DETERMINING THE SUSCEPTIBILITY AND CROSS-RESISTANCE IN A VIP3A RESISTANT STRAIN OF FALL ARMYWORM *SPODOPTERA FRUGIPERDA*, TO PURIFIED BT PROTEINS, BT CORN, AND BT COTTON

A Thesis

by

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ABSTRACT

The fall armyworm, *Spodoptera frugiperda* is one of the major target pests of Bt corn and cotton in the U.S. Current control strategies for FAW rely heavily on transgenic plants. Negative side effects of extensive use have resulted in field-evolved resistance. Gene pyramiding has been used to delay these resistance issues; however, the durability of this technique can be greatly reduced by cross-resistance. In this study, we investigated the susceptibility and cross-resistance of different genotypes of fall armyworm carrying Vip3A resistant alleles to purified Bt proteins, Bt corn and Bt cotton.

Purified Bt protein assays, utilized to determine cross-resistance to other proteins, indicate that the resistant (RR) larvae tested 39.5 fold more resistant to the Vip3Aa51 toxin when compared to the susceptible (SS) strain, and tested highly susceptible to all other Bt proteins. SS and the heterozygote (RS) larvae were highly susceptible to all proteins. To confirm the susceptibility found in the protein bioassay, and to determine how the genotypes behave on Bt plants, corn leaves and whole plant corn bioassays were used. This bioassay found that RR survive well on non-Bt and Vip3111 (Vip3a, Cry1Ab) corn. RR had moderate survivorship on Herculex (Cry1F) corn, however survivorship diminished on all other technologies. These data suggest that RR may have some low level resistance to Cry1F. SS showed high survivorship on non-Bt but no survivorship on any other technology.

Cotton leaf and square bioassays were utilized to determine the cross-crop resistance of Vip3A resistant FAW. During the leaf bioassay RR, RS, and SS genotypes

had low survivorship across all varieties. However, in the square bioassay RR showed high survivorship across Bollgard III (Cry1Ac, Cry2Ab, Vip3A), Bollgard II (Cry1Ac, Cry2Ab), Widestrike (Cry1F, Cry1Ac), Widestrike 3 (Cry1F, Cry1Ac, Vip3A), regardless of technology. RS and SS showed similar results with high survivorship on non-Bt, Bollgard II (Cry1Ac, Cry2Ab), and Widestrike (Cry1F, Cry1Ac), however very little or no survivorship on Bollgard III (Cry1Ac, Cry2Ab, Vip3A), and Widestrike 3 (Cry1F, Cry1Ac, Vip3A). Results generated from these studies provided important information for insect pest management and aid in developing effective resistance management strategies for the sustainable use of Vip3A technology.

DEDICATION

This thesis is dedicated to my family and friends who have been behind me and pulling for me this entire time. It has been a long while, but I am almost done.

ACKNOWLEDGEMENTS

I would like to start by thanking my parents. Without your love and support, none of this would be possible. You have both been there to help me through the tough times and the good times and taught me that I can indeed do anything I put my mind to and that I will not stop until I have it done my way. I would also like to thank the wonderful woman I have met along the way that has pushed me, guided me, and helped keep me on track when life would pull me in all directions, Jessica Baber.

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CONTRIBUTORS AND FUNDING SOURCES

This work was supervised be a thesis committee consisting of Dr. David Kerns of Department of Entomology from Texas A&M University, Dr. Charles Suh Department of Entomology at the USDA ARS in College Station, Texas, and Dr. Ronnie Schnell Department of Soil and Crop Science at Texas A&M University.

The design and the data analysis was assisted by Dr. Fei Yang of the Department of Entomology at Texas A&M University.

All other work conducted for this thesis was completed by Ryan Gilreath independently.

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I would like to acknowledge Syngenta for supplying the Bt toxin Vip3Aa19 that assisted with developing the resistant fall armyworm colony, and BASF for suppling Vip3A51 which was used during the bioassay portions of this thesis.

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CHAPTER I

INTRODUCTION

Biology of *Spodoptera frugiperda*

The fall armywom *Spodoptera frugiperda* (J.E. Smith) (Insecta: Lepidoptera: Noctuidae) (FAW) is a globally distributed destructive pest. It has been classified as sporadic pest due to its migratory behavior. The native range of this pest is the tropical regions of the western hemisphere from the United States down to Argentina. FAW had stayed confined to the western Hemisphere until recently when reports have surfaced of the identification of FAW in western and central Africa (Goergen et al. 2016). It has now been documented to be in more than 30 countries in Africa. In 2018, India reported discovery of FAW, and most recently this pest was reported in China (Fas 2019). The adult moth is a strong flier and can cover great distances during its migration. Due to the fact this species has no diapause mechanism, migration in the U.S. is typically from the warmer southern regions northward.

The life cycle for this pest is usually requires approximately 30 days, consisting of egg, six larval instars, pupa and adult (Lunginbill 1928). The life cycle slows down during the spring and fall periods to around 60 days, and during the winter months about 80 to 90 days. Adult moths lay eggs on the surface of leaves in masses of 150 – 200 eggs. Total egg production for one female may vary, however it can average about 1,000 to a maximum of 2,000. Females will also deposit a layer of scales between the eggs and over the entire mass.

Newly hatched neonates are usually white to yellowish with a black head capsule. The next two instars the color of the larvae will darken (Luginbill 1928), but in the last three instars the typical dark brown color emerges. The color patters may vary depending upon diet. The late instars display a prominent inverted Y on the front of the head capsule. Larvae also have distinct pattern on the eighth abdominal segment of four black dots.

Once the larvae reach the critical size and weight, pupation is initiated. During this stage the larvae construct a cocoon around themselves where they will molt into adult moths. When the moths emerge there is a preoviposition period of three to four days, once this period ends females will begin to lay their eggs. It has been estimated that the life span of adult FAW is on average ten days.

FAW is a highly polyphagous insect that has a very wide host range of more than 80 host species that encompasses 23 families (Pashley 1988). FAW consist of two host-associated strains: the corn strain is primarily found on corn, sorghum type forages, and cotton; and a rice strain that primarily feeds on rice, turf grasses, and annual grasses. Larvae feeding on these hosts cause great damage by consuming foliage, fruiting structures, and grain. Early instar larvae begin by chewing through the leaf creating pin holes. Injury to cotton by early instars can skeletonize the leaves where the egg mass is located (Hardke et al. 2015a). Late instars may feed and destroy terminals of young cotton plants, and may also feed on bracts, large squares and young bolls (Leigh et al. 1996). During the pre-bloom stages of growth in cotton, FAW can cause defoliation. Freshly hatched neonates infesting corn will drop down into the whorl of the plants

feeding on the foliage until they are large enough where the damage becomes present (Harrison 1986). Second to 3rd instar larvae consume foliage creating large holes. Late instars can cause complete defoliation of plants.

Damage occurring in corn can be very similar to cotton. FAW can be found early in the season feeding down in the whorl of the plant causing pinholes or even long lesions. As the plant matures larvae can be found feeding on the feeding in the ear of the plant as well.

Management Strategies

The wide host range and geographical distribution of this destructive pest creates a challenge for monitoring and maintaining population sizes. Moth populations can be sampled with black light and pheromone traps. Collections from these traps are not good indictors of population size but can represent presence in the field. Monitoring efforts should focus in part on blooms and late in the season, or on stressed cotton that may have lower Bt toxin expression. Scouting methods in corn can be more difficult due to the sporadic nature of the pest. Corn plants along with other grasses that may be present in the field should be check for larvae and egg clusters (AgriLife Extension: Managing Insects and Mite Pests of Texas Corn, p.16).

The lack of a diapause mechanism makes conventional cultural control methods ineffective. Reduced tillage scenarios have been shown to be less attractive to migrating moths, thus reducing the need for chemical suppression (Roberts et al. 1993). Antibiosis and antixenosis can be key mechanisms for host plant resistance in corn hybrids (Sparks 1986); however, they are inadequate for complete control. The most adopted cultural

practice among growers is to plant and harvest early to avoid higher FAW populations that may develop later in the season (Mitchell 1978).

Braconids, Ichneumonids, and Tachinids account for 85% of the 53 species of natural enemies known to parasitize FAW (Ashley 1979). Within those 53 species, 43 genera and 10 families are included. Common examples include species from the order

Table 1. Insecticides recommended for the control of fall armyworm

Insecticide	Trade name	Lb of A.i. per acre	Recommendation per acre	Mode of Action Group (IRAC[2])
Chlorantrailiprole	Prevathon	0.047-0.09	14-27 fl oz	28
Chlorantraniliprole, λ-cyhalothrin	Besiege	0.835, 0.417 (respectively)	8-12.5 fl oz	28, 3
Emamectin benzoate	Denim	0.01-0.015	8-12 floz	
Indoxacarb	Stewerd EC	0.09-0.11	9.2-11.3 fl oz	22A
Methomyl	Lannate LV	0.45-0.68	24-36 fl oz	1A
Methoxyfenozide	Intrepid 2	0.06-0.16	4-10 fl oz	
Acephate	Orthene 97	0.974	16 oz	1B
Spinosad	Blackhawk	0.054-0.072	2.4-3.2 fl oz	5
Spinotram	Radiant SC	0.033-0.625	4.25-8 fl oz	
Novaluron	Diamond 0.83	0.0389-0.0778	6-12 fl oz	15

[1]Rates will vary depending on crop, product and formulation

[2]IRAC = 1A-Carbamate, 1B-Organophosphate, 3-Pyrethroids, 5-Spinosyn, 22A-Oxadiazines, 28-Diamides, 15-Benzoylureas

Insect Control Recommendations for Field Crops (Stewart, McClure 2013)

of Hemiptera and Coleoptera. These predators can be found preying upon the FAW eggs and larvae. There have been several entomopathogenic viruses such as nucleopolyhedrovirus, studied for controlling FAW. Some of the major drawbacks of viruses is the significant amount of damage allowed before killing the insect and inconsistent efficacy (Sparks 1986).

Chemical control in corn and cotton has traditionally been one of the most relied upon methods for controlling FAW (Vyavhare et al 2018, Porter et al. 2005; Table 1). Current recommendations range between 5 different IRAC (Insecticide Resistance Action Committee) classifications (Sparks et al. 2015). Efficient and successful control of FAW usually necessitates the use of the upper range of labeled insecticides (Adamcyzk and Sumerford 2000). Oftentimes dispersion of FAW within the canopy of plants can reduce the efficacy of the insecticide application due to the inability of the insecticide to penetrate deep into the lower regions of the canopy (Mink and Luttrell 1989, Cook et al. 2004). Furthermore, crops such as corn and sorghum present difficulties controlling early infestations due to the ability of the FAW being hidden from any contact to insecticides down in the whorl of the plant. However, Young (1980) found that with conventional application methods there is not enough water provided to penetrate deep enough into the whorl of the , and is not economical to apply sufficient amounts of water to increase efficacy. There is also a relationship between larval size and LD_{50} values, as larval size increases the amount of insecticide needed to kill the larvae increases (Yu 1991). In addition to being difficult to manage from the practical

point of view, FAW has become resistant to several classes of insecticides. Classes include pyrethroids, carbamates, and organophosphates (Yu 1991).

Bacillus thuringiensis

Difficulties found when using insecticides, whether due to resistance issues or difficulty making contact with the insect, have resulted in heavy reliance on Bt technologies. *Bacillus thuingiensis* (Bt) is a soil bacterium that produces a crystalline structure that dissolves in the larval midgut, once dissolved it will release one or more insecticidal crystal proteins called enodotoxins (Hofte and Whiteley 1989). Most of these proteins are protoxins and are broken down and converted into smaller toxic polypeptides by the insects own protein-digesting enzymes. Thus, by its own digestive action, the insect exposes itself to the toxin (Nation 2016). Once the toxin has been broken down in size, it then binds to the plasma membrane receptor where the action of the toxins generate small pores in the membrane itself. With the creation of these pores, the result will lead to colloid-osmotic lysis (Knowles and Ellar 1987).

There are several Bt proteins utilized for the control of FAW; Cry1A.105, Cry1F, Cry2Ab, Cry2Ae, and Vip3A. During the early stages of development of Bt, there were multiple different Cry genes that controlled multiple insect species. However, throughout several years of repeated use in multiple crops, Bt resistance in FAW has begun to emerge. Field evolved resistance to Cry1F has been documented in areas of Puerto Rico, Brazil, and southeastern areas of the United States (Storer et al. 2010, Farias et al. 2014, Huang et al. 2014).

Failures with Cry1F technologies has placed increased selection pressure on Cry2 technologies. These technologies have been around and widely adopted for more than 20 years. However, Cry1F resistant FAW have been selected for resistance against the Cry2Ab protein in laboratory settings (Santos-Amaya et al. 2015). Fortunately, the newest Bt technology, Vip3A, provides a different mode of action relative to the Cry1 and Cry2 proteins.

Unlike the Cry proteins that are synthesized during the onset of sporulation and during the stationary growth phase as parasporal crystalline inclusions, Vip protein is produced during the vegetative stage of growth. Vip also shares no similar binding sites and no sequence homology with Cry proteins (Chakroun et al. 2016, Lee et al. 2003, Estruch et al. 1996). Currently there are 4 main Vip proteins that target different species of insects. Vip1 and Vip2 act as binary toxins for species within the families of Coleoptera and Hemiptera. Vip3 is more specialized in targeting Lepidopteran species. At this point, there are 15 Vip1 proteins known, 20 Vip2 proteins, 101 Vip3 proteins and 1 Vip4 protein (Chakroun et al. 2016).

Symptomology of insects after ingesting the Vip protein, resembles similar observations of insects ingesting Cry proteins. Feeding is abruptly stopped, loss of gut peristalsis, and overall paralysis of the insect (Chakroun et al. 2016). Examined sections of the gut after ingestions reveals major damage to the midgut, disrupted and swollen epithelial cells and leakage of cellular material in the lumen of the insect. Eventually complete death for susceptible insects.

Vip has been introduced successfully to both corn and cotton. The transformation even in cotton is the COT102 event which produces the Vip3Aa19 protein. In corn the transformation event is MIR162 and produces the Vip3Aa20 protein.

*Cotton (*Gossypium hirsutum*)*

Gossypium hirsutum also known as upland cotton, from the family Malvaceae, is an indeterminate plant that is planted as an annual crop. This specific species of cotton is one of the most widely planted species of cotton globally. Cotton can reach heights greater than 1m tall with alternating leaves that are almost round at the base and contain 3-5 lobes towards the tip. Developmental stages of cotton can be broken up into 5 main growth stages: germination and emergence, seedling establishment, leaf area and canopy development, flowering and boll development, and maturation. Cotton has many uses from clothing, plastics, and feed products. Annually cotton is the number one value-added crop in the U.S. (NCC 2018). Like many other agronomic crops, cotton is highly susceptible to yield loss through insect damage and has a wide range of insect pests. To avoid insect damage and increase yield while reducing chemical control methods cotton has been genetically improved to express multiple different Bt technologies.

*Corn (*Zea mays*)*

Corn is an annual grass from the family *Graminae* (*Poaceae*). This is a tall monecious grass with overlapping sheaths and broad blades. Stamen is produced at the top of the plant in long spikelete form and pistillate inflorescences are in the leaf axils.

The pistillate (ear) is enclosed in numerous large bracts and a mass of long styles protrude from the tip as a mass of silky threads (silks) (Hitcock et al. 1971). Approximately 90 million acres of corn are planted annually within the U.S. Corn is the primary feed grain and accounts for more than 95 percent of total feed grain production in the U.S. (USDA-ERS 2018). There are several other uses for corn such as human food products and bio-fuels. Corn is susceptible to insects during each stage of growth from establishment to maturation.

Domesticated some 10,000 years ago, corn is one of the oldest domesticated crops. Modern technology and breeding techniques have greatly increased our ability to manipulate corn genetics for several beneficial reasons, to increase yield, become more heat tolerant, drought resistant and ultimately insect resistant. To control insects corn has been bred and genetically modified to express *Bacillus thuringensis* (Bt) crystal proteins. Since the mid 1990's corn has been modified to express Bt toxins beginning with Cry1 proteins. Currently corn can be found expressing every major Bt protein Cry1Ab, Cry1A.105, Cry1F, Cry2Ab, and Vip3A.

CHAPTER II

DETERMINE THE CROSS-RESISTANCE PATTERNS OF VIP3A RESISTANT FALL ARMYWORMS *SPODOPTERA FRUGIPERDA* ON OTHER PURIFIED BT PROTEINS

Introduction

Fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (FAW) has become a major target pest to multiple transgenic crops expressing *Bacillus thuringiensis* (Bt) in multiple countries. Although previously restricted to the western Hemisphere in recent years FAW in western and central Africa and 15 provinces in China (Goergen et al. 2016, FAS 2019). The recent outbreak of this pest has caused 90,000 hectares of damage.

Control for FAW can be difficult due to the sporadic behavior of the pest. Cultural control methods can be futile due to the lack of the diapause mechanism. Chemical control can be effective when applied in a timely manner under optimal conditions. However, problems may arise with insecticides due to the difficulty of reaching the insect in some crops such as corn or sorghum where the FAW feed down in the whorl of the plant making control efforts very difficult. Other issues with insecticides include resistance. FAW have become resistant to several classes of insecticides including pyrethroids, organophosphates, and carbamates (Yu et. al 1991).

Genetically manipulated plants that express the entomopathogenic bacteria *Bacillus thuringiensis* (Bt) is the preferred method used for controlling FAW. Bt crops have been used in commercial agriculture for several decades, beginning with the Cry1

proteins introduced in the late 90's to Cry2 proteins, and then the latest Vip3A. The adoption and use of Bt crops has risen significantly since their first release in 1996. With such widespread use resistance issues have arose again. Documented cases of Cry1F resistance have already been reported (Storer et al. 2010, Farias et al. 2014, Huang et al. 2014).

Gene pyramiding is one of the major IRM strategies that is used to delay the evolution of resistance to Bt toxins (Zhao et al. 2003). However, the efficacy of these pyramided proteins can be greatly reduced by cross-resistance (Brevault et al. 2013). Numerous studies have shown cross-resistance frequently occurs among several closely related Bt proteins (Carriere et al. 2016, Tabashnik et al. 2009). Patterns of cross-resistance can vary among different Bt technologies and insects, therefore understanding this cross-resistance will provide information about the mechanisms of resistance and assist in the design of Bt crops.

Recently a Vip3A resistant strain of FAW was established using an F_2 screen of two-parent families collected from Louisiana, U.S. (Yang et al., 2017). This is of concern because resistance to such newly released technology could jeopardize the sustainable use of this technology for future. The goal of this objective is to determine if Vip3A resistance can cause cross-resistance to other Bt proteins.

Materials and Methods

Diet Bioassays

Repeater pipets were used to dispense 0.8 ml per well of liquid diet (Southland Product, Inc. Lake Village, AR) into 128-well bioassay trays (C-D International, Pitman, NJ). The diet was allowed to cool and solidify before Bt protein solution was overlaid onto the diet surface of each well. The protein solution was then allowed to air dry. Four proteins were tested, Cry1F (Corteva Agriscience), Cry2Ab2 (Bayer), Cry2Ae, and Vip3A. BASF Company (Research Triangle Park, NC) provided the Vip3A and Cry2Ae proteins. The concentrations for each protein ranged from 0.1 to 31.6µg/cm². Each protein solution was suspended in 0.1% Triton-X100 (Micro Essential Laboratory, Inc.) and overlaid onto the diet surface of each well and allowed to air dry. A constant volume of 40µl Bt protein solution was overlaid for Cry1F and Cry2Ae proteins while a volume of 200µl Bt protein solution was overlaid for Cry2Ab2.

One neonate (< 24hr) was placed on the surface of the diet of each well. Each genotype was replicated four times per Bt protein concentration, with 16 larvae in each replication. Once infested the trays were placed in a growth chamber maintained at 27 ± 1° C, 50% RH and 14:10 (L:D) photoperiod. Mortality and larval development was assessed after seven days. Larvae were considered dead if they were still in the first instar stage or not moving after gentle prodding.

Insect Sources

A Vip3A resistant strain of FAW (RR) has been established using an F_2 screen described by Yang et al. (2108) from larvae collected from Bollgard 2 cotton in Rapides Parish, Louisiana in 2016. A susceptible strain (SS) of FAW was established from larvae collected from non-Bt corn near Weslaco, Texas in 2013. SS has been documented to be susceptible to Cry1F, Cry1A.105, Cry2Ab2, Cry2Ae, and Vip3A proteins in artificial diet, as well as corn and cotton plants expressing these Bt proteins (Huang et al., 2014, Yang et al., 2016). In addition to the RR and SS, a heterozygous (RS) strain of FAW were produced from reciprocal crossings between the RR and SS.

Statistical Analysis

Larval mortality was calculated as a percentage (%) = [100* (number of dead larvae + number of larvae at the first instar stage / total number of insects assayed)]. Larval mortality was corrected at each concentration based on the control using the Abbotts method (Abbott 1925). Larval mortality was analyzed using a two-way analysis of variance (ANOVA) with insect genotype and protein concentration as the two main factors (SAS Institute 2010). To meet the normality assumptions the data was transformed using arcsine ($X^{0.5}$). Treatment means will then be separated using Tukey's HSD with $\alpha = 0.05$ (SAS Institute 2010). Probit analysis was used to determine the median lethal concentration (LC50) that caused 50% mortality and the corresponding 95% confidence limit (CL) (SAS Institute 2010). Resistance ratios were calculated using the LC_{50} of one (RR, RS) population divided by the LC_{50} of the SS population.

Results

SS was susceptible to Vip3Aa51 with a mortality greater than 80% at 0.316 μg/cm^2 concentration and reached 100% at the 1 μg/cm^2 concentration (Figure 2). The LC_{50} values for SS against the Vip3A protein was 0.08 with a 95% CL of 0.06-0.1 μg/cm^2 (Table 2). RS showed similar results with greater than 80% at 0.316 μg/cm^2 reached 100% at the 1 μg/cm^2 concentration. The estimated LC_{50} values for RS against Vip3A was 0.13 μg/cm^2 with a 95% CL of 0.10-0.20 μg/cm^2. Percent mortality ratings for RR was very low and was significantly lower at the 0.1-10 μg/cm^2 concentrations compared to the other two genotypes. Probit analysis showed that the LC_{50} value for the Vip3A resistant strain was estimated to be higher than the tested dose.

Compared to the Vip3A51 protein, Cry1F, Cry2Ab2, and Cry2Ae were more toxic to RR, and just as toxic to the other genotypes. In the Cry1F bioassay mortality for the RR genotype was 67% at the 0.316 μg/cm^2 concentration and neared 100% at the 1 μg/cm^2 concentration (Figure 1). The estimated LC_{50} for Cry1F on RR was 0.14 with a 95% CL of 0.10-0.18 μg/cm^2. RS and SS genotype followed very similar patterns in respect to percent mortality and LC_{50} values. The resistant ratio for the RR genotype on the Vip3Aa51 toxin was 39.5, and RS had a resistant ratio of -5.71 showing extreme susceptibly to the toxin.

Compared to Cry1F, Cry2Ab2 and Cry2Ae, were both effective against all three genotypes. Generally speaking, the dose response for the two proteins was similar. However, all three genotypes appeared to be more susceptible to Cry2Ae than to Cry2Ab2 (Figures 3 and 4). For example, RR had 56% mortality at the 1 μg/cm^2

concentration of Cry2Ab2 but showed a 92% mortality for the same concentration of Cry2Ae (Figure 4). Resistant ratios for RR and RS on all Cry proteins tested showed both genotypes to be highly susceptible. The measurements of these bioassays showed that the RR genotype was highly resistant to Vip3A, but does not show signs of cross-resistance to Cry1F, Cry2Ab2 or Cry2Ae.

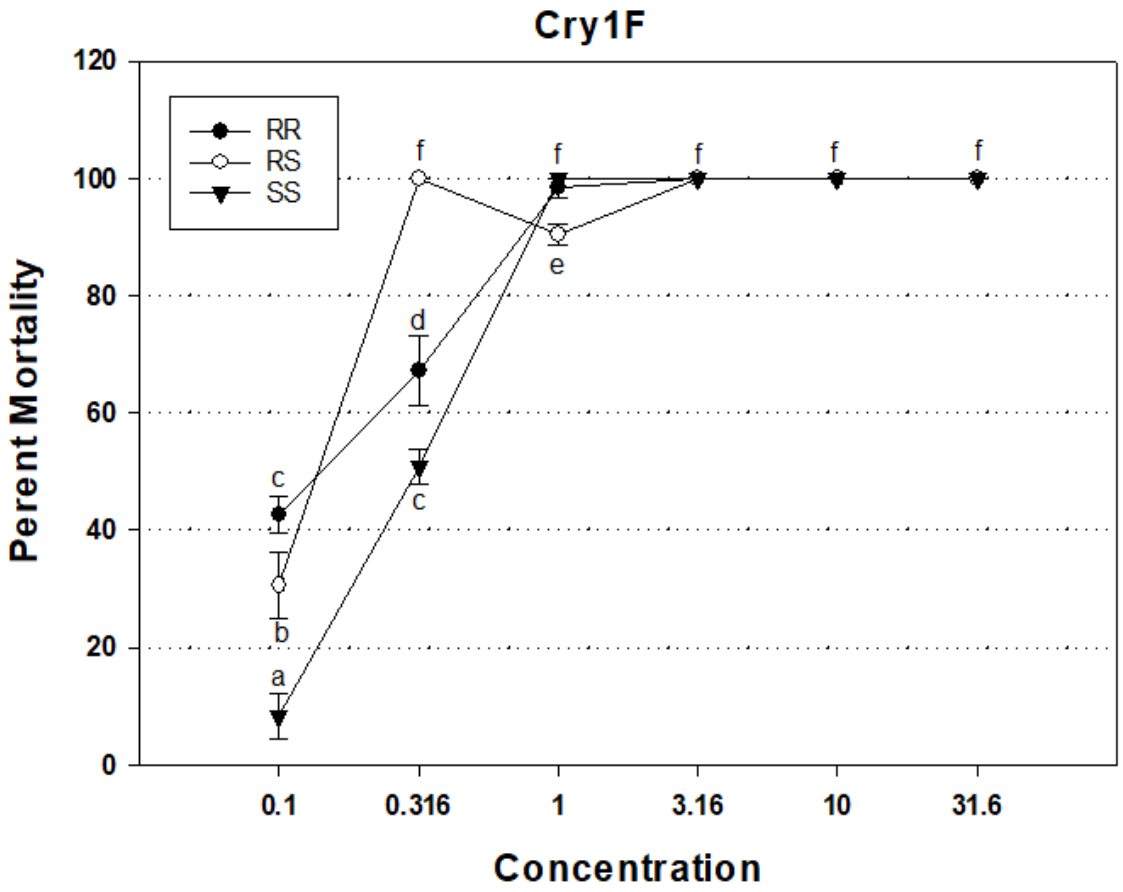

Figure 1. Concentration-larval mortality response to Cry1F. Mean values in figure followed by the same letter are not significantly different based on a two-way ANOVA (Tukey's HSD P > 0.05). RR = Resistant larvae, RS = Heterozygote larvae, SS= Susceptible larvae

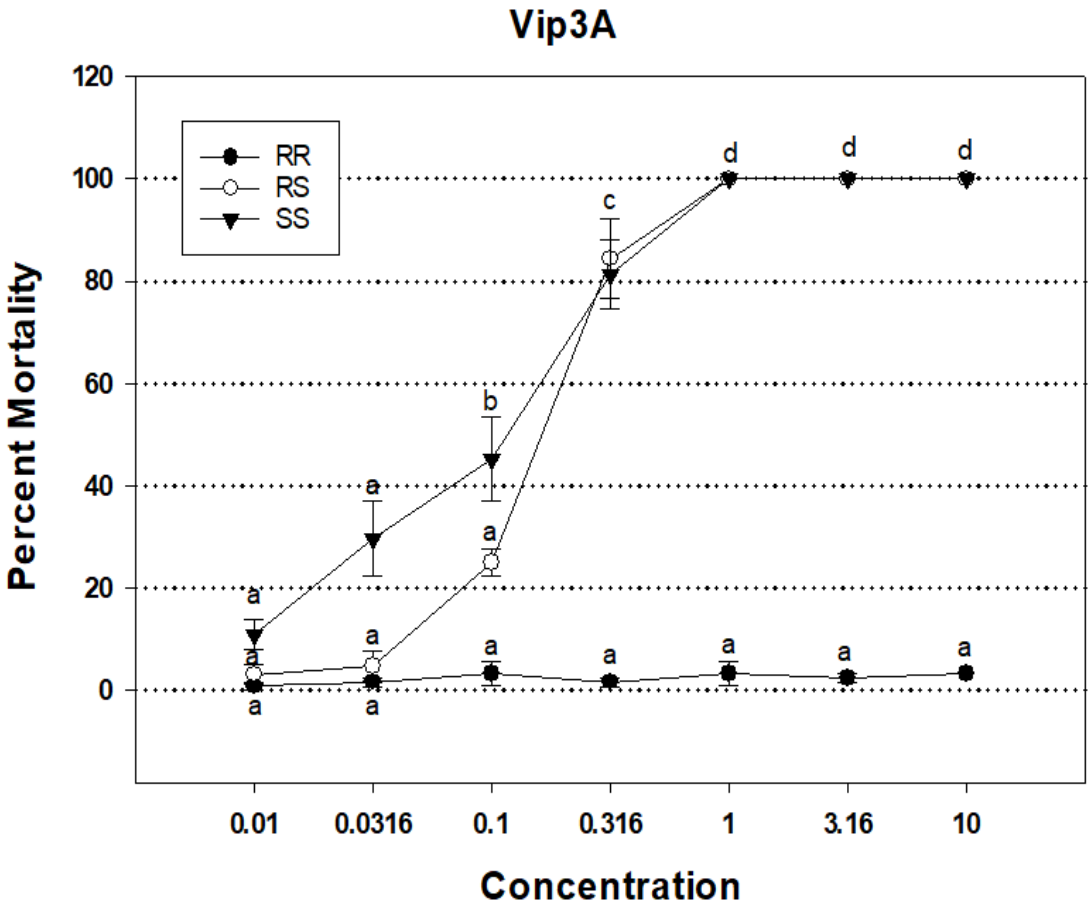

Figure 2. Concentration-larval mortality response to Vip3A. Mean values in figure followed by the same letter are not significantly different based on a two-way ANOVA (Tukey's HSD $P > 0.05$). RR = Resistant larvae, RS = Heterozygote larvae, SS= Susceptible larvae

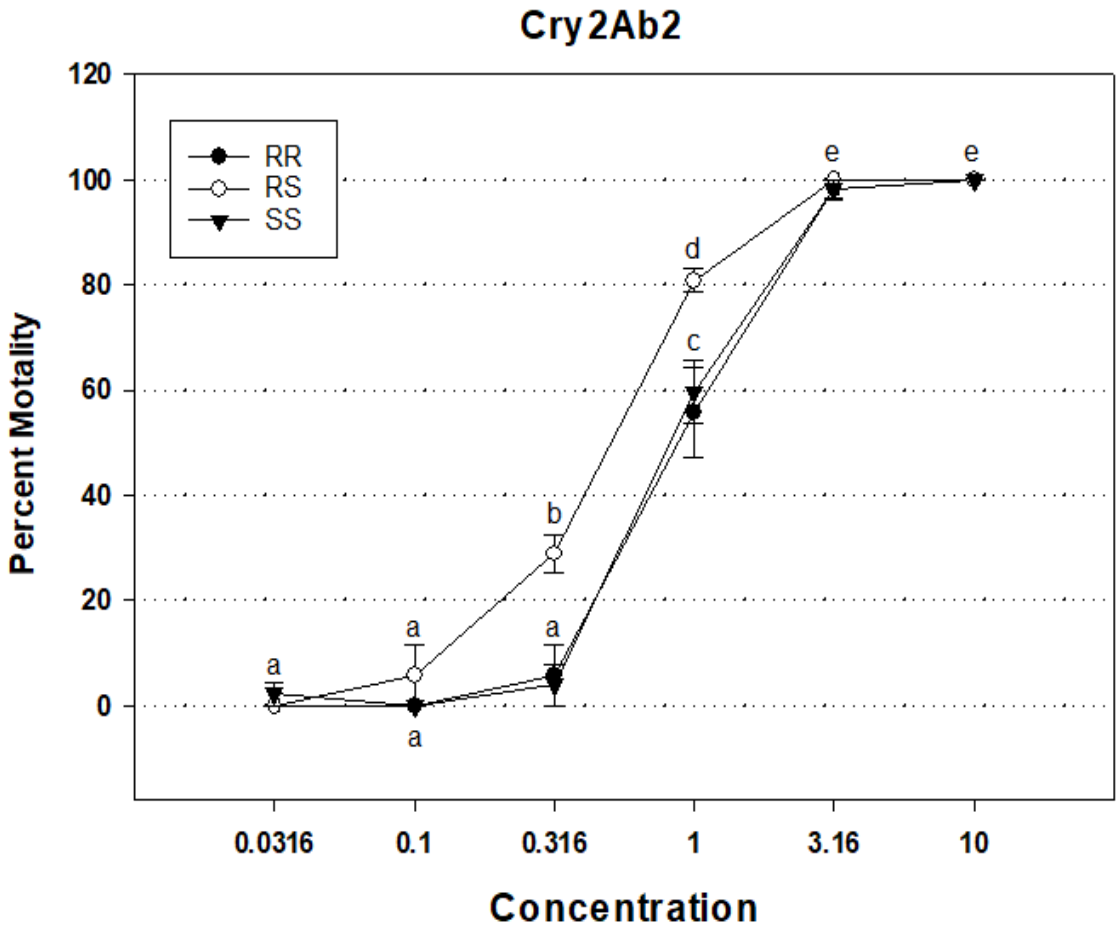

Figure 3. Concentration-larval mortality response to Cry2Ab2. Mean values in figure followed by the same letter are not significantly different based on a two-way ANOVA (Tukey's HSD P > 0.05). RR = Resistant larvae, RS = Heterozygote larvae, SS= Susceptible larvae

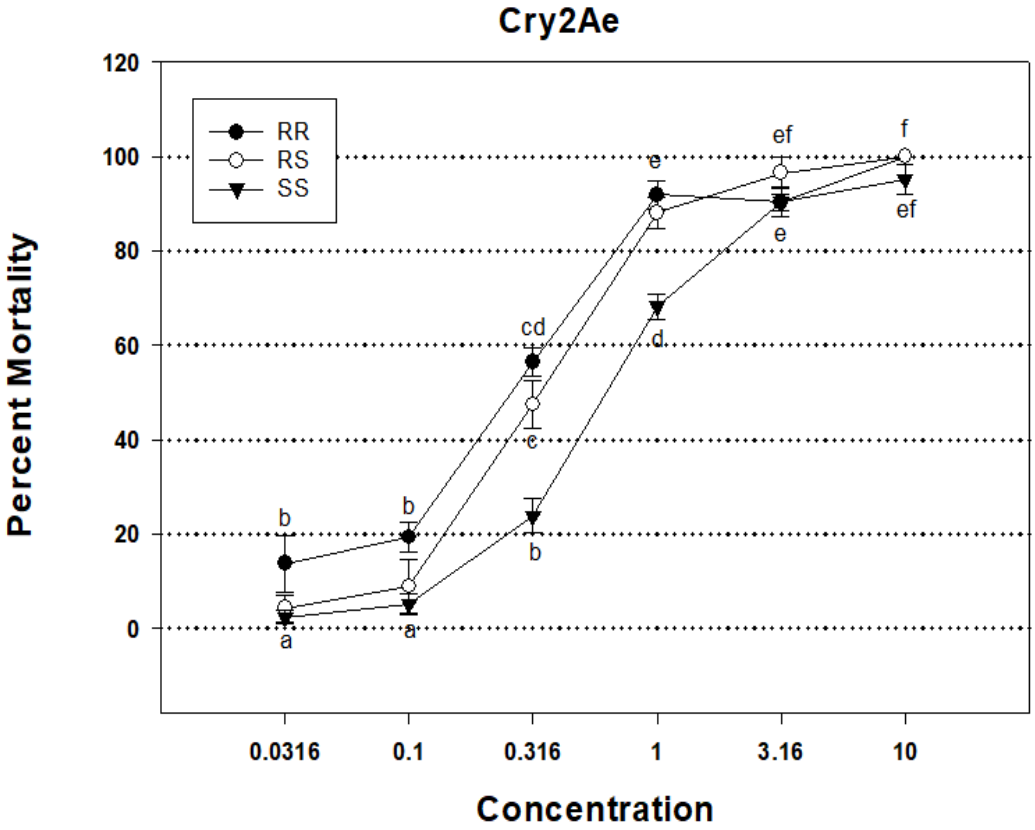

Figure 4. Concentration-larval mortality response to Cry2Ae. Mean values in figure followed by the same letter are not significantly different based on a two-way ANOVA (Tukey's HSD P > 0.05). RR = Resistant larvae, RS = Heterozygote larvae, SS= Susceptible larvae

Discussion

Insect mortality data have been the most widespread and commonly used method for measuring the toxicity of insecticides and Bt toxins. Studies conducted on insects resistant to Vip3A technology has been very limited with only six reports; including *Spodoptera frugiperda* in United States (Yang et al. 2017), *S. frugiperda* in Brazil (Bernardi et al. 2015, Bernardi et al. 2016), *S. litura* in India (Barkhade et al. 2010), *Helicoverpa armigera* and *Helicoverpa punctigera* in Australia (Chakroun et al. 2016, Mahon et al. 2012), and *Heliothis vierscens* in the United States (Pickett et al. 2017). This study examined the susceptibility of a Vip3A resistant strain of FAW and two other FAW genotypes (SS, RS) to four Bt proteins (Vip3A, Cry1F, Cry2Ab2, and Cry2Ae) which are expressed in both Bt corn and Bt cotton. The results showed the RR strain of FAW is highly susceptible to Cry2Ae, Cry2Ab2, and Cry1F. These findings will aid future studies for Vip3A resistance along with Bt resistance management.

Despite the susceptibility of the Vip3A resistant strain of FAW in the current study to Cry1F protein, field evaluations suggest there are major issues concerning the use of this protein. Several cases of field evolved resistance has been reported in several countries (Storer et al. 2010, Farias et al. 2014, Huang et al. 2014). If Cry1F resistance becomes prevalent, high selection pressure would be placed upon Cry2 and Vip3A. Because Cry1Ab is often pyramided with other proteins, it is important to note that Cry1Ab is ineffective for the control of FAW. In field scenarios, cotton and second generation corn products that contain the Cry1F protein may not, in terms of IRM

strategy, be considered as a pyramided technology. Fortunately, this study suggests that the Vip3A strain of FAW shows no cross-resistance to other proteins.

The lack of cross-resistance to Cry1F, Cry2Ae, and Cry2Ab2 was expected because Vip3A has no shared biding sites or sequencing homology with any of the Cry proteins (Chakroun et al. 2016, Kurtz 2010, Sena et al. 2009). There are multiple case studies that conducted experiments on multiple insect species , *Helicoverpa armigera*, *H. punctigera*, *H. virescens*, and *Spodoptera frugiperda*, that were documented as resistant to one or multiple Cry1 or Cry2 proteins but showed no cross-resistance to Vip3A (Mahon et al. 2016, Wei et al. 2017, Jackson et al. 2007, Huang et al. 2014, Yang et al. 2017). These data and the data found in this study, suggest that the lack of cross-resistance between Cry proteins and Vip3A is common amongst multiple insect species.

Table 2. Median lethal concentration (LC_{50}) of Vip3A resistant (RR) Vip3A susceptible (SS) and heterozygote (RS) genotypes of *Spodoptera frugiperda* to four purified Bt proteins in diet-overlay bioassays

Bt protein	Insect genotype	N[1]	LC_{50} (95% CL) (µg g^{-1})[2]	Slope ± SE	X^2	df	Resistance Ratio[3]
Cry1F	RR	448	0.14 (0.10, 0.18)	2.03 ± 0.28	11.84	22	0.5036
	RS		0.14 (0.09, 0.19)	2.29 ± 0.41	46.11	22	0.5036
	SS		0.278 (0.24, 0.33)	3.57 ± 0.42	10.45	22	1.00
Cry2Ab2	RR	448	0.89 (0.76, 1.05)	3.62 ± 0.41	20.48	22	1.37
	RS		0.46 (0.39, 0.56)	2.71 ± 0.27	15.70	22	0.7078
	SS		0.65 (0.33, 1.35)	2.01 ± 0.45	59.29	22	1.00
Cry2Ae	RR	448	0.24 (0.18, 0.31)	1.5 ± 0.13	26.62	22	0.3582
	RS		0.33 (0.25, 0.44)	2.06 ± 0.22	26.68	22	0.4925
	SS		0.67 (0.53, 0.85)	1.73 ± 0.14	20.58	22	1.00
Vip3Aa51	RR	448	>31.6	0.12 ± 0.13	13.25	26	39.5
	RS		0.13 (0.10, 0.2)	2.45 ±0.35	31.6	26	1.625
	SS		0.08 (0.06, 0.1)	1.6 ± 0.14	30.90	26	1.00

[1]Total number of insects assayed

[2]Median lethal concentration (LC_{50}) that caused 50% mortality and the corresponding 95% confidence limit

[3]Resistance ratios were calculated using the LC_{50} of RR or RS population divided by the LC_{50} of the SS population

CHAPTER III

DETERMINE CROSS-RESISTANCE OF VIP3A RESISTANT STRAIN OF FALL ARMYWORMS *SPODOPTERA FRUGIPERDA* IN CORN BT TECHNOLOGIES USING LEAF TISSUES AND WHOLE PLANT BIOASSAYS

Introduction

The fall armyworm, *Spodoptera frugiperda* (J.E. Smith; FAW) is one of the major target pests of Bt corn. FAW has been classified as a sporadic pest due to its migratory behavior (Hardke et al. 2015b). They do not enter diapause, so annual migration northward begins from warm climates zones such as southern Florida, Texas, Georgia, Alabama, Louisiana, and other southern coastal areas across the U.S. (Hardke et al. 2015b). FAW has a wide variety of host plants ranging from corn, sorghum, forage grasses, turf grasses, rice, cotton, peanuts, and has been reported on over 80 different species in 23 families (Pashley 1988; Hardke et al. 2015b). Invertebrate pest causes up to 15 percent of damage of agricultural production, costing the U.S. approximately $8 billion.

There are several control methods for FAW in corn. Cultural methods include host plant resistance such as antibiosis. Suppressing overwintering habitats could be useful, however, since the FAW does not possess a diapause mechanism suppression could be futile. Many labeled insecticides provide control for this species such as chlorantraniliprole, emamectin benzoate, methoxyfenozide, and several others. However, FAW have been demonstrated to develop resistance to several classes of

insecticides including pyrethroids, organophosphates, and carbamates (Hardke et al. 2015b).

Genetically engineered plants that express *Bacillus thuringiensis* (Bt) have become a major tool to control insect pest in corn, cotton and soybeans (James 2015). Global use of these genetically engineered plants has risen from 1.1 million hectares in 1996 to 98.5 million in 2016 (Tabashnik 2017). In 2016, 94 million acres of corn was planted in the U.S. and produced 14.3 billion bushels that profited over 51 billion U.S dollars (NASS 2017). Of the 94 million acres of corn planted 92 percent contained Bt (NASS 2017). With this extensive use of Bt crops, field resistance has occurred in several target species in several different countries (Yang et al. 2017). The evolution of resistance to Bt proteins in insects, is becoming the main threat to the suitable use of this technology (Yang et al. 2017).

With field resistance to many insecticides the use of Bt technology has been heavily relied upon. There are currently three different groups of Bt proteins that are utilized for the control of FAW which are categorized as Cry1, Cry2, and Vip3A (Yang et al. 2017). Field resistance to Cry1F has been reported in corn for multiple locations including Puerto Rico, Brazil, and southeastern areas of the U.S. (Storer et al. 2010, Farias et al. 2014, Huang et al. 2014). Cry2 proteins have been commercially used for a number of years and may face many challenges in future years because, documented cases of Cry1F resistant larvae have been selected for resistance to Cry2 proteins in laboratory settings (Santos-Amaya et al. 2015). Because of the risk associated with potential resistance to Cry2 proteins, preservation of Vip3A susceptibility is critical.

Trtikova et al. (2015) found that there are several factors that can go into the level of expression and concentration of Bt proteins within transgenic corn. It was concluded that not only is the Bt content controlled by the transgene expression but it could also be dependent on the corn hybrid. Added to that, under stressful conditions the concentration of the Bt protein being expressed is very difficult to predict. The biological interaction between insects and leaf tissues may also vary depending upon if the insects is placed on a leaf that has been excised or placed into the whorl of a plant.

Because of the variability of expression due to several factors, the objectives of this study was to determine if there is cross-resistance between our Vip3A resistant FAW to other Bt proteins occurs and if survivorship changes on whole corn plants is comparable to the bioassays. To determine if cross-resistance occurs, leaf tissue bioassays were used to ensure the larvae feed on leaves containing a particular Bt technology, because such data cannot be guaranteed using whole plants with respect of the mobility of the insect. Comparing the survivorship between whole plants to leaf bioassays will aid in confirming what may be found in the leaf bioassays and provide a better representation of possible scenarios in the field. This study also determined if other pyramided Bt proteins were capable of managing Vip3A resistant FAW.

Materials and Methods

Insect Sources

A Vip3A resistant strain of FAW (RR) has been established using an F_2 screen described by Yang et al. (2018) from larvae collected from Bollgard 2 cotton in Rapides Parish, Louisiana in 2016. A susceptible strain (SS) of FAW was established from larvae collected from non-Bt corn near Weslaco, Texas in 2013. SS has been documented to be susceptible to Cry1F, Cry1A.105, Cry2Ab2, Cry2Ae, and Vip3A proteins in artificial diet, as well as corn and cotton plants expressing these Bt proteins (Huang et al. 2014, Yang et al. 2016). In addition to the RR and SS, a heterozygous (RS) strain of FAW were produced from reciprocal crossings between the RR and SS.

Leaf Bioassay

Corn was grown in a greenhouse located at the USDA Southern Plains Agricultural Research Center: College Station, Texas. Seeds were planted in 18.9-liter plastic pots filled with standard potting mixture. Maintaining 2-3 plants per pot with regular irrigation and fertilization as described by Niu et al. (2014) and Yang et al. (2016). The corn hybrids used were DKC 62-08 (SmartStax) (Bayer CropScience), DKC 67-72 (VT Double Pro) (Bayer CropScience), M78S-3111 (Agrisure Viptera 3111) (Syngenta), 1319 HR (Herculex) (Bayer CropScience), 1319VYHR (Leptra) (Bayer CropScience), DKC 62-95 (nont-Bt) (Bayer CropScience), N78N-GT (non-Bt) (Syngenta), 1319 (non-Bt) (Bayer CropScience). Table 3 shows the hybrids used and the Bt proteins that express within each.

Table 3. Corn seed selection

Corn Hybrids								
Technology	Herculex	SmartStax	VT Double Pro	Agrisure Viptera 3111	Leptra	Non-Bt	Non-Bt	Non-Bt
Hybrid	1319 HR	DKC 62-08	DKC 67-72	M78S-3111	1319VYHR	1319[a]	N78N-GT[b]	DKC 62-95[c]
Bt Technology	Cry1F	Cry1F, Cry2Ab2, Cry1A.105	Cry1A.105, Cry2Ab2	Cry1Ab, Vip3A	Cry1Ab, Cry1F, Vip3A	Non-Bt	Non-Bt	Non-Bt

[a]Isoline of 1319 HR/VYHR
[b]Isoline of Agrisure Viptera 3111
[c]Isoline of DKC 62-08/67-72

When the plants reached the V5-V7 growth stages leaves were excised, and brought to lab for assay preparations. Leaves were washed and cut into roughly 76.2×7.62 mm squares and placed into a sterile petri dish (100×15 mm), lined with moistened Whatman 90 mm (#1) filter paper. 5 neonates (<24hr old) were placed on the leaf surface of each petri dish and enclosed with a lid. The dishes were then be placed into a growth chamber at 27 ± 1° C, 50% RH and a 14:10 (L: D) photoperiod. Leaves were changed every 1-2 days, while filter paper was re-moistened daily and changed when needed. Mortality and larval development was then assessed 7 days after the infestation. Larvae were considered dead if there was no movement after gently prodding. Developmental data included, weight of surviving insects, and instar classification. The experimental design was a randomized complete block design using 4 replications by genotype and hybrid.

Whole Plant Corn Bioassay

Corn hybrids mentioned above were planted and grown at the USDA Southern Plains Agricultural Research Center: College Station, Texas. Two seeds were planted in 18.9-liter plastic pots filled with standard potting soil as described by Niu et al. (2014). 5 neonates (<24 hr old) were placed down into the whorl of the plant at V3-V4 plant stages. Plants were maintained with regular water and fertilization. Only the RR and SS genotypes were used. Each combination of genotype and corn hybrid was replicated 4 times with 2 pots (3-4 plants) per replication in a randomized complete block design. The Davis scale of 1 (no damage or few pinholes) to 9 (most leaves with long lesions)

was used to evaluate leaf injury on the 10^{th} day after infestation (Davis 1992). Larval development and percentage of plants containing live larvae was recorded immediately after damage ratings. Larvae were considered dead if there was no movement after gently prodding. Developmental data included, weight of surviving insects, and instar classification.

Statistical Analysis

Leaf injury was transformed using the log(x+1) scale, while percentage of plants containing live larvae was transformed using arcsine of ($x^{0.5}$) to normalize treatment variances. . Larval mortality was corrected for each hybrid based on the non-Bt using the Abbotts method (Abbott 1925). Data was then analyzed using two-way analysis of variance (ANOVA) with insect strain and corn hybrid as the two main factors (SAS Institute 2010). Treatments were separated using Tukey's HSD at $\alpha = 0.5$ level (SAS Institute 2010).

Results

Corn Leaf Bioassay

No statistical differences were found between any of the non-Bt hybrids therefore the data was pooled together. The leaf bioassays showed all three genotypes had high survival on non-Bt corn with around 70 percent survivorship (Table 4). RR larvae survived well on Viptera 3111, which contains Cry1Ab and Vip3A, with 72 percent survivorship and showed no statistical difference compared to non-Bt. Moderate survivorship occurred on Herculex (Cry1F) corn with the RR genotype. This suggests

that there may be some moderate resistance to the Cry1F protein. RS and SS had some survivors on Herculex as well, although not statistically different from other hybrids containing Cry1F, Cry1A.105 or Cry2Ab2 proteins. Pyramided proteins containing Cry1F, Cry1A.105 or Cry2Ab2 proteins negated the resistance mechanism of RR which suggests that these technologies are capable of managing the Vip3A resistant strain of fall armyworms. Larval development on non-Bt was normal with larvae reaching on average the 4th instar (Figure 5). RR larvae averaged almost 3rd instar on Agrisure Viptera 3111 and was statistically different from non-Bt, which suggest that there is incomplete resistance. Larval weights mirrored average instars with high weights in all three genotypes on non-Bt and very low weights on all other hybrids (Figure 6).

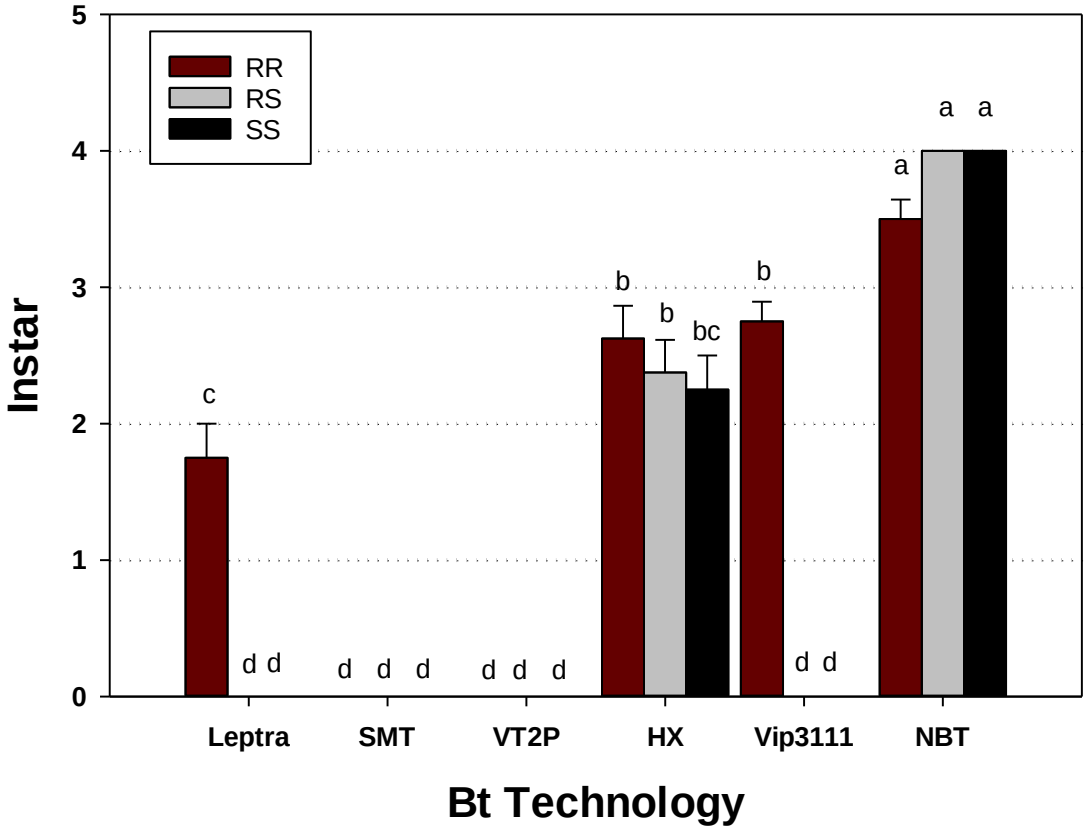

Figure 5. Average instar for surviving larvae on corn leaves. Mean values in figure followed by the same letter are not significantly different based on a two-way ANOVA (Tukey's HSD P > 0.05) RR = Resistant larvae, RS = Heterozygote larvae, SS= Susceptible larvae. Agrisure Viptera 3111- Cry1Ab, Vip3A, Herculex – Cry1F, Leptra – Cry1Ab, Cry1F, Vip3A, SmartStax – Cry1F, Cry2Ab2, Cry1A.105, VT Double Pro – Cry1A.105, Cry2Ab2

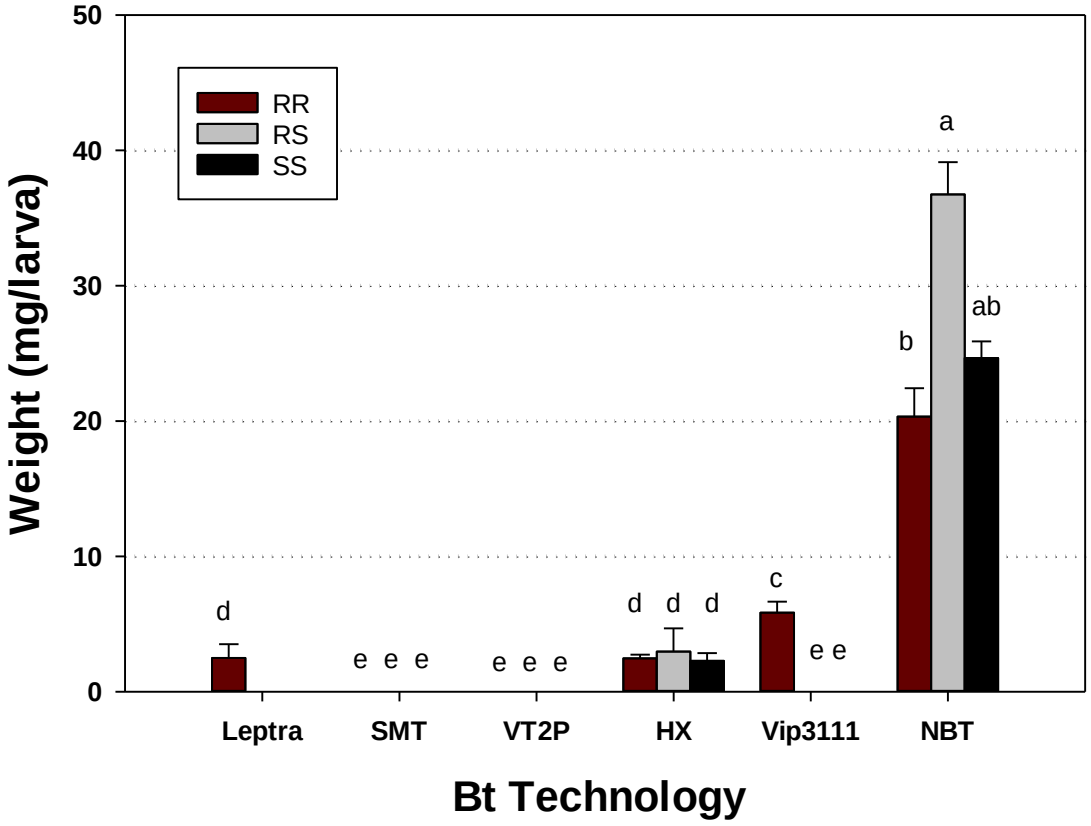

Figure 6. Average weights for surviving larvae on corn leaves. Mean values in figure followed by the same letter are not significantly different based on a two-way ANOVA (Tukey's HSD P > 0.05) RR = Resistant larvae, RS = Heterozygote larvae, SS= Susceptible larvae. Agrisure Viptera 3111- Cry1Ab, Vip3A, Herculex – Cry1F, Leptra – Cry1Ab, Cry1F, Vip3A, SmartStax – Cry1F, Cry2Ab2, Cry1A.105, VT Double Pro – Cry1A.105, Cry2Ab2

Table 4. Percent survivorship of different genotypes of *Spodoptera frugiperda* on corn leaves.

Insect Genotype[1]	Survivorship (%)					
	Non-Bt	Agrisure Viptera 3111	Herculex	Leptra	SmartStax	VT Double Pro
RR	75.42 ± 3.66a	71.25 ± 5.54a	33.75 ± 8.00b	8.75 ± 2.39bc	0.00 ± 0.00c	0.00 ± 0.00c
RS	77.08 ± 4.06a	0.00 ± 0.00c	12.50 ± 3.23bc	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00c
SS	70.00 ± 5.81a	0.00 ± 0.00c	12.50 ± 9.46bc	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00c

Means in a column or row followed by the same letter are not significantly different based on a two-way ANOVA (Tukey's HSD $P > 0.05$)
[1]RR = Resistant larvae, RS = Heterozygote larvae, SS= Susceptible larvae. Agrisure Viptera 3111- Cry1Ab, Vip3A, Herculex – Cry1F, Leptra – Cry1Ab, Cry1F, Vip3A, SmartStax – Cry1F, Cry2Ab2, Cry1A.105, VT Double Pro – Cry1A.105, Cry2Ab2

Whole Plant Corn Bioassay

Non-Bt corn had 100% of the plants containing live larvae of both RR and SS genotypes after 10 days (Figure 7). Vip3111, which contains Cry1Ab and Vip3A proteins, had approximately 90% of the plants containing live RR larvae and no plants containing any SS live larvae. Herculex, which contains the Cry1F protein, had approximately 50% of the plants containing live RR larvae. Leptra, which contains Cry1Ab, Cry1F, and Vip3A had 0% of the plants containing any live RR or SS larvae. No genotypes survived on any of the other Bt technologies. Damage ratings showed similar results as the percentage of plants containing live larvae with damage scores around 7 in the non-Bt (Figure 8) for both RR and SS genotypes. Vip3111 was statistically different from the non-Bt damage ratings for RR genotype. Herculex, which had 50% of the plants contain live larvae of RR, had a damage rating of 2. While 50% of the plants contained live larvae, if the parameters were to exclude 1st and 2nd instar larvae the percentage of plants with live larvae would decrease. All other Bt technologies had very low damage ratings.

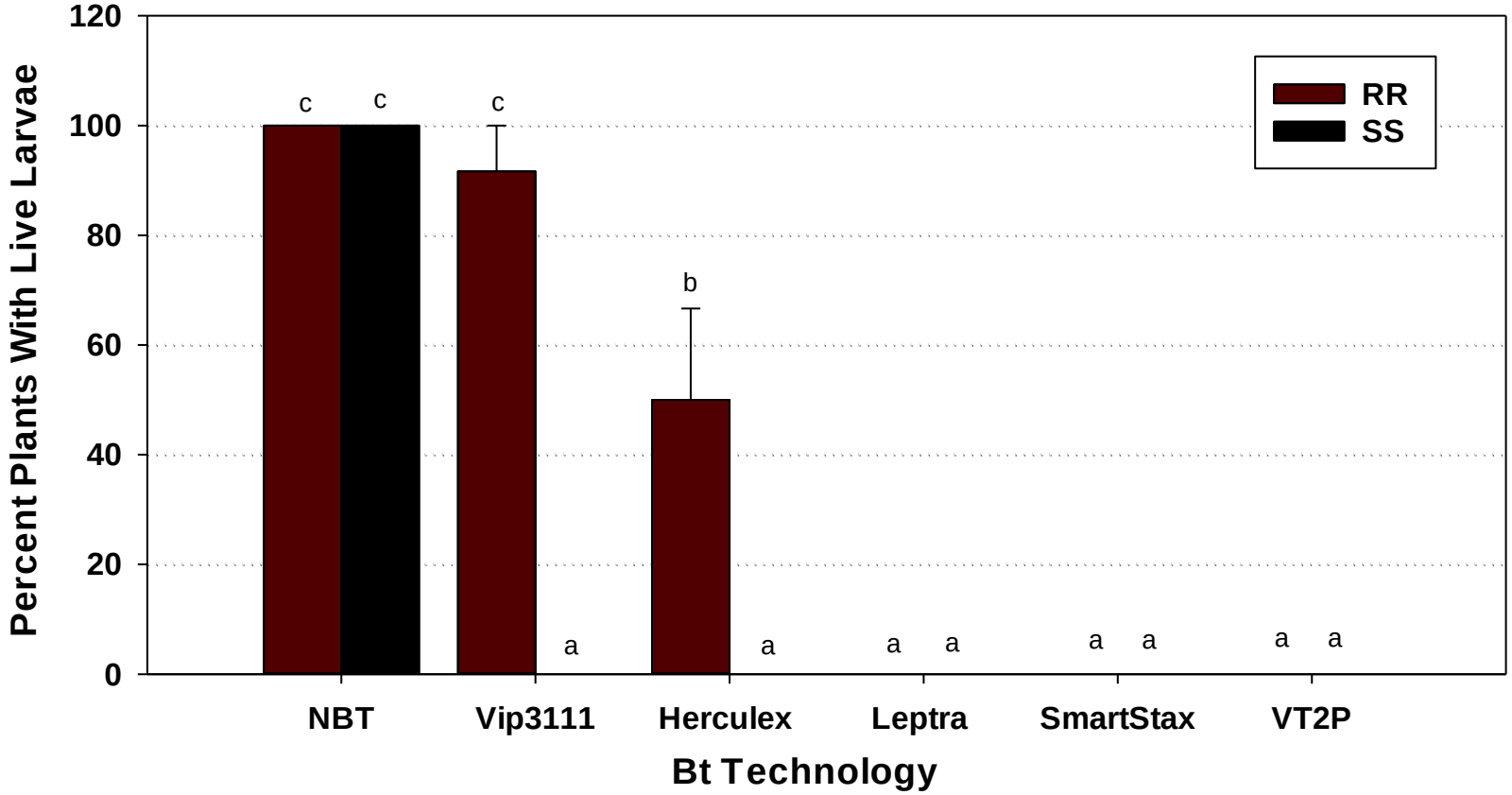

Figure 7. Percentage of plants with live larvae after 10 days. Mean values in figured followed by the same letter are not significantly different based on a two-way ANOVA (Tukey's HSD P> 0.05) RR = Resistant larvae, SS = Susceptible larvae. Agrisure Viptera 3111- Cry1Ab, Vip3A, Herculex – Cry1F, Leptra – Cry1Ab, Cry1F, Vip3A, SmartStax – Cry1F, Cry2Ab2, Cry1A.105, VT Double Pro – Cry1A.105, Cry2Ab2

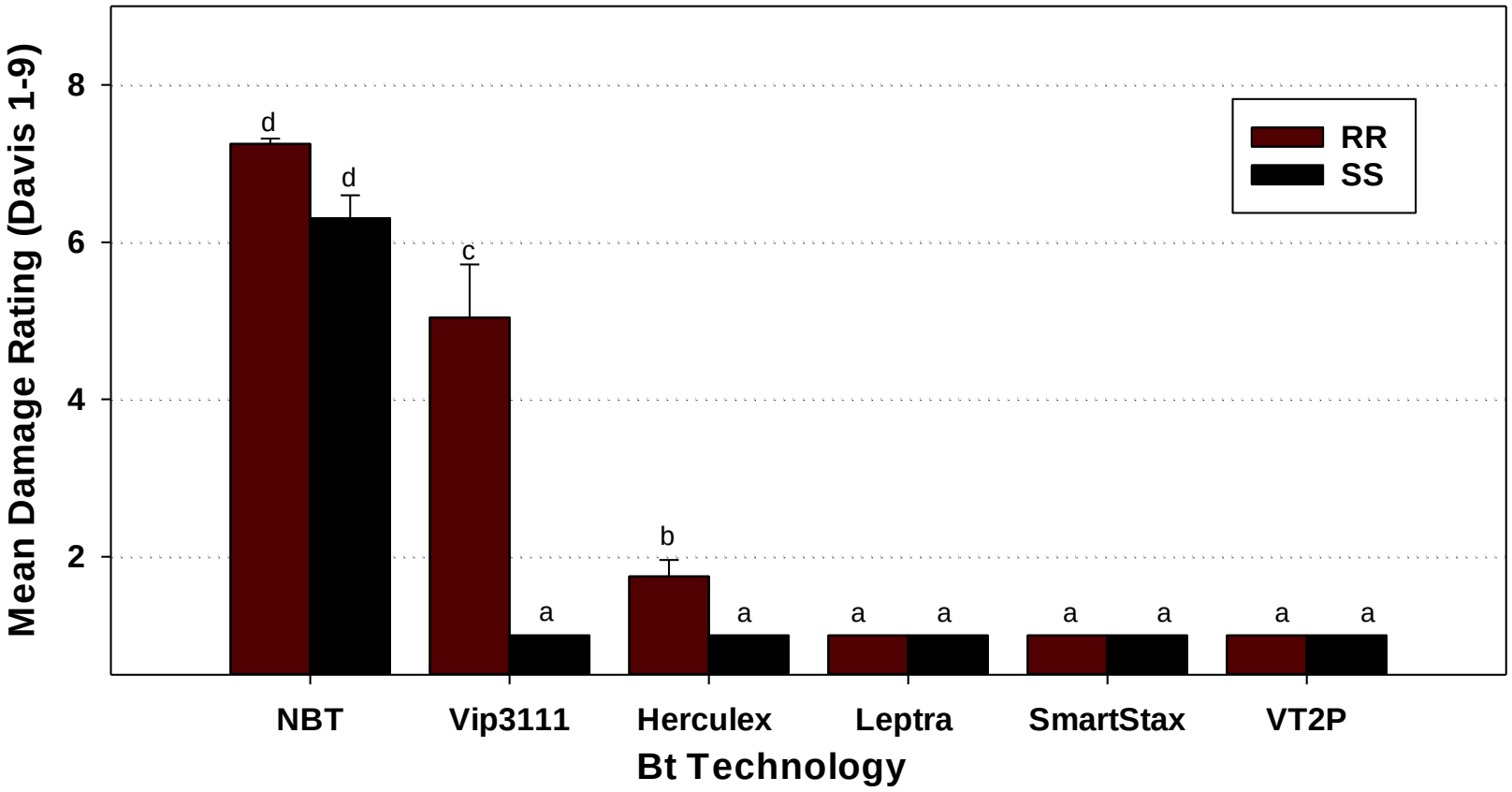

Figure 8. Mean damage ratings after 10 days. Mean values in figured followed by the same letter are not significantly different based on a two-way ANOVA (Tukey's HSD P> 0.05) RR = Resistant larvae, SS = Susceptible larvae. Agrisure Viptera 3111- Cry1Ab, Vip3A, Herculex – Cry1F, Leptra – Cry1Ab, Cry1F, Vip3A, SmartStax – Cry1F, Cry2Ab2, Cry1A.105, VT Double Pro – Cry1A.105, Cry2Ab2

Discussion

Vip3A protein is crucial to the sustainability of Bt technologies and has been incorporated into almost all third generation Bt corn products for the control of several insect species. The results of this study showed pyramided corn products that contain Cry1 and Cry2 proteins kept survivorship to a minimum. Herculex corn had roughly 34% survivorship in the leaf bioassays and 50% of plants in the whole plant bioassay contained live larvae, which showed that the RR population may have some moderate resistance to Cry1F. This is documented by several cases of field-evolved resistance to Cry1F (Huang et al. 2014, Farias et al. 2014).

With moderate resistance to Cry1F, and understanding that Cry1Ab is ineffective against FAW, it is understandable to see roughly 10 percent survivorship of RR genotype during the leaf bioassay on Leptra corn (Cry1Ab, Cry1F, and Vip3A). However, when placed on whole plant material, none of the Leptra plants contained live RR larvae. This data would agree with Trtikova et al. (2015) that there are several factors to be considered when examining expression levels of Bt genes. Damage ratings during the whole plant bioassay were statistically lower on the Vip3111 than the non-Bt for RR larvae, which suggests that the resistance is incomplete. Yang et al. (2018) conducted an experiment on the Vip3A RR population and found there to be no maternal or sex linkage involved with this population. The leaf bioassay conducted in the current study found similar results because of the low survivorship of the RS larvae on Vip3111 leaves. Data from both studies suggests that pyramided corn products containing Cry1 or Cry2 proteins are still capable of managing Vip3A resistant fall armyworms.

CHAPTER IV

DETERMINE CROSS-CROP RESISTANCE OF VIP3A RESISTANT STRAIN OF FALL ARMYWORMS *SPODOPTERA FRUGIPERDA* TO COTTON BT TECHNOLOGIES USING PLANT TISSUE BIOASSAYS

Introduction

Currently, the fall armyworm *Spodoptera frugiperda* (J.E. Smith) (FAW) is one of the major target pests of Bt cotton. FAW has been classified as a sporadic pest due to its migratory behavior, which begins from warmer climates in the southern U.S. moving northward (Hardke et al. 2015b). FAW is a highly polyphagous insect and can colonize over 80 host species including corn and cotton (Pashley et al. 1986). This pest can cause up to 15% of damage of agricultural production, costing the U.S. approximately 8 billion dollars, 17.7 billion U.S. dollars in Brazil, and 359.8 million U.S. dollars in Australia (Zhou et al.2017).

Controlling FAW can become difficult due to the behavior of this pest. Cultural practices such as suppressing overwintering habitats may be futile due to the lack of a diapause mechanism. There are a number of insecticides labeled for the control of FAW, however, with the heavy reliance and usage, resistance in FAW has been developed against many insecticides (Hardke et al. 2015b). With difficulty controlling FAW using cultural and insecticidal approaches, heavy reliance has been shifted towards the use of Bt technology.

Transgenic crops that express the entomopathogenic bacteria *Bacillus thuringiensis* (Bt), have been used in production agriculture since 1996 (James 2016). Many benefits are associated with the use of this technology such as, reduced use of chemical insecticides, reduced pest populations, and increased yields and profits for growers (Hutchison et al 2010). Global use of this technology has risen from 1.1 million hectare to 98.5 million hectares in roughly one decade (Tabashnik 2017). Upland cotton accounted for 5.38 million hectares, of which 94 percent was planted to a biotech variety (NASS 2018). Originally, the Cry1F and Cry2 proteins showed great efficacy for control of FAW. However, with the rapid adoption and extensive use, field evolved resistance to the Cry1F protein are beginning to surface (Storer et al. 2010, Farias et al. 2014, Huang et al. 2014). Field evolved resistance to Bt technologies is the main threat to the sustainability and future use of this technology (Yang et al. 2017). However, the newest technology, Vip3A is of different mode of actions relative to Cry proteins. It not only has efficacy against FAW but also shows great control of the cotton bollworm *Helicoverpa zea* (Burkness et al. 2010).

Available data suggests that the genetic elements involved in the making of Bt crops can be different from crop to crop. For example, the Vip3A gene in cotton is the result of the transformation event COT102, which produces the Vip3Aa19 protein, while in corn the transformation event is MIR162 produces the Vip3Aa20 protein. Because different Vip3A proteins are being utilized between crops, resistance to one protein may not confer resistance to the other protein. In addition, Bt expression in cotton can vary among varieties, plant age, plant parts, and type of genes and gene insertion sites (Dong

Li 2007, Carriere et al. 2019). Among cotton tissues, in one study the leaves were found to have the highest levels of Cry1Ac expression followed by squares, bolls, and then flowers (Kranthi et al. 2005).

The objective of this study was to determine the cross-crop resistance of FAW selected with Bt corn to Bt cotton using leaf bioassays. Since expression levels may differ in different structures of the plant, compounded with the biological interactions of FAW on different structures, cotton squares will be assayed to provide information of the performance of between leaves and squares.

Material and Methods

Insect Sources

A Vip3A resistant strain of FAW (RR) has been established using an F_2 screen described by Yang et al. (2108) from larvae collected from Bollgard 2 cotton in Rapides Parish, Louisiana in 2016. A susceptible strain (SS) of FAW was established from larvae collected from non-Bt corn near Weslaco, Texas in 2013. SS has been documented to be susceptible to Cry1F, Cry1A.105, Cry2Ab2, Cry2Ae, and Vip3A proteins in artificial diet, as well as corn and cotton plants expressing these Bt proteins (Huang et al., 2014; Yang et al., 2016, 2017b). In addition to the RR and SS, a heterozygous (RS) strain of FAW will be produced from reciprocal crossings between the RR and SS.

Cotton Leaf Bioassays

Cotton varieties PHY 312RF (WideStrike) (Corteva Agriscience), PHY 490 WRF3 (WideStrike3) (Corteva Agriscience), DP 1522 B2XF (Bollgard II) (Bayer CropSceince), DP 16R338B3XF (Bollgard 3) (Bayer CropSceince), ST 4949 TL (TwinLink) (BASF), FM 1953GLTP (TwinLink Plus) (BASF), DP1441RF (non-Bt) (Bayer CropSceince) were planted in a greenhouse located at the USDA Southern Plains Agricultural Research Center: College Station, Texas. Table 5 shows the varieties used and the Bt proteins that make up each. Once cotton reached 7-8 nodes, fully expanded leaves were excised and brought to the lab for assay preparations. Leaves were washed and cut into roughly 76.2×76.2 mm squares and placed into a sterile petri dish (100×15 mm), lined with moistened Whatman 90 mm (#1) filter paper. Five neonates (<24hr old) were placed on the leaf surface of each petri dish and sealed with a lid. The dishes were then be placed into a growth chamber at 27 ± 1° C, 50% RH and a 14:10 (L: D) photoperiod. Leaves were changed every 1-2 days, while filter paper was re-moistened daily and changed when needed. Mortality and larval development was then assessed 7 days after infestation. Larvae were considered dead if there was no movement after gently prodding. Developmental data included, weight of surviving insects, and instar classification. The experimental design was a randomized complete block design using 4 replications by genotype and variety.

Table 5. Cotton seed selection

Cotton Varieties							
Technology	WideStrike	WideStrike 3	Bollgard II	Bollgard III	TwinLink	TwinkLink Plus	Non-Bt
Variety	PHY 312RF	PHY 490WRF3	DP 1522B2XF	DP 16R338B3XF	ST 4949TL	FM 1953GLTP	DP 1441RF
Bt Technology	Cry1F, Cry1Ac	Cry1F, Cry1Ac, Vip3A	Cry1Ac, Cry2Ab2	Cry1Ac, Cry2Ab2, Vip3A	Cry1Ab, Cry2Ae	Cry1Ab, Cry2Ae, Vip3A	Non-Bt

Cotton Squares Bioassay

Seeds from 5 different cotton varieties were planted in the field at the USDA Southern Plains Agricultural Research Center: College Station, Texas. All varieties used during the leaf bioassay were used for the square bioassay except for TwinLink and TwinLink Plus. When match head to medium size squares were present they were then excised and brought to the laboratory. One square was placed directly into 30 mL Dart clear portion containers (Dart Container Corporation, Mason MI). Two early 2nd instar larvae were then placed on the square. There were four replications for each combination of genotype and cotton variety. Within each replication, there was approximately 15 squares. Squares were replaced every 1-2 days. Once infested the containers were placed in a growth chamber maintained at 27 ± 1° C, 50% RH and a 14:10 (L: D) photoperiod. Larval survival, growth, and development was recorded 7 days after infestation. Larvae were considered dead if there was no movement after gently prodding. Developmental data included weight of surviving insects, and instar classification.

Statistical Analysis

Data on insect survival was transformed using an arcsine square-root transformation, while data on larval instar and weight was transformed using a log, ln (x + 1) transformation for normal distributions. Larval mortality was corrected for each variety based on the non-Bt using the Abbotts method (Abbott 1925). Transformed data was then analyzed using two-way analysis of variance (ANOVA) with insect strain and varieties as the two main factors (SAS Institute 2010). Survivorship was calculated as a

percent =100* (number of surviving larvae / number of total larvae assayed). Treatments were then be separated using Tukey's HSD at $\alpha = 0.5$ level (SAS Institute 2010).

Results

Cotton Leaf Bioassay

All genotypes had high survival on non-Bt cotton, with 80 percent and higher survivorship (Table 6). All three genotypes had low survivorship on varieties containing Cry1F, Cry2Ab2 and Cry2Ae toxins. All the varieties, other than non-Bt, had low survivorship in all three genotypes with 20 percent survivorship and less. Any of the surviving larvae from the pyramided technologies, showed hindered development. All three genotypes developed well on non-Bt averaging over 3rd instar (Figure 9). All varieties with surviving larvae averaged 2nd instar, and weighed less than 2 mg (Figure 10).

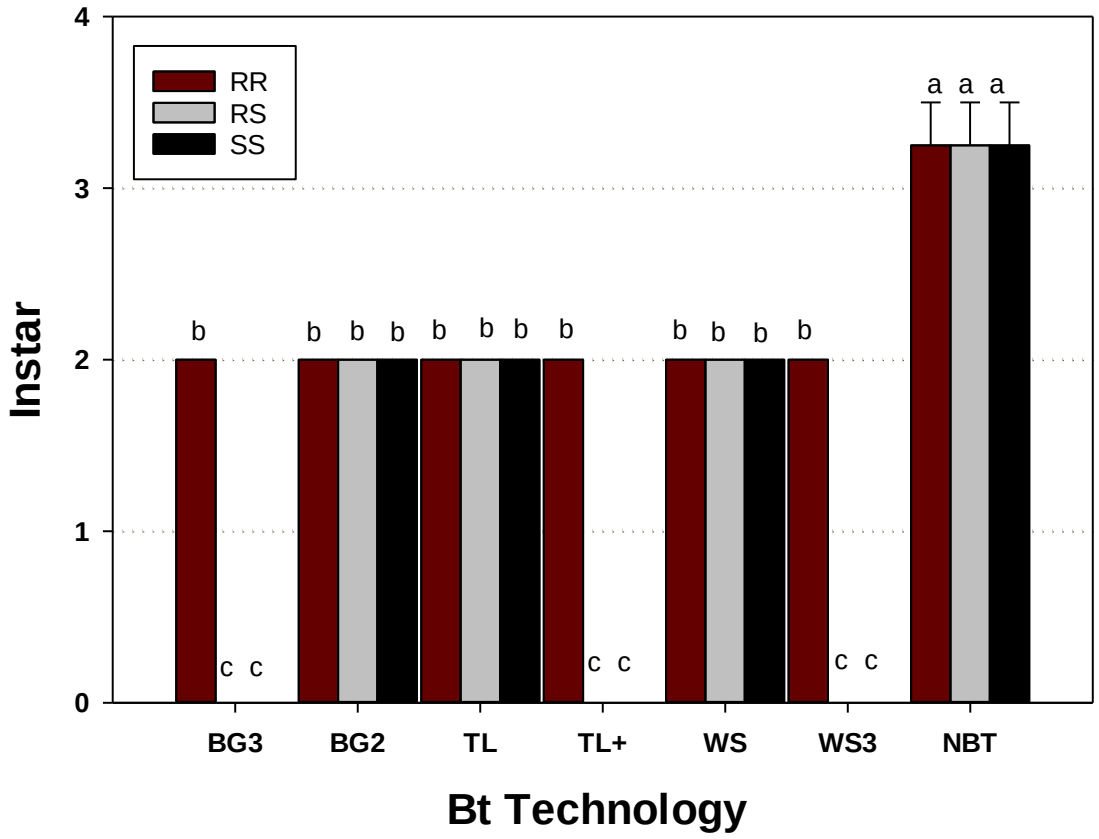

Figure 9. Average instar for surviving larvae on cotton leaves after 7 days. Mean values in figure followed by the same letter are not significantly different based on a two-way ANOVA (Tukey's HSD P > 0.05) RR = Resistant larvae, RS = Heterozygote larvae, SS= Susceptible larvae
(WS3) Widestrike 3-Cry1F, Cry1Ac, Vip3A (WS) Widestrike- Cry1F, Cry1Ac (TL+) Twinlik Plus-Cry1Ab, Cry2Ae, Vip3A (TL) Twinlink- Cry1Ab, Cry2Ae (BG3) Bollgard III- Cry1Ac, Cry2Ab2, Vip3A (BG2)Bollgard II- Cry1Ac, Cry2Ab2

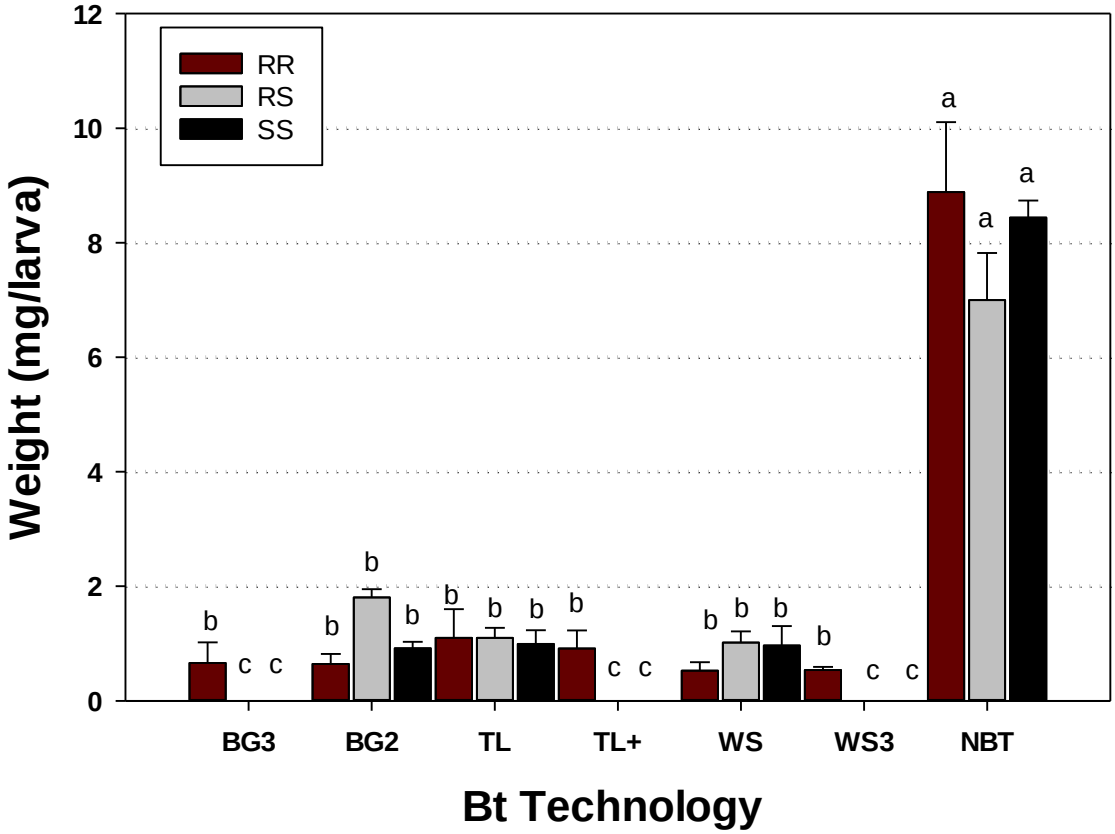

Figure 10. Average weights for surviving larvae on cotton leaves after 7 days. Mean values in figure followed by the same letter are not significantly different based on a two-way ANOVA (Tukey's HSD P > 0.05) RR = Resistant larvae, RS = Heterozygote larvae, SS= S Susceptible larvae
(WS3) Widestrike 3-Cry1F, Cry1Ac, Vip3A (WS) Widestrike- Cry1F, Cry1Ac (TL+) Twinlik Plus-Cry1Ab, Cry2Ae, Vip3A (TL) Twinlink- Cry1Ab, Cry2Ae (BG3) Bollgard III- Cry1Ac, Cry2Ab2, Vip3A (BG2)Bollgard II- Cry1Ac, Cry2Ab2

Table 6. Survivorship of different genotypes of *Spodoptera frugiperda* on cotton leaves after 7 days.

Insect Genotype[1]	Survivorship (%)						
	Non-Bt	WideStrike3	WideStrike	TwinLink Plus	TwinLink	Bollgard III	Bollgard II
RR	83.75 ± 9.21a	12.50 ± 3.23bc	18.75 ± 3.75b	16.25 ± 5.15bc	2.50 ± 1.44c	7.50 ± 3.23bc	11.25 ± 1.50bc
RS	81.25 ± 2.39a	0.00 ± 0.00c	7.5 ± 4.33bc	0.00 ± 0.00c	3.75 ± 1.25c	0.00 ± 0.00c	17.50 ± 2.50bc
SS	90.00 ± 2.04a	0.00 ± 0.00c	3.75 ± 1.25c	0.00 ± 0.00c	10.00 ± 4.08bc	0.00 ± 0.00c	12.50 ± 2.50bc

Means in a column or row followed by the same letter are not significantly different based on a two-way ANOVA (Tukey's HSD P > 0.05)
[1]RR = Resistant larvae, RS = Heterozygote larvae, SS= Susceptible larvae
Widestrike 3-Cry1F, Cry1Ac, Vip3A Widestrike- Cry1F, Cry1Ac Twinlik Plus- Cry1Ab, Cry2Ae, Vip3A Twinlink- Cry1Ab, Cry2Ae Bollgard III- Cry1Ac, Cry2Ab2, Vip3A Bollgard II- Cry1Ac, Cry2Ab2

Cotton Square Bioassay

The RR genotype had high survivorship on non-Bt squares at roughly 70% (Table 7). RR also showed high survivorship with an average of survivorship of 55% for all five varieties; and there were no differences across any of the varieties. RS had 70% survivorship and SS had 73% survivorship on non-Bt which did not differ from RR. RS genotype showed 55% and 63% survivorship on Bollgard II and Widestrike, respectively and did not show significant differences compared to non-Bt. Similarly, SS genotype showed 80% survivorship on Bollgard II and 38% on Widestrike and showed no significant differences compared to non-Bt. Despite the survivorship on Bollgard II and Widestrike, survivorship was diminished on Bollgard III and Widestrike 3, which contains the Vip3A protein for both RS and SS genotypes. Developmental data showed no statistical differences and no growth inhibition of the surviving larvae on any of the varieties in this study (Figure 11).

Table 7. Survivorship of *Spodoptera frugiperda* on cotton squares after 7 days.

Insect Genotype	Survivorship (%)				
	Non-Bt	**Bollgard ll**	**Bollgard lll**	**Widestrike**	**Widstrike 3**
RR	67.5 ± 4.98 abc	63.33 ± 3.6 abc	64.17 ± 2.85 abc	36.67 ± 3.04 c	40.83 ± 4.38 bc
RS	70.00 ± 3.33 abc	55.00 ± 9.08 abc	0.83 ± 0.83 d	63.33 ± 2.36 abc	12.5 ± 8.65 d
SS	73.33 ± 5.93 ab	80.00 ± 5.27 a	0.00 ± 0.00 d	38.30 ± 4.19 bc	11.60 ± 8.66 d

Means in a column or row followed by the same letter are not significantly different based on a two-way ANOVA (Tukey's HSD $P > 0.05$)
Squares were infested with 2nd instar larvae
RR = Resistant larvae, RS = Heterozygote larvae, SS = Susceptible larvae
Widestrike 3-Cry1F, Cry1Ac, Vip3A Widestrike- Cry1F, Cry1Ac Bollgard III- Cry1Ac, Cry2Ab2, Vip3A Bollgard II- Cry1Ac, Cry2Ab2

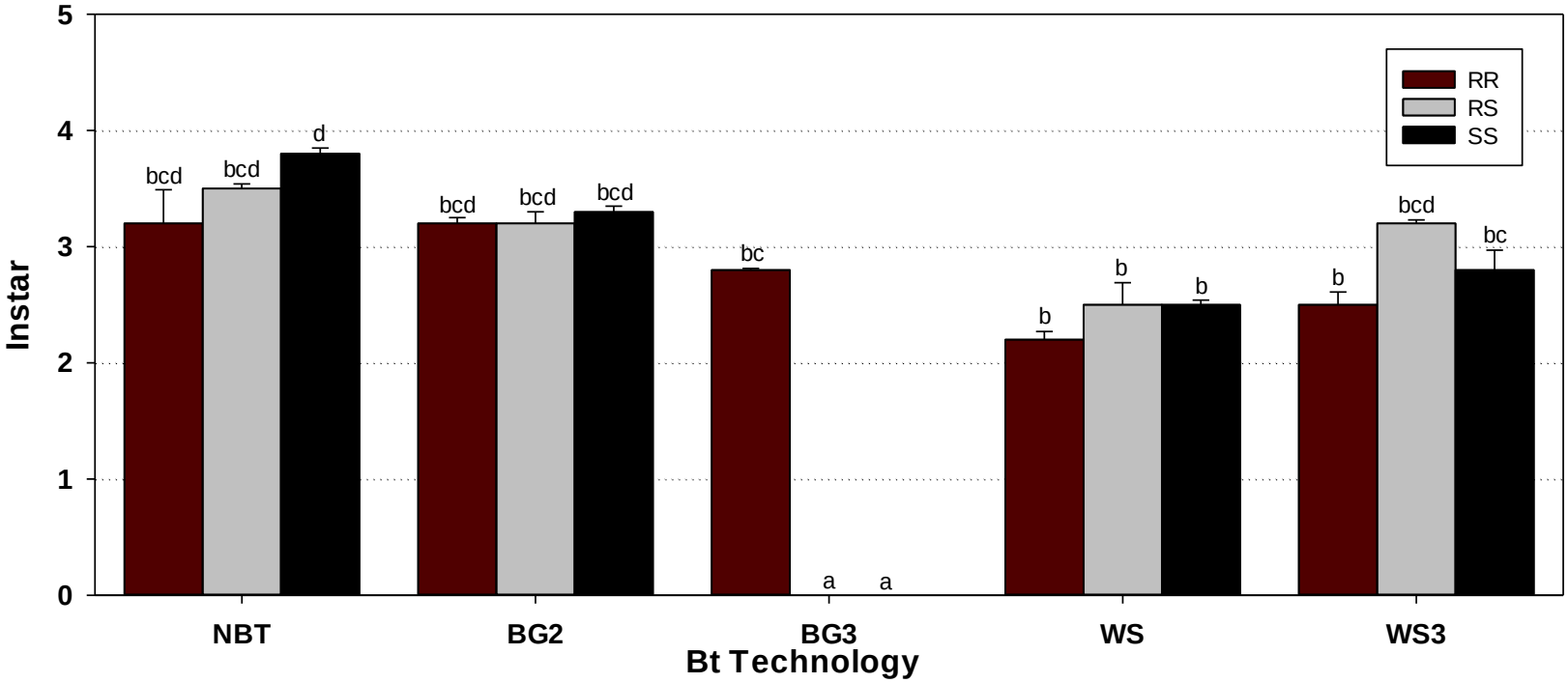

Figure 11. Average instar for surviving larvae on cotton squares after 7 days. Mean values in figure followed by the same letter are not significantly different based on a two-way ANOVA (Tukey's HSD P > 0.05) RR = Resistant larvae, RS = Heterozygote larvae, SS= Susceptible larvae (WS3) Widestrike 3-Cry1F, Cry1Ac, Vip3A (WS) Widestrike- Cry1F, Cry1Ac (BG3) Bollgard III- Cry1Ac, Cry2Ab2, Vip3A (BG2) Bollgard II- Cry1Ac, Cry2Ab2

Discussion

Since the development of Bt technology in 1996 cotton varieties can be categorized into three generations. The first generation Bt cotton only contained one Bt gene (Cry1Ac). The second generation contained the first pyramided proteins with Cry2Ab and Cry1Ac (Bollgard II), or Cry2Ae with Cry1Ab (TwinLink), or Cry1Ac and Cry1F (Widestrike). The last and most recent generation now possess the Vip3A protein pyramided amongst the other proteins. In this study, we evaluated the survivorship of different genotypes of *S. frugiperda* carrying Vip3A resistant alleles. The results of this study show that current pyramided Bt technologies containing Cry1F, Cry1Ac, Cry2Ae or Cry2Ab proteins are still capable of managing Vip3A resistant fall armyworms.

Previous studies conducted by Yang et al. (2017), found Cry2Ab2 resistant FAW to be resistant to pyramided Bt cotton that contained Cry1Ac and Cry2Ab2, or Cry1Ab and Cry2Ae but highly susceptible to Vip3A cotton. The results from the current cotton leaf bioassay found Vip3A RR larvae to have low to moderate survivorship on Bollgard II, Bollgard III, TwinLink, TwinLink Plus, Widestrike, and Widestrik 3 cotton varieties and were significantly lower than the non-Bt. The results from the current study suggest that cotton varieties possessing Cry1Ac, Cry1F, Cry2Ab, and Cry2Ae and not Vip3A have moderate control when any FAW genotype fed on the leaves of the plant.

However, as documented by Kranthi et al. (2005), Bt expression levels vary from plant tissues within the plant, with the leaf having the highest expression levels followed by the squares and then bolls. Cotton square data suggests expression levels within Bollgard II and Widestrike varieties are not high enough and probably violate the high-

dose/refuge strategy (Huang et al. 2011), because the SS genotype had high survivorship and no statistical differences between non-Bt. In spite of the high survivorship on squares, survivorship on leaf discs was much lower which would suggest the expression level of the Bt toxin would be higher in the leaf.

Hardke et al. (2015b) found no significant differences of third instar FAW feeding on either Bollgard or non-Bt cotton squares, however square damages were significantly different on Bollgard II when compared to the non-Bt, and reached complete larval mortality on Widestrike cotton squares. Results from the current study observed no differences in survivorship for the RR genotype on Bollgard II, Bollgard III, Widestrike, Widestrike 3 and non-Bt squares. The survivorship of the RR genotype on multiple Bt cotton varieties coincides with the reports from Adamczyk et al. (2001), and Kranthi et al. (2005) that the expression of Bt toxins within the plant can vary greatly due to several factors. The lack of efficacy provided by Bollgard II, Bollgard III, Widestrike, and Widestrike 3 in the square bioassay, suggests that future efficacy of these technologies could be jeopardized by Vip3A resistant FAW under certain field conditions where expression levels may be reduced. The data from the current study suggests that future cotton varieties may require additional and novel proteins to be efficacious towards Vip3A resistant FAW.

CHAPTER V

CONCLUSIONS

The fall armyworm *Spodoptera frugiperda* (J.E. Smith); FAW) has become one of the most destructive agricultural pest on the global scale. Recent reports and documentation of FAW in countries such as Africa, India, and China make this pest a high priority for developing IRM strategies. As reported previously in chapter one this pest can overwinter in warm regions and colonize several host species, to be ready to invade agricultural crops.

Data from the diet overlay bioassays in chapter two showed that when FAW alleles possess Vip3A resistance genes they are still highly susceptible to Cry1F, Cry2Ab2, and Cry2Ae Bt proteins and were not significantly different than the documented susceptible strain of FAW. The RS genotype was highly to susceptible to Cry1F, Cry2Ab2, Cry2Ae, and Vip3A suggesting the resistance gene is indeed not sex-linked.

Confirming what was found during the Bt protein bioassays, the corn leaf and whole corn plants expressed similar results. However, with one exception RR showed moderate survivorship on Herculex (Cry1F) leaves regardless if placed upon excised corn leaves or within the whorl of the plant. This can be expected due to the Cry1F resistant alleles being so widespread throughout much of the Americas (Storer et al. 2010, Farias et al. 2014, Huang et al. 2014). RR showed good survivorship on Agrisure

Viptera 3111 (Cry1Ab, Vip3A), but it is important to note that the development of the larvae was hindered. This suggests that the resistance is incomplete.

To determine what the cross-crop resistance of the Vip3A resistant FAW would be, cotton leaves and square bioassays were used in chapter 4. The leaf tissues followed similar results as compared to the previous two chapters and showed very low survivorship across all varieties tested. Generally speaking, FAW do not develop on cotton leaves as well as they do on corn leaves however, all three genotypes did survive and develop well on non-Bt cotton leaves suggesting the proteins hindered survivorship. While survivorship was diminished during the leaf bioassays the square bioassays showed conflicting results.

Just as Trtikova et al. (2015) pointed out that there are several factors that can influence Bt expression in plants, square bioassay showed that SS, RS, and RR survival on squares was markedly different from survival on leaf tissues. In the square bioassay survivorship RR ranged from 36-64% across all varieties tested. Unfortunately, not all varieties tested in the leaf tissue bioassay (TwinLink, TwinLink Plus) were tested during the square bioassay. Additionally, growing conditions were not ideal for producing squares for the bioassays, and the late planted cotton was exposed to less than optimal growing conditions. The conditions may have influenced the level of Bt expression. Thus, differences in Bt expression between leaves and squares likely played a major role in the lack of survivorship of the FAW genotypes on the squares of some varieties. While RS and SS had very little survivorship on Bollgard II and Widestrike leaves interestingly, RS and SS survived well on squares from Bollgard II and Widestrike with

55%, 63%, and 80%, 38% respectively. This may explain why when scouting cotton most often FAW are found feeding on fruiting structures.

Overall under optimal conditions current commercial Bt technologies available on the market are still capable of managing Vip3A resistant FAW. The results generated for this thesis provide crucial information that will aid in decision making for growers across the world. Thus, IRM strategies that incorporate pyramided products will reduce resistant alleles and ensure the sustainable use of this Bt technology for the future.

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