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

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Abstract

The fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (FAW), is one of the major target pests of Bt 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, 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, but tested highly susceptible to Cry1F, Cry2Ab, and Cry2Ae purified Bt proteins. To confirm the susceptibility found in the protein bioassay, cotton leaf and square bioassays were utilized to determine the cross-crop resistance of Vip3A resistant FAW. During the leaf bioassay all three genotypes had low survivorship across all varieties except non-Bt. However, in the square bioassay, RR showed high survivorship across Bollgard III (Cry1Ac, Cry2Ab, Vip3A), Bollgard II (Cry1Ac, Cry2Ab), Widestrike (Cry1F, Cry1Ac), and Widestrike 3 (Cry1F, Cry1Ac, Vip3A). RS and SS showed similar results with survivorship on Bollgard II, and Widestrike, however very little survivorship on Bollgard III, and Widestrike 3. 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.

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. FAW control can be difficult due to the sporadic behavior of the pest. Chemical control can be effective when applied in a timely manner and under optimal conditions. However, FAW have become resistant to several classes of insecticides including pyrethroids, organophosphates, and carbamates (Yu et. al 1991). Additionally, insecticides are generally ineffective in some crops such as corn or sorghum because of the feeding behavior of FAW, which reduces their exposure to insecticides. Cultural control methods targeting overwintering FAW are generally futile because this pest lacks a diapause mechanism.

Consequently, genetically-modified plants that express the entomopathogenic bacteria *Bacillus thuringiensis* (*Bt*) is the preferred and most effective method used for controlling FAW. The adoption and use of *Bt* crops have risen significantly since their first release in 1996. With such widespread use, resistance issues have emerged, and 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 Insect Resistant Management strategies 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 the mechanism(s) of resistance will assist in the selection or pyramiding of toxins incorporated in *Bt* crops to minimize cross-resistance.

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 major concern because resistance to such newly released technology could jeopardize the sustainable use of this technology in the future. The objective of this study is to determine if Vip3A resistant FAW have any cross-resistance to other *Bt* proteins and determine the cross-crop resistance of Vip3A resistant FAW to *Bt* cotton using square and leaf tissue 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.

Methods and Methods

Insect Sources

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

Diet Bioassays

Repeater pipets were used to dispense 0.8 ml of liquid diet in each well (Southland Product, Inc. Lake Village, AR) of a 128-well bioassay tray (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 CropScience), 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. The protein was overlaid and not incorporated into the diet to assure the insect would ingest the protein at the given concentration before digesting any diet. 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 did not move after gentle prodding.

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). Prior to analysis, the percentage data were transformed using arcsine ($X^{0.5}$) to meet the normality assumptions. Treatment means were separated using Tukey's HSD with $\alpha = 0.05$ (SAS Institute 2010). Probit analysis was used to determine the median lethal concentration (LC_{50}) and corresponding 95% confidence limits (CL) (SAS Institute 2010). Resistance ratios were calculated using the LC_{50} of one (RR or RS) population divided by the LC_{50} of the SS population.

Cotton Leaf and Square Bioassay

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.

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. 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 were 15 squares. Squares were replaced every 1-2 days. Once infested the containers were sealed with a lid and placed in a growth chamber maintained at 27 ± 1° C, 50% RH and a 14:10 (L: D) photoperiod.

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

Diet Bioassays

SS larvae were 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 1). RS showed similar results with greater than 80% at 0.316 µg/cm^2 reached 100% at the 1 µg/cm^2 concentration. Percent mortality ratings for RR was significantly lower at the 0.1-10 µg/cm^2 concentrations compared to the other two genotypes. Compared to the Vip3A51 protein, Cry1F, Cry2Ab2, and Cry2Ae were more toxic to RR, and just as toxic to the other genotypes. RS and SS genotype followed very similar patterns in respect to percent mortality.

Cotton Leaf and Square Bioassay

All genotypes had high survival on non-Bt cotton leaves, with 80 percent and higher survivorship (Table1). All three genotypes had low survivorship on leaves 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. The RR genotype had high survivorship on non-Bt squares at roughly 70% (Table 2). 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.

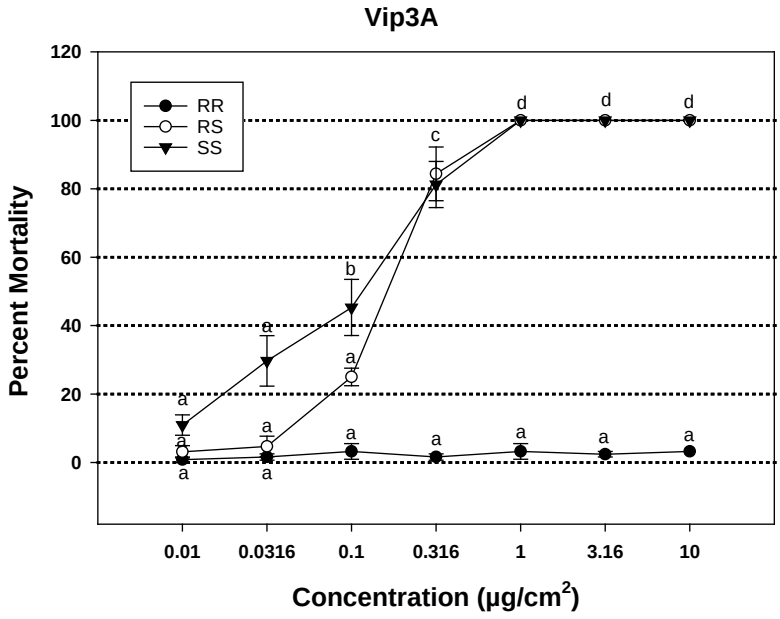

Figure 1. Concentration-larval mortality response to Vip3A. Mean values in figure followed by the same letter are not significantly different based on a two-way ANOVA (Tukey's HSD P > 0.05). RR = Resistant larvae, RS = Heterozygote larvae, SS= Susceptible larvae

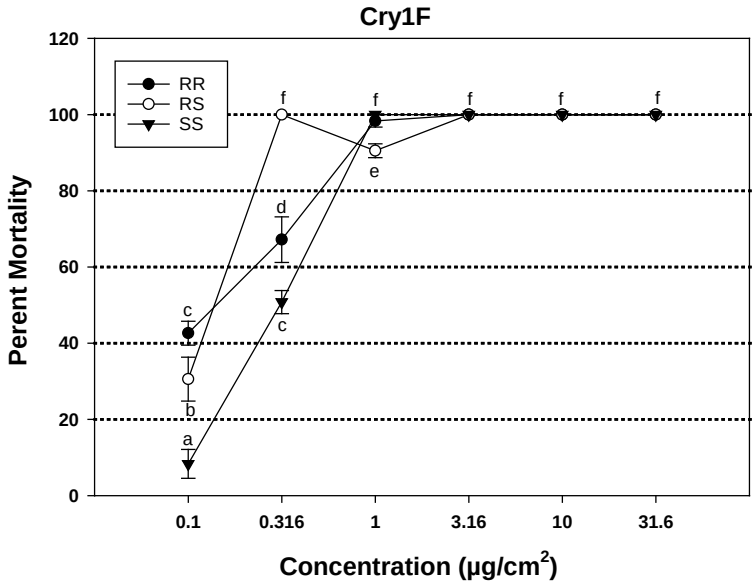

Figure 2. Concentration-larval mortality response to Cry1F. Mean values in figure followed by the same letter are not significantly different based on a two-way ANOVA (Tukey's HSD P > 0.05). RR = Resistant larvae, RS = Heterozygote larvae, SS= Susceptible larvae

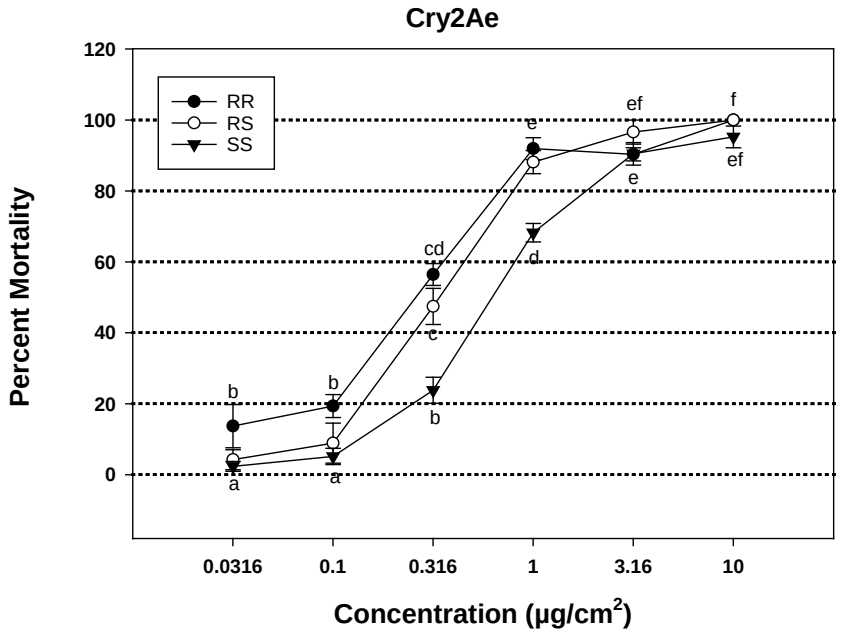

Figure 3. Concentration-larval mortality response to Cry2Ae. Mean values in figure followed by the same letter are not significantly different based on a two-way ANOVA (Tukey's HSD P > 0.05). RR = Resistant larvae, RS = Heterozygote larvae, SS= Susceptible larvae

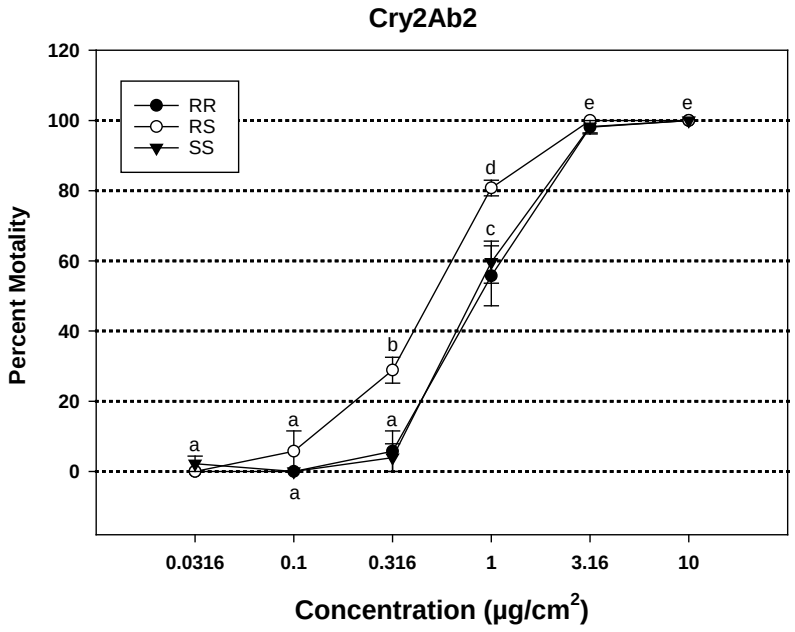

Figure 4. Concentration-larval mortality response to Cry2Ab2. Mean values in figure followed same letter are not significantly different based on a two-way ANOVA (Tukey's HSD P > 0.05). Resistant larvae, RS = Heterozygote larvae, SS= Susceptible larvae

Table 1. Survivorship of different genotypes of *Spodoptera frugiperda* on cotton leaves after 7

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

Table 2. Survivorship of *Spodoptera frugiperda* on cotton squares after 7 days.

Insect Genotype[1]	Survivorship (%)				
	Non-*Bt*	Bollgard ll	Bollgard lll	Widestrike	Widestrike 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)

[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

Discussion

The results from the current cotton leaf bioassay found Vip3A RR larvae to have low survivorship on cotton varieties tested. 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 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.

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 other reports 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.

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