and 100X imidacloprid solution + 400 µg CYP450 6BQ13 dsRNA.

No.	Name	Ct	Rep. Ct	Std. Dev.
1	10X I CYP	19.53	19.69	0.15
2	10X I CYP	19.72		
3	10X I CYP	19.82		
4	10X II CYP	20.35	20.47	0.25
5	10X II CYP	20.30		
6	10X II CYP	20.75		

7	10X III CYP	20.68	20.36	0.29
8	10X III CYP	20.22		
9	10X III CYP	20.16		
10	100X I CYP	18.19	18.76	0.49
11	100X I CYP	19.07		
12	100X I CYP	19.00		
13	100X II CYP	16.84	18.44	1.77
14	100X II CYP	18.14		
15	100X II CYP	20.34		
16	100X III CYP	21.07	20.86	0.21
17	100X III CYP	20.87		
18	100X III CYP	20.64		
19	CDSRNA	20.85	21.25	1.17
20	CDSRNA	20.33		
21	CDSRNA	22.57		
22	NTRC CYP	18.57	18.71	0.52
23	NTRC CYP	18.28		
24	NTRC CYP	19.29		
25	10X I 18S	15.37	15.28	0.31
26	10X I 18S	14.93		
27	10X I 18S	15.53		
28	10X II 18S	15.01	15.21	0.22
29	10X II 18S	15.17		
30	10X II 18S	15.46		
31	10X III 18S	14.65	14.6	0.25
32	10X III 18S	14.83		
33	10X III 18S	14.32		
34	1OOX I 18S	15.60	15.58	0.14
35	1OOX I 18S	15.44		
36	1OOX I 18S	15.71		
37	1OOX II 18S	15.19	15.45	0.23
38	1OOX II 18S	15.59		
39	1OOX II 18S	15.58		
40	1OOX III 18S	15.54	15.17	0.32
41	1OOX III 18S	15.04		
42	1OOX III 18S	14.94		
43	CDSRNA 18S	14.83	15.24	0.37
44	CDSRNA 18S	15.56		
45	CDSRNA 18S	15.32		
46	NTRC 18S	15.28	15.31	0.14
47	NTRC 18S	15.46		

48	NTRC 18S	15.19		
49	NTEC CYP	23.60	26.36	3.83
50	NTEC CYP	24.75		
51	NTEC CYP	30.73		
52	NTEC 18S	18.57	16.93	1.43
53	NTEC 18S	15.92		
54	NTEC 18S	16.29		

4. Discussion

In this study, a protocol to study the synergism between RNAi through oral acquisition of dsRNA against one of the xenobiotic metabolizing genes, CYP450 6BQ13, and commonly used insecticide, imidacloprid towards *B. cockerelli* was attempted. We hypothesized that, the systemically administered dsRNA against CYP450 6BQ13 gene would downregulate the CYP450 6BQ13 gene, and the *B. cockerelli* receiving the dsRNA would not able to oxidize the supplied insecticide, imidacloprid, hence the toxicity due to this chemical would be more effectively increases and causes more psyllid mortality. Our first attempt suggested that there was no such significant downregulation of CYP450 6BQ13 gene, after systemic acquisition of dsRNA construct targeted against CYP450 6BQ13 gene. However, there are a many possible reasons behind it. RNAi occurs only when the dsRNA is delivered or up taken into the cells of the targeted tissue (Yu et al., 2012). Another limiting factor of the RNAi could be the concentration or amount of administered dsRNA construct.

Though the significant downregulation of CYP450 6BQ13 gene could not achieved in this study, the oral acquisition of two different treatments, 10X imidacloprid solution + CYP450 6BQ13 dsRNA and 100X imidacloprid solution + CYP4506BQ13 dsRNA caused 100% mortality of potato psyllid, which is greater than the mortality caused due to single treatment, 10X imidacloprid solution (Mortality = 93% ± 3.41% SEM) and 100X imidacloprid solution

(Mortality =97% ± 3.41% SEM). There is no antagonism effects for two different treatments otherwise the mortality due to both treatments, 10X imidacloprid solution + CYP450 6BQ13 dsRNA and 100X imidacloprid solution + CYP4506BQ13 dsRNA, would have caused lower mortality than that of the single treatment, 10X imidacloprid solution and 100X imidacloprid solution.

Enzyme-based metabolism of xenobiotics consists of involvement of the various detoxifying enzymes, such as esterases, cytochrome P450s (CYP5450s), and glutathione S-transferases (GSTs) (Hardy 2014; Li et al., 2013). Cytochrome P450s (CYP450s) are a large class of enzymes involve in the detoxification and/or activation of xenobiotics (Liu et al., 2015). Studies have revealed that multiple CYP450s genes are involved in the xenobiotics metabolism or detoxification. Hence, in this study we have targeted only one gene from CYP450s family, therefore this could be one of the limitations of this study. For the future study, multiple genes involved in the xenobiotic metabolism from CYP450s family, GSTs family and esterases family should be targeted to observe the significant synergism between insecticides application and RNAi against xenobiotic detoxifying genes.

Microbial communities, especially the secondary endosymbionts in the potato psyllids are responsible for providing resistance to insecticides (Arp et al., 2014; Hail et al., 2012). Bautista et al. (2008) mentioned that, in insect species the cytochrome family of enzymes (Cytochrome P450s) is highly involved in insecticide metabolism. In insects the increase in expression of cytochrome P450s may enhance the xenobiotic metabolism of insecticides, and lead to the resistance towards various insecticides (Carino et al., 1992; Liu and Scott 1997; Li et al., 2006).

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Chapter Five
Conclusions and Future Research

Plant-feeding insects utilizing a plant's nutrition can cause nutritional deficiency and mechanical injuries to the host plants. These pests can also be associated with the transmission of various phytopathogens. Nowadays, this phenomenon is globally affecting the agricultural industries. The crop loss due to insect pests is causing a threats in global food security. Currently, major insect pests are attempted to manage by insecticide application; however, the use of chemical pesticides can pollute the surrounding ecosystem when chemical persist in the environment and they can kill beneficial insects and off-target insects. Also, pests may develop resistance against the chemical pesticides overtime. It is therefore important to develop a targeted long-term management treatment against insect pests.

These studies aimed to investigate the biological management tool against the potato/tomato psyllid (*Bactericera cockerelli* Sulc.), which is an economically important insect pest that prefers to feed on solaneceous crops. Feeding by *B. cockerelli* can cause the condition known as psyllid yellows, this disease can cause crop loss of potato. *B. cockerelli* is also associated with the transmission of *Candidatus* Liberibacter solanacearum, the causal agent of Zebra Chip disease, in potato.

RNA interference (RNAi) has been widely used in entomological research in a variety of insects and has a potential for RNAi-based pest management technique. Introduction of exogenous double-stranded RNA, which is complementary to the endogenous messenger RNA (mRNA) triggers the RNA interference process by silencing the mRNA expression. Utilizing this cellular process, RNAi technology has been developed against *B. cockerelli*. Various sources of genomic database of *B. cockerelli* were utilized to identify some potential genes, which has

essential role in fitness, phenotypic characteristics and common cellular activities. Double stranded RNA (dsRNA) constructs were designed against the mRNA of the potential genes. These dsRNA constructs were orally fed to the potato psyllid. To deliver the dsRNA constructs we have developed various delivery system and tested their efficiencies. Successful delivery of dsRNA constructs using the delivery systems was achieved, also the introduced dsRNA caused significant mortality of psyllid and induced gene silencing through downregulation of endogenous mRNA level.

Moreover, insecticide metabolizing genes could be one of the best targets, hence, downregulation of such genes would make insects more susceptible towards the insecticides and application of even low doses of insecticides could incapacitate or kill the insects. Utilizing this information, this study also attempted investigation of identifying genes responsible for insecticide metabolism. Double stranded RNA was constructed against one of the genes from the family of insecticide metabolizing gene and offered to potato psyllids along with insecticides.

This study should be replicated and focused on identification of more potential genes. Future study also could estimate the lowest dsRNA concentration needed to incapacitate or kill a potato psyllid. In this study, according to the delivery systems and treatments, the average concentration of dsRNA ranged from 25 µg to 400 µg. The total costs associated with the *in vitro* synthesis of dsRNA is way high when compared with the amounts of dsRNA needed to treat a single plant in the wild or even massively high to treat the entire fields. Another possible area of research should also be focused on determining the approximate time the plant takes to distribute the dsRNA constructs throughout different parts and eventually clearing from all points of plant.

In conclusion, the success in the management of one insect species, *B. cockerelli*, through RNAi technology will have potential application to other insect pests. This could be an effective

ways to mitigate the loss associated with the insect pests. The downregulation of insecticides metabolism through RNAi technology could be one of the efficient tool towards integrated pest management technique.