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Resistance of fall armyworm, *Spodoptera frugiperda* (J.E. Smith) to *Bacillus thuringiensis*

Ying Niu

Louisiana State University and Agricultural and Mechanical College

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RESISTANCE OF FALL ARMYWORM, *SPODOPTERA*
FRUGIPERDA (J.E. SMITH) TO *BACILLUS THURINGIENSIS*

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Department of Entomology

by

Ying Niu

B.S. Southwest University, 2010

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ABSTRACT

The fall armyworm, *Spodoptera frugiperda* (J.E. Smith), is a target pest of *Bacillus thuringiensis* (Bt) corn in North and South America. In this study, multiple tests were conducted in the laboratory and greenhouse to 1) determine the susceptibility of two field populations of *S. frugiperda* collected from Florida (FL) and Puerto Rico (PR) to purified Cry1F protein and Bt corn leaf tissue and 2) evaluate larval survival of and plant injury by Cry1F-susceptible, -resistant, and -heterozygous genotypes of *S. frugiperda* on whole plants of transgenic corn containing single and pyramided Bt genes. Corn hybrids evaluated in this study included five non-Bt corn hybrids, two single-gene Bt corn products: Herculex[®]I (Cry1F corn) and YieldGard[®] (Cry1Ab corn), and four pyramided Bt corn traits: Genuity[®]VT Double Pro[™], VT Triple Pro[™], SmartStax[™], and Agrisure[®] Viptera[™] 3111. Diet-incorporated bioassays showed that FL was susceptible to Cry1F protein with a LC₅₀ value of 0.13-0.23 µg/g, while PR was highly resistant to Cry1F (>137-fold). Leaf tissue bioassays also exhibited that FL was susceptible to all Bt corn hybrids, while PR was highly resistant to Cry1F corn leaf tissue. Both FL and PR could not survive on leaf tissue of Viptera[™] 3111. However, PR exhibited a significant cross-resistance to the leaf tissue of the other three pyramided Bt corn traits. In greenhouse whole plant tests, larvae of the three insect genotypes on non-Bt corn hybrids survived well and caused serious plant damage. Cry1Ab corn was ineffective against all three insect genotypes. On Cry1F corn plants, resistant larvae survived on 72.9% plants after 12-15 d and caused significant leaf injury. In contrast, no live larvae and little or no leaf injury were observed on the Cry1F corn plants that were infested with susceptible or heterozygous genotypes, or on the pyramided Bt plants infested with the three

insect genotypes. The results demonstrated that the Puerto Rico population of *S. frugiperda* was highly resistant to both purified Cry1F protein and Cry1F corn plants and the resistance was recessive. Corn hybrids containing any one of the four pyramided Bt traits are effective for managing the Cry1F resistance in *S. frugiperda*.

CHAPTER 1. INTRODUCTION

1.1 Corn Production in the United States

Field corn (*Zea mays* L.) is the most widely planted field crop in the United States. In 2013, corn acreage in the United States was 97.4 million acres and the total harvest was 89.3 million acres with a production value of \$63 billion (NASS, 2013). In 2013, Bt corn accounted for 76% of the total acres (NASS, 2013). Corn also contributes significant value to the agriculture in Louisiana. In 2012, 540,000 acres of corn was planted in Louisiana with a yield of 75,000 tons valued at \$632.6 million (NASS, 2013).

1.2 Major Corn Insect Pests

There are various arthropod pests that damage field corn. A majority of these pests are generally divided into two groups, including the above-ground group and the below-ground group. Lepidopteran species are the major above-ground pests of corn plants, while coleopteran species are major pests that attack below ground plant tissues. The major lepidopteran species which damage corn in the U.S. mid-southern region include the corn earworm (*Helicoverpa zea* (Boddie)), fall armyworm (*Spodoptera frugiperda* (J.E. Smith)) and a complex of corn stalk borers (Siebert et al., 2012). Across the north central and mid-western region, European corn borer (*Ostrinia nubilalis* (Hubner)) and southwestern corn borer (*Diatraea grandiosella* Dyar) are the two major corn borer species (Ostlie et al., 1997, Huang et al., 2011). Yield losses of traditional non-Bt corn by a corn borer complex of the sugarcane borer, *Diatraea saccharalis* (F.) and *D. grandiosella* are estimated at up to 28% in mid-southern states (Sankula and Blumenthal, 2004). Recently, it is also reported that *S. frugiperda* infestations occur frequently across the Southern region of the U.S. in

conventional non-Bt and Bt corn varieties, especially when fields are planted after the optimum seeding dates (Hardke et al., 2011).

1.3 *Spodoptera frugiperda* (J.E. Smith)

S. frugiperda is native to the tropical regions of the western hemisphere from the United States to Argentina (Pashley et al., 1985; Adamczyk et al., 1999). It normally overwinters successfully in southern Florida and southern Texas of the United States. *S. frugiperda* is a strong flier, and disperses long distances annually during the summer months (Belay et al., 2012). It can be found in most U.S. states. However, as a regular and serious pest, its range tends to be mostly the southeastern states. The life cycle is completed in about 30 days during the summer, but 60 days in the spring and fall, and 80 to 90 days during the winter. The number of generations varies with the appearance of the dispersing adults in an area (Pitre and Hogg, 1983; Ashley et al., 1989; Sparks, 1979). *S. frugiperda* has historically been one of the most common pests of field corn in the Southern U.S. (Pitre and Hogg, 1983; Buntin, 1986; Buntin et al., 2004). This pest has a wide host range of more than 80 plant species. It does not overwinter in most of the corn-production regions of the United States (Wyckhuys and O'Neil, 2006).

1.4 Management of *Spodoptera frugiperda*

Traditional chemical control strategies often provide unsatisfactory control of *S. frugiperda* in field corn. Almost immediately after larval hatching, neonates move into the whorl region of corn plants where they are protected from foliar insecticide sprays (Harrison, 1986; Castro, 2002; Bokonon-Ganta et al., 2003; Siebert et al., 2008a). Those insecticides which are generally effective against other pests, such as the corn earworm, *Helicoverpa zea*

(Boddie), typically provide only limited control of *S. frugiperda* (Young, 1979; Guillebeau and All, 1990). Regional populations of *S. frugiperda* have developed resistance to several classes of insecticides including carbamates, organophosphates, and pyrethroids (Adamczyk et al., 1999). Thus, transgenic corn varieties have become a more viable option for controlling *S. frugiperda*.

1.5 Transgenic Bt Corn Technology

The ability to transfer foreign genes to desired plant genomes represents a major technological advance in modern agriculture (James, 2011). *Bacillus thuringiensis* (Bt) is a rod shaped soil bacterium that produces specific crystalline (Cry) endotoxin during the reproductive stages and vegetative insecticidal proteins (VIP) during the vegetative growth stages that are toxic to specific insect species (Vaeck et al., 1989, Gasser and Fraley, 1989). The sotto disease that killed vast populations of silkworms *Bombyx mori* (L.), in Japan in 1901 made bacteriologist Shigetane Ishiwata discover, isolate and name the soil bacterium (Ishiwata, 1901). Later, a German biologist, Ernst Berliner, confirmed this discovery while isolating the bacterium that had caused the death of the Mediterranean flour moth, *Ephesia kuehniella* (Zell), in 1911 (Berliner, 1915; Siegel, 2000; Sanahuja et al., 2011). Such insecticidal proteins produced by Bt have been used by farmers for insect-pest control under various trade names including Sporeine[®], Thuricide[®], Able[™], Biobit[®], and Dipel[®] (Baum et al., 1999; Kaur et al., 2000; NPTN, 2000). Pesticides with Bt formulation are considered as friendly to the environment, people, soil decomposers, pollinators, parasitoids, and wildlife. Bt toxins are highly diverse, highly effective, and relatively cheap. These merits have made it

widely used all over the world for controlling lepidopteran, coleopteran larvae and several dipteran pests (Baum et al., 1999; Kaur, 2000).

The primary target pests of Bt are specific insect species. Bt controls insects with toxins called insecticidal crystal proteins or delta endotoxins. When insects ingest toxin crystals, which are then dissolved and cut with proteases in the highly alkaline of insect midgut, making the cry toxin release from the crystal. The Cry toxin is then inserted into the insect gut cell membrane, paralyzing the digestive tract and forming a pore, which makes the insect stop eating and starve to death (Dean, 1984).

Bt crops are the plants which can express Bt proteins. Transgenic Bt tobacco was the first plant modified to express δ - endotoxins with Cry1Ab gene in 1987 in Belgium (Vaeck et al., 1989). Bt potatoes were first developed for the control of Colorado potato beetle, *Leptinotarsa decemlineata* (Perlak et al., 1993). Bt corn were first commercialized in the U.S. in 1996 primarily for management of *O. nubilalis* and *D. grandiosella* (Ostlie et al., 1997). Since then, many Bt corn products have been produced for controlling *Diabrotica spp.*, *H. zea*, and *S. frugiperda*. Bt corn expressing a single protein (Cry1Ab) was introduced in the U.S. southern States and commercially planted in 1999 (Buntin et al., 2000, 2004; Huang et al., 2006).

1.6 Bt Resistance in *Spodoptera frugiperda*

Resistance development in target pest populations has been a big challenge for the sustainable use of transgenic Bt crops (Alstad and Andow, 1995; Ostlie et al., 1997; Gould, 1998; Tabashnik et al., 2008). Resistance to Bt insecticides were earlier detected and reported in field populations of diamondback moth, *Plutella xylostella* (L.) (Tabashnik, 1994),

and cabbage looper, *Trichoplusia ni* (Hubner) in Canada (Kain et al., 2004). Major resistance genes to Bt crops have been found in laboratory selections in tobacco budworm, *Heliothis virescens* (Fabricius), (Gould et al., 1995, 1997), pink bollworm, *Pectinophora gossypiella* (Saunders) (Tabashnik et al., 2000), poplar leaf beetle, *Chrysomela populi* (L.) (G é nissel et al., 2003), *D. saccharalis* to Cry1Ab corn (Huang et al., 2007a, b, 2008, 2009), *O. nubilalis* to Cry 1F corn (Pereira et al., 2008), *H. zea* to Cry1Ac cotton in the U.S (Tabashnik et al., 2008; Moar et al., 2008) and *Helicoverpa armigera* (Hübner) to Cry1Ac cotton in Australia (Akhurst et al., 2003; Downes et al., 2007; Mahon et al., 2007) and China (Li et al., 2004; Xu et al., 2009).

Resistance of *S. frugiperda* to Cry1F corn observed in 2006 in Puerto Rico was the first documented field resistance to Bt crops in the world (Matten et al., 2008; Storer et al., 2010). Since then another three cases of field resistances to Bt crops have been reported, which are the resistance of the African stem borer, *Busseola fusca* (Fuller), to Cry1Ab corn in South Africa in 2007 (Van Rensburg, 2007), resistance of *P. gossypiella*, to Cry1Ac cotton in western India (Monsanto, 2010a; Dhurua and Gujar, 2011), and recently resistance of western corn rootworms, *Diabrotica virgifera* to Cry3Bb1 corn in the United States (Gassmann et al., 2011).

Cry1F-expressed corn was registered in 2001 in United States to control the major and secondary Lepidoptera pests. In 2003, Cry1F corn was cultivated in Puerto Rico to control *S. frugiperda* which is the most important corn pest in Puerto Rico (Hardke et al., 2011). A document has revealed that resistance to Cry1F corn in *S. frugiperda* occurred in late 2006. There might have several factors that had contributed to this resistance (Storer et

al., 2010; Huang et al., 2011). Among these, the lack of a high Bt dose expressed in Herculex[®] I hybrids for *S. frugiperda* could be a major reason for the resistance development. In addition, the isolated island geography of Puerto Rico, tropical environment, and limited availability of alternate hosts favorable for *S. frugiperda* could intensify *S. frugiperda* infestations (Huang et al., 2011). In 2006, large populations of *S. frugiperda* were recorded in Puerto Rico that caused severe damage to traditional non-Bt corn, and a serious drought from October 2006 to April 2007 also forced the populations to rely more on the irrigated crops such as the Cry1F corn (Storer et al., 2010).

1.7 Bt Resistance Management

The wide use of Bt corn demands an effective insecticide resistance management (IRM) plan to ensure the sustainable use of Bt corn technologies (Ostlie et al., 1997; Gould, 1998; US EPA, 1998, 2001; Baute, 2004). To delay resistance development, the United States and Canada have implemented an IRM plan named the ‘high dose/refuge’ strategy for planting Bt crops (Ostlie et al., 1997; Gould, 1998; Baute, 2004). This strategy firstly aims to use ‘high-dose’ Bt plants to kill resistant heterozygotes of the target pests (US EPA, 2001). Thus the resistance alleles of resistant heterozygous insects can’t be transmitted into the next generation. Secondly, the remaining area is planted to non-Bt varieties that serve as a refuge for susceptible insects. The susceptible insects emerged from the non-Bt crop should mate with the rare resistant homozygous individuals that have survived on the Bt crop. If the frequency of resistance is very low (e.g. 0.001), majority of offspring carrying resistance alleles will be heterozygous and the heterozygotes should be killed by the high does Bt crops (Huang et al., 2011). Through this strategy, the resistance allele frequency in the target pest

populations can be maintained at low levels for a long-period of time. There are three key assumptions for the success of the “high dose/refuge” IRM strategy (Huang et al., 2011). First, the Bt crops should produce a high dose of Bt proteins that can kill the individuals of the target species that carry one copy of the resistance allele. In other words, the resistance should be functionally recessive. Second, the initial resistance allele frequency should be very low, usually <0.001 . And finally, the rare survivors that are homozygous for resistance can mate with the susceptible individuals from the non-Bt refuge plants (Ostlie et al., 1997; US EPA, 2001). Previous studies have demonstrated that resistance development to Bt crops in target pest populations can be significantly delayed if these three assumptions are met (Huang et al., 2011). For example, in North America, the major target pest species of Bt corn, *O. nubilalis* and *D. grandiosella* and the major targets of Bt cotton, *H. virescens* and *P. gossypiella* are still very susceptible and have not shown any resistance to Bt corn or Bt cotton after 16 years of intensive use of transgenic Bt crops in the U.S and Canada (Huang et al., 2011). Analysis of these cases showed that the three assumptions of the “high dose/refuge” strategy are met for all of the four species. In contrast, at least one of the three assumptions was not met in the four cases of documented field resistance to Bt crops. For example, none of the Bt crops associated with the four cases expressed a “high dose” of Bt proteins against the target species as required in the “high dose/refuge” IRM strategy (Huang et al., 2011).

Previous studies have shown that neither the Cry1Ab nor Cry1F Bt corn expresses a ‘high dose’ against *S. frugiperda*. In addition, with the increased planting of Bt crops in the United States and Canada, compliance rates with the requirements of the “high dose/structural

refuge” IRM strategy have decreased significantly in both countries. During the early commercialization of Bt corn, grower compliance to refuge requirements was reported to be high. For example, an early report indicated that >85% corn growers in the United States complied with the refuge planting requirements of the strategy (ABSTC, 2002). From 2001 to 2006, compliance rates dropped as low as 72% (ABSTC, 2005; Goldberger et al., 2005). By 2007 and 2008, the compliance rate with the refuge planting in the United States was 74-80% (US EPA, 2010). A similar decrease in the compliance rate in refuge planting requirements was also reported in Canada (Canadian Corn Pest Coalition, 2005; Dunlop, 2009).

Furthermore, modeling has shown that target insect pests could develop resistance more rapidly to single gene Bt crops than to multiple toxins (Roush, 1998, Zhao et al., 2003). To delay resistance development, a gene-pyramiding strategy has been employed to develop transgenic plants that express multiple Bt toxins for targeting a same group of insect pests.

The first commercialized pyramided Bt corn technologies in the U.S. for managing lepidopteran pests are Genuity® VT Double Pro™, Triple Pro™, SmartStax™, and Agrisure® Viptera™ 3111. Corn hybrids containing these pyramided Bt traits were first commercially planted in the United States during the 2010 crop season (Monsanto, 2010b). It is expected that the use of pyramided Bt corn should delay resistance development in field (Roush, 1998; Zhao et al., 2003; Monsanto, 2010b).

1.8 Objectives

1. To determine the susceptibility of two field populations of *Spodoptera frugiperda* collected from Florida and Puerto Rico to purified Cry1F protein and corn leaf tissue containing single and pyramided Bt genes; and

2. To evaluate larval survival and plant injury of Cry1F-susceptible, -resistant, and -heterozygous genotypes of *Spodoptera frugiperda* on transgenic corn containing single and pyramided Bt genes.

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CHAPTER 2. SUSCEPTIBILITY OF FIELD POPULATIONS OF THE FALL ARMYWORM (LEPIDOPTERA: NOCTUIDAE) FROM FLORIDA AND PUERTO RICO TO PUERIFIED CRY1F PROTEIN AND CORN LEAF TISSUE CONTAINING SINGLE AND PYRAMIDED BT GENES

2.1 Introduction

Transgenic corn hybrids expressing *Bacillus thuringiensis* (Bt) proteins were initially developed to reduce injury from corn stalk borers such as the European corn borer, *Ostrinia nubilalis* (Hübner) and southwestern corn borer, *Diatraea grandiosella* (Dyar). Fall armyworm, *Spodoptera frugiperda* (J.E. Smith), is an important pest of corn in both North and South America (Pashley et al., 1985; Pashley, 1986; Buntin et al., 2004; Chilcutt et al., 2007). Several studies have evaluated the field efficacy of first generation single gene Bt corn products (e.g. YieldGard[®] Corn Borer, Herculex[®]I) against *S. frugiperda* (Buntin et al., 2000; 2004; Buntin, 2008; Siebert et al., 2008). Results of these studies showed that the single gene Bt corn also could suppress *S. frugiperda* but the suppression levels were usually not high enough to qualify as “high dose”. For this reason, *S. frugiperda* is not listed as a target species of the first generation Bt corn technologies except Herculex[®]I expressing the Cry1F protein (US-EPA, 2001a).

Herculex[®]I Cry1F corn was first registered in 2001 in the United States and later became commercially available in the United States and Puerto Rico to control stalk borers and some Noctuidae moths including *S. frugiperda*. This insect has been reported as the most important corn pest in Puerto Rico (US-EPA, 2007; Storer et al., 2010). Unfortunately, field resistance to Cry1F corn was observed in *S. frugiperda* populations in Puerto Rico in 2006 (Storer et al., 2010). This became the first example of field resistance to commercial Bt crops

in the world (US-EPA, 2007; Matten et al., 2008; Storer et al., 2010). Besides intensive plantings of Cry1F corn in Puerto Rico, several other factors might have contributed to the development of field resistance (US-EPA, 2007; Storer et al., 2010; Huang et al., 2011). To delay resistance development and broaden the target spectrum, a gene-pyramiding strategy has been utilized to develop transgenic plants that express multiple Bt proteins with dissimilar modes of action for targeting a same group of insect pests (Ghimire et al., 2011). The first commercialized pyramided Bt corn technologies for managing lepidopteran pests in the United States included Genuity®VT Double Pro™, Genuity®VT Triple Pro™, Genuity® SmartStax™, and Agrisure® Viptera™ 3111. Compared to the first generation single-gene Bt corn, the pyramided Bt corn products are more effective for controlling some Noctuidae species including *S. frugiperda* (Burkness et al., 2010) and thus *S. frugiperda* is listed as a target in all pyramided Bt corn traits that have been commercialized for managing above-ground lepidopteran corn pests in the United States (US-EPA, 2009; 2010; Monsanto, 2012; Syngenta, 2012).

During 2011, two field populations of *S. frugiperda* were established from larvae collected from corn fields in Florida and Puerto Rico, respectively. Preliminary studies showed that the Puerto Rico population was highly resistant to Cry1F corn plants, while the Florida population was still susceptible to the Cry1F corn. Therefore, these two populations of *S. frugiperda* should provide great value for analyzing cross-resistance to other Bt corn technologies, especially to the recently commercialized pyramided Bt corn. The objectives of this study were to 1) document the resistance of the field population of *S. frugiperda* from

Puerto Rico to purified Cry1F protein and commercial Cry1F corn and 2) to determine the cross-resistance of this population to pyramided corn products.

2.2 Materials and Methods

2.2.1 Insect Sources

Two field populations of *S. frugiperda* were established from larvae collected from corn fields in Florida and Puerto Rico, respectively, in 2011. The Florida population (FL) was initiated from 96 larvae sampled from Hendry County in south Florida and the Puerto Rico population (PR) was developed from >300 larvae collected in southern Puerto Rico. Field-collected larvae were reared individually on a meridic diet (Ward's Stonefly Heliothis diet, Rochester, NY) in 30-ml plastic cups (Fill-Rite, Newark, NJ) until the pupal stage. The larval-rearing cups were held in 30-well trays (Bio-Serv, Frenchtown, NJ) and placed under room conditions until pupation. Pupae of each population were placed in 3.8-L paper containers (Huhtamaki Foodservice, De Soto, KS) containing ~100g of vermiculite (Sun Gro, Pine Bluff, AR) for adult emergence, mating, and oviposition. Insect populations had been maintained in the laboratory for two generations for FL and three generations for PR when this study was initiated.

Susceptibility of *S. frugiperda* was evaluated using two approaches: 1) a diet incorporating bioassay with purified Cry1F protein and 2) testing on leaf tissue of Bt and non-Bt corn hybrids. There were two independent trials for each test approach. For the diet incorporation bioassays, the first trial used the original two populations (FL and PR) that were established from larvae collected from fields without further selection in the laboratory. In the first trial with leaf tissue bioassays, larval mortality was evaluated for three insect

populations including FL, PR, and an F₁ population (FL x PR) that was generated by crossing FL and PR. Results of the first trial showed that compared to FL, the PR population was highly resistant to both the purified Cry1F protein and Cry1F corn leaf tissue (see below). After the first trial with Cry1F protein and corn leaf tissue, the original PR larvae were selected on Cry1F corn (Pioneer 31D59) leaf tissue for two generations. In the selection process, 2-3 pieces of leaf tissue were placed in each well of 32-well C-D International trays (Bio-Ba-32, C-D International, Pitman, NJ). Approximately 5-10 newly hatched larvae were released in each well. For each generation, >1000 neonates were selected on Cry1F corn leaf tissue. After 7 days, the survivors were transferred into the diet. After each selection, approximately 120-180 survivors were reared until the next generation. If the number of survivors was more than enough (e.g. >180 larvae), only the survivors with a relatively bigger body size were used to develop the next generation. The Cry1F corn leaf tissue selected-PR populations were then backcrossed with the FL population and reselected for Cry1F resistance in F₂ generations on Cry1F corn leaf tissue. The procedures of the reselections for Cry1F resistance in the F₂ generations of the backcrosses were the same as described above. Thereafter, the backcrossed and reselected population was referred as Cry1F-RR. The Cry1F-RR population had been continuously selected on Cry1F corn leaf tissue for at least two more generations before it was used for this study. In addition, another F₁ population (Cry1F-RS) was developed by crossing individuals from FL and Cry1F-RR. In the second trial, susceptibility of *S. frugiperda* was evaluated for all three populations including FL, Cry1F-RR, and Cry1F-RS in both diet incorporation and leaf tissue bioassays.

2.2.2 Source of Cry1F Protein and Corn Leaf Tissue

In the diet incorporation bioassays, purified trypsin-activated (99.9%) Cry1F protein was obtained from Case Western Reserve University, Cleveland, OH (Huang et al. 2007). The Cry proteins were produced using recombinant *Escherichia coli* culture and were subsequently activated with trypsin. The activated Cry proteins were lyophilized before they were used in the bioassays. The purity of Cry1F proteins was determined using high-performance liquid chromatography and sodium dodecyl sulfate polyacrylamide gel electrophoresis (Pusztai-Carey et al., 1995; Masson et al., 1998).

In the leaf tissue tests, susceptibility of *S. frugiperda* was evaluated on leaf tissue of five non-Bt and seven Bt corn hybrids (Table 2.1). The seven Bt corn hybrids represent five Bt corn traits, which include one single-gene Bt corn product, Herculex[®]I and four pyramided Bt corn products, Genuity[®]VT Double Pro[™], Genuity[®]VT Triple Pro[™], Genuity[®]SmartStax[™], and Agrisure[®] Viptera[™] 3111. Herculex[®]I contains a single Bt gene, Cry1F (Event TC1507), effective for above-ground lepidopteran insects. VT Double Pro[™] expresses two Cry proteins, Cry1A.105 and Cry2Ab2 (Event MON89034) and both proteins are effective against above-ground lepidopteran species including *S. frugiperda* (Monsanto, 2012). VT Triple Pro[™] contains the same two Cry proteins in VT-2P plus Cry3Bb1 (MON88017) which is effective against below-ground corn rootworms *Diabrotica* spp. (Coleoptera: Chrysomelidae) (Monsanto, 2012). SmartStax[™] produces six Bt proteins including the three Bt proteins of VT Triple Pro[™] plus Cry1F (Event TC1507) targeting lepidopteran species and Cry34/35Ab1 (Event DAS-59122) against rootworms (Monsanto 2012). Viptera[™] 3111 expresses three Bt proteins including Vip3A (Event MIR162) and

Table 2.1. Hybrids used in evaluation of susceptibility of *Spodoptera frugiperda* to Bt corn

Traits	Corn hybrid	Event	Used in	Abbreviation in the figures	Bt genes	Major target pests
Non-Bt	DKC 61-22	--	Trial 1	NonBt-1	closely related to DKC 61-21	--
Non-Bt	DKC 67-86	--	Trial 1	NonBt-2	closely related to DKC 67-88	--
Non-Bt	DKC 63-45	--	Trial 2	NonBt-1	closely related to DKC 61-21	--
Non-Bt	N78N-GT	--	Trial 2	NonBt-2	closely related to N78N-3111	--
Non-Bt	Pioneer 31G66	--	Trial 2	NonBt-3	closely related to Pioneer 31D59	--
Herculex [®] I	Pioneer 31D59	TC1507	Trial 1&2	HX1	Cry1F	lepidoptera
Genuity [®] VT Double Pro [™]	DKC 64-04	MON89034	Trial 1	VT-2P	Cry1A.105, Cry2Ab2	lepidoptera
	DKC 63-87	MON89034	Trial 2	VT-2P	Cry1A.105, Cry2Ab2	lepidoptera
Genuity [®] VT Triple Pro [™]	DKC 67-88	MON89034+ MON 88017	Trial 1	VT-3P	Cry1A.105, Cry2Ab2, Cry3Bb1	lepidoptera &rootworms
	DKC 62-97	MON89034+ MON 86017	Trial 2	VT-3P	Cry1A.105, Cry2Ab2, Cry3Bb1	lepidoptera &rootworms
Genuity [®] SmartStax [®]	DKC 61-21	MON89034+ MON88017+TC 1507+ DAS- 59122	Trial 1&2	SmartStax	Cry1A.105, Cry2Ab, Cry1F, Cry3Bb1, Cry34/35Ab	lepidoptera & rootworms
Agrisure [®] Viptera [™] 3111	N78N-3111	MIR162+Bt11+ MIR604	Trial 1&2	VIP	Vip3A,Cry1Ab,mCry3A	lepidoptera & rootworms

Cry1Ab (Event Bt11) for controlling lepidopteran species and mCry3A (Event MIR604) for managing rootworms (DiFonzo and Collen, 2012). The five non-Bt corn hybrids were genetically closely related to one or two of the seven Bt corn hybrids. In each of the two trials, larval mortality of *S. frugiperda* was evaluated on corn leaf tissue of five Bt corn hybrids representing five Bt corn technologies along with two (1st trial) or three (2nd trial) non-Bt corn hybrids (Table 2.1). Expression (or not expression) of Bt proteins in plants was confirmed using ELISA-based assays (EnviroLogix, Quantiplate™ kits, Portland, ME).

2.2.3 Diet Incorporation Assays

Larval susceptibility of *S. frugiperda* to purified Cry1F protein was individually assayed using a diet incorporation procedure in 128-cell trays (C-D International, Pitman, NJ). In each bioassay, 6-8 Cry1F concentrations were used. Cry1F concentrations used in each bioassay were slightly different depending on the insect population and amount of Cry1F protein available. In the first trial, Cry1F concentrations of 0, 0.1, 0.316, 1, 3.16, 10, and 31.6µg/g were used to assay both FL and PR populations. Based on the results of the first trial, Cry1F concentrations used in the 2nd trial were modified to 0, 0.0316, 0.1, 0.316, 1, 3.16, and 10µg/g in assaying FL. In addition, concentrations of 31.6 (for both PR and Cry1F-RS) and 100µg/g (for PR only) were also included in the second bioassays. To prepare the appropriate concentrations of Bt diet, purified Cry1F protein was first suspended in distilled water at the room temperature and stirred completely using an iron stick to ensure that the protein was uniformly distributed in the solution. The Cry1F solutions were then mixed with a meridic diet (WARD'S Stonefly *Heliothis* diet) just prior to placing the diet into individual cells of the 128-cell trays. In the bioassay, approximately 1 g of treated diet was placed into

each cell. One neonate (< 24 h) was released on the diet in each cell. After larval inoculation, cells were covered with vented lids (C-D International, Pitman, NJ). The bioassay trays were placed in environmental chambers maintained at 28 °C, 50% RH, and a 16:8 (L: D) h photoperiod. Larval mortality was recorded on the 7th day after inoculation. Larvae were considered dead if they did not respond after being touched with a camel hair brush. In a bioassay, each combination of insect population by Cry1F concentration was replicated four times with 16-32 larvae in each replicate.

2.2.4 Leaf Tissue Test

Fully expanded leaf tissues of Bt and non-Bt corn plants were removed from greenhouse grown V5-V8 stage plants. In the bioassays, 2-3 pieces of leaf tissue were placed in each well of a 32-well C-D International tray (Bio-Ba-32, C-D International, Pitman, NJ). In each of the two trials, four neonates (<24 h old) of each of three populations were placed on the surface of the leaf tissue in each well. Bioassay trays containing leaf tissues and neonates were placed in growth chambers maintained at the same conditions as for the diet incorporation bioassays. Larval mortality was recorded on the 7th day after release of neonates. As mentioned above, larvae were considered dead if they did not respond after being touched with a camel hair brush. In each trial, there were four replications for each combination of corn hybrid and insect population and each replication included 32 neonates in eight wells (n = 128).

2.2.5 Data Analysis

In the diet incorporation bioassay, larval mortality of *S. frugiperda* at a Cry1F concentration was corrected with mortality on the control diet using the method as described

in Abbott (1925). The corrected concentration/mortality data were then subjected to a probit analysis to determine the Cry1F concentration that produced a 50% mortality value (LC_{50}) and the corresponding 95% confidence interval (CI) (Finney, 1971; SAS Institute, 2010). For each bioassay, the Cry1F concentrations used in the probit analysis included the highest concentration that produced zero mortality, the lowest concentration that resulted in 100% mortality, and all results between those extremes (Huang et al., 2007). In the bioassays with PR and Cry1F-RR, no significant larval mortality was observed even at the highest Cry1F concentrations tested, and thus the LC_{50} values for these two populations were considered to be greater than the highest Cry1F concentrations used in the bioassays. Resistance ratios for each Cry protein were calculated using the LC_{50} value of PR, Cry1F-RR, or Cry1F-RS divided by the LC_{50} of the FL population.

Because the LC_{50} values of the PR and Cry1F-RR populations couldn't be calculated with the probit analysis, larval mortality data, after transformed by arcsine of $(x)^{0.5}$, were also subjected to a two-way analysis of variance (ANOVA) with Cry1F concentration and insect population as the two main factors. Similarly, in the two trials using corn leaf tissue, percentage larval mortalities were first transformed by arcsine of $(x)^{0.5}$ and then analyzed using a two way ANOVA with corn hybrid and insect population as the two main factors (SAS Institute, 2010). Treatment means in all ANOVAs were separated with LSMEANS test at $\alpha = 0.05$ level (SAS Institute, 2010).

In addition, the dominance level of Cry1F resistance in *S. frugiperda* was estimated using two approaches. The first approach involved the use of the Stone's dominance "D" value. The LC_{50} values estimated in the 2nd diet incorporation bioassays were used to

calculate the dominance “D” value using the formula described in Stone (1968).

$$D = \frac{2\log LC_{RS} - \log LC_{RR} - \log LC_{SS}}{\log LC_{RR} - \log LC_{SS}}$$

The “D” value ranges from -1 to 1: a value of -1 indicating resistance is completely recessive; a value of 0 suggesting resistance is additive; and a value of 1 implying resistance is completely dominant. The dominance level of Cry1F resistance in *S. frugiperda* was also estimated as “effective dominance”, D_{ML} , using the method as described in Bourguet et al. (2000). D_{ML} ranges between 0 and 1. $D_{ML} = 0$ refers to a completely recessive resistance and $D_{ML} = 1$ means the resistance is completely dominant. In this study, D_{ML} was estimated using the mortality data of the three insect populations recorded in each of the two trials on corn leaf tissue of four Bt corn technologies. D_{ML} for VipteraTM3111 couldn’t be calculated because all insect populations exhibited 100% mortality on the Bt corn leaf tissue in both trials.

2.3 Results

2.3.1 Susceptibility of Field Populations from Florida and Puerto Rico to Purified Cry1F Protein: Trial One

The FL population was susceptible to the purified Cry1F protein with a LC_{50} of 0.23 µg/g and a 95% CI of 0.11-0.37 µg/g (Table 2.2). Relative to FL, PR was highly resistant to the Cry1F protein. No significant larval mortality ($\leq 13.7\%$, corrected mortality) of PR was observed across all the Cry1F concentrations assayed and thus the LC_{50} value of this population was estimated to be > 31.6 µg/g, which corresponded a resistance ratio of > 137 -fold. Two-way ANOVA showed that the main effects of both Cry1F concentration and insect populations on the 7-day larval mortality were significant ($F = 31.31$; $df = 6, 41$; $P <$

Table 2.2. LC₅₀s and 95% confidence intervals (CI) based on larval mortality of *Spodoptera frugiperda* neonates on diet treated with purified Cry1F *Bacillus thuringiensis* protein *

Population	N [#]	Slope ± SE	LC ₅₀ (95% CI) (µg/g)	χ ²	df	Resistance ratio
Trial 1						
FL	503	1.13 ± 0.18	0.23(0.11-0.37)	45.87	18	---
PR		---	>31.6	---	---	>137
Trial 2						
FL	553	1.38 ± 0.22	0.13(0.07-0.20)	71.62	18	---
Cry1F-RS	675	1.47 ± 0.16	1.07(0.76-1.50)	64.08	22	8.2
Cry1F-RR		---	>100	---	---	>769

[#]Total number of neonates assayed.

[†]Resistance ratio of an insect population was calculated by dividing the LC₅₀ value of the population by that of the FL population.

0.0001 and $F = 308.16$; $df = 1, 41$; $P < 0.0001$, respectively). The effect of the interaction of the two factors was also significant ($F = 26.82$; $df = 6, 41$; $P < 0.0001$). Significant larval mortality (50.2%) of the FL population occurred at 0.1 $\mu\text{g/g}$, the lowest concentration assayed, and the mortality reached 95.4% at 3.16 $\mu\text{g/g}$ and 100% at 10 $\mu\text{g/g}$ (Figure 2.1). In contrast, Cry1F at all tested concentrations did not cause any significantly greater mortality to PR than those observed on non-Bt control diet.

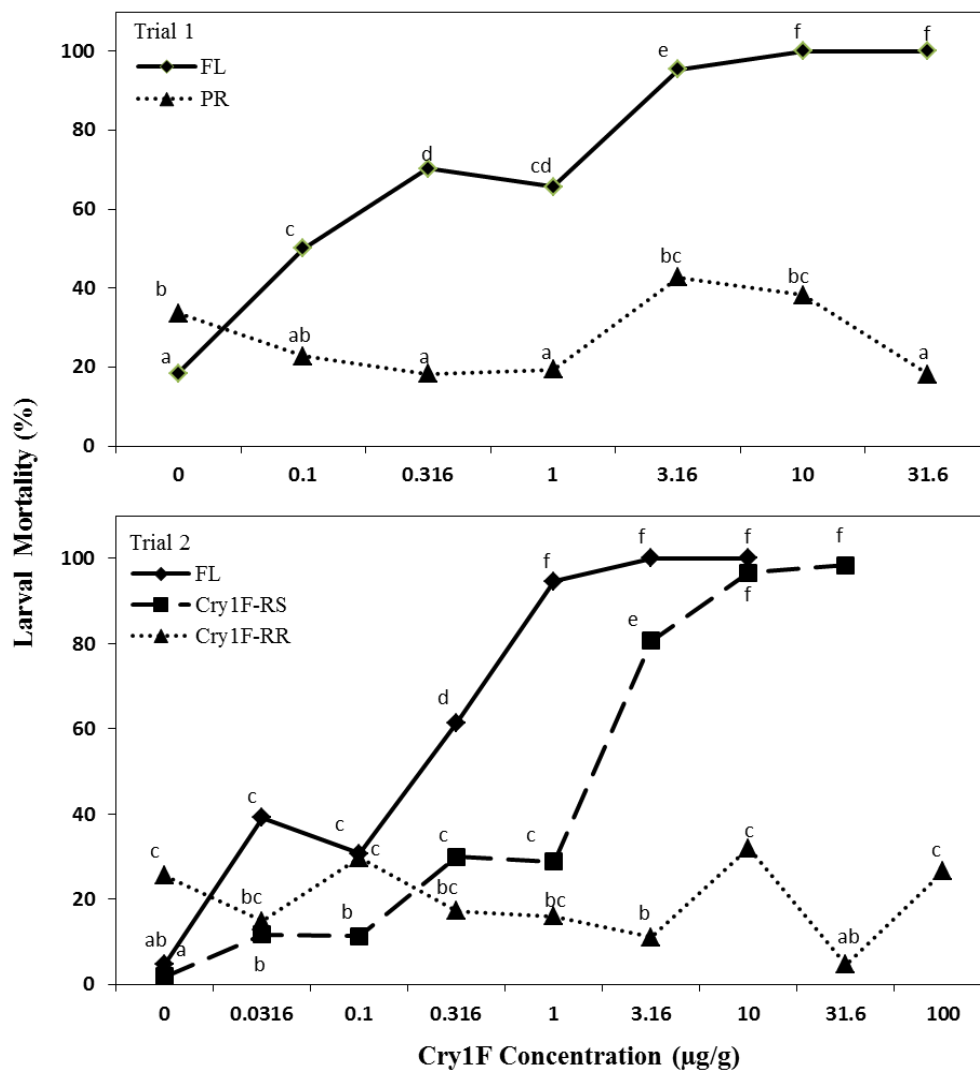


Figure 2.1. Larval mortality of *Spodoptera frugiperda* after 7 days on diet treated with different concentrations of purified Cry1F protein. Mean values across all treatments in each figure followed by a same letter are not significantly different ($P < 0.05$; LSMEANS test)

2.3.2 Susceptibility of FL, Cry1F-RR, and Cry1F-RS Populations to Purified Cry1F Protein: Trial Two

Similarly as observed in the first trial, the FL population was still susceptible to the purified Cry1F protein with a LC_{50} of $0.13\mu\text{g/g}$ and a 95% CI of $0.07\text{-}0.20\mu\text{g/g}$, which was not significantly different compared to the LC_{50} value calculated in the first trial based on the overlapped 95% CIs (Table 2.2). The backcrossed and reselected population, Cry1F-RR, was also highly resistant to the purified Cry1F protein. Again, no significant mortality ($\leq 8.7\%$, corrected mortality) of Cry1F-RR was recorded at the tested concentration range (up to $100\mu\text{g/g}$) and thus the LC_{50} value was estimated to be $> 100\mu\text{g/g}$, which was at least 769-fold greater than the LC_{50} of FL. The LC_{50} value of Cry1F-RS, the F_1 population of the cross between FL and Cry1F-RR, was $1.07\mu\text{g/g}$ with a 95% CI of $0.76\text{-}1.50\mu\text{g/g}$, which was significantly greater than the LC_{50} of FL based on the non-overlapped 95% CIs. However, the value of $1.07\mu\text{g/g}$ was considerably less than the LC_{50} of Cry1F-RR.

Two-way ANOVA also showed that effects of Cry1F concentration, insect population, and their interaction on 7-day larval mortality were all significant ($F = 56.79$; $df = 8, 72$; $P < 0.0001$ for, $F = 130.88$; $df = 2, 72$; $P < 0.0001$, and $F = 32.15$; $df = 13, 72$; $P < 0.0001$, respectively). Compared to the non-treated control diet, Cry1F at the tested concentrations did not result in any significant levels of mortality against the Cry1F-RR population, even at the highest concentration evaluated in the bioassays ($100\mu\text{g/g}$) (Figure 2.1). In contrast, significant mortality (39.1%) was observed for the FL population at the lowest concentration tested ($0.0316\mu\text{g/g}$). Mortality of FL reached 94.6% at $1\mu\text{g/g}$ and 100% at $3.16\mu\text{g/g}$. Mortality of the Cry1F-RS population at $\leq 1\mu\text{g/g}$ was low, between 11.3 ($0.0316\mu\text{g/g}$) and 30% ($1.0\mu\text{g/g}$). The mortality values were significantly less ($P < 0.05$) than those of the FL

population, but in general were not significantly different compared to the mortalities observed for the Cry1F-RR population ($P > 0.05$). At $\geq 3.16 \mu\text{g/g}$, mortality of Cry1F-RS increased significantly as the Cry1F concentration increased and reached 80.7% at $3.16 \mu\text{g/g}$ and 96.6% at $10 \mu\text{g/g}$. The mortality of Cry1F-RS at $10 \mu\text{g/g}$ was not significantly different from 100% that was observed for the FL population.

2.3.3 Susceptibility of FL, PR, and FL x PR Populations to Bt Corn Leaf Tissue: Trial One

The effect of corn hybrid, insect population, and their interaction on larval mortality at 7 days was significant ($F = 82.42$; $\text{df} = 6, 63$; $P < 0.0001$, $F = 35.91$; $\text{df} = 2, 63$; $P < 0.0001$, and $F = 4.22$; $\text{df} = 12, 63$; $P < 0.0001$, respectively). Larval mortality of the three insect populations on leaf tissue of the two non-Bt corn hybrids after 7 days varied significantly, ranging from 9.4% for PR to 66.4% for FL on DKC 67-86 (Figure 2.2). Except for these two extremes, there was generally no significant difference in larval mortality on the two non-Bt corn hybrids. A high mortality (96.9%) of FL larvae was observed on leaf tissue of Herculex[®]I expressing the Cry1F protein, which was significantly greater than the mortality observed on the non-Bt corn leaf tissue. In contrast, the PR larvae appeared to be highly resistant to Cry1F corn leaf tissue, with a 7-day mortality of only 39.1%. This mortality level was similar to the average mortality (38.5%) of the three populations on the two non-Bt corn leaf tissue. The F_1 population of the cross between FL x PR was susceptible to Cry1F leaf tissue, producing a 7-day mortality of 83.6% (Figure 2.2). This was significantly greater ($P < 0.05$) than that observed for PR but significantly less ($P < 0.05$) than the mortality of FL. However, all three insect populations were susceptible to leaf tissue of the four pyramided Bt corn hybrids. No survivors of FL were observed after 7 days on the

four pyramided Bt corn hybrids. Mortality of the FL x PR population was also high on these pyramided Bt corn products (>95%), which was not significantly different ($P > 0.05$) than that observed for the FL population.

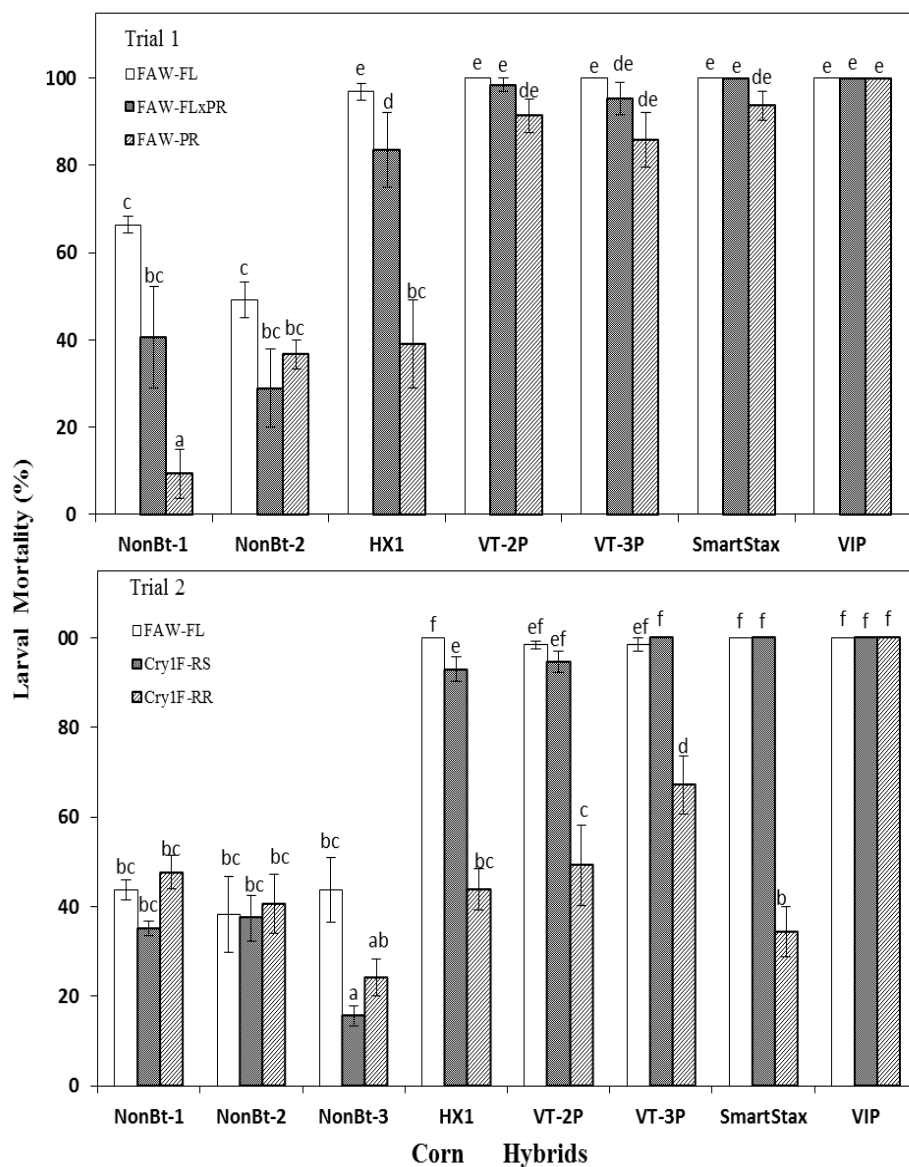


Figure 2.2. Larval mortality of *Spodoptera frugiperda* after 7 days feeding on leaf tissue removed from non-Bt and Bt corn plants. Mean values across all treatments in each figure followed by a same letter are not significantly different ($P < 0.05$; LSMEANS test). NonBt-1 in trial one = DKC 61-22, NonBt-1 in trial two= DKC 63-45, NonBt-2 in trial one= DKC 67-86, NonBt-2 in trial two= N78N-GT, NonBt-3 in trial two= Pioneer 31G66, HX1= Pioneer 31D59, VT-2P in trial one=DKC 64-04, VT-2P in trial two=DKC 63-87, VT-3P in trial one=DKC 67-88 VT-3P in trial two=DKC 62-97, SmartStax= DKC 61-21, VIP= N78N-3111

PR larvae couldn't survive on Agrisure® Viptera™ 3111 leaf tissue, while a few larvae (e.g. 6.2-14.1%) of PR survived on the other three pyramided Bt corn hybrids (Genuity®VT Double Pro™, VT Double Pro™, and SmartStax™).

2.3.4 Susceptibility of FL, Cry1F-RR, and Cry1F-RS Populations to Bt Corn Leaf Tissue: Trial Two

As observed in the first trial, the effect of corn hybrid, insect population, and their interaction on larval mortality was all significant ($F = 147.88$; $df = 7, 71$; $P < 0.0001$, $F = 120.67$; $df = 2, 71$; $P < 0.0001$, and $F = 21.27$; $df = 14, 71$; $P < 0.0001$, respectively). The 7-day larval mortality was in general similar ($P > 0.05$) on leaf tissue of the three non-Bt corn hybrids across the three insect populations with an average mortality of 36.3% (Figure 2.2). Again, larval mortality of the FL population was high and not significantly different ($P > 0.05$) on the five Bt corn hybrids, ranging from 98.4-100%. The Cry1F-RS population was also susceptible to the five Bt corn hybrids with a mortality range of 93.0 to 100%. The mortality (93.0%) of Cry1F-RS on Herculex I was similar to that (94.5%) observed on the VT Double Pro™ but was significantly less than the mortalities (100%) on VT Triple Pro™, SmartStax, and Viptera3111. The backcrossed and reselected Cry1F-RR population was also not able to survive on the Viptera 3111 hybrid. In contrast, larvae of the Cry1F-RR population survived well on the Cry1F corn hybrid with a 7-day mortality of 43.8%, which was not significantly different compared to the mortalities observed on the three non-Bt corn hybrids. However, unlike the performance of the PR population observed in the first trial, larvae of Cry1F-RR survived well on the other three pyramided Bt corn hybrids. The 7-day mortality of Cry1F-RR was only 34.4% on SmartStax™, which was not significantly different than the

mortalities observed on the three non-Bt corn hybrids. The mortality (49.2%) of Cry1F-RR on VT Double ProTM was also not significantly different compared to those values recorded on the non-Bt corn leaf tissue. Mortality (67.2%) of Cry1F-RR on the VT Triple ProTM hybrid was significantly greater ($P < 0.005$) than the mortality on non-Bt corn leaf tissue but significantly less ($P < 0.001$) than that of FL and Cry1F-RS populations on Bt corn hybrids.

2.3.5 Dominance Level of Cry1F Resistance in *S. frugiperda*

Dominance level “D” measured using Stone’s method (Stone, 1968) based on the LC₅₀ values of FL, Cry1F-RR, and Cry1F-RS populations was < -0.37 , suggesting that Cry1F resistance in Cry1F-RR was recessive or incompletely recessive (Table 2.3). Because all three populations could not survive on leaf tissue of the VipteraTM 3111 hybrid (N78N-3111) in both trials, effective dominance level, D_{ML}, couldn’t been calculated for this Bt corn product. D_{ML} values measured based on larval mortality on leaf tissue of the other six Bt corn hybrids were consistent in the two trials, ranging from 0 to 0.33 in trial one and from 0 to 0.22 in trial two. The results suggested that the Cry1F resistance in *S. frugiperda* was functionally recessive to incompletely recessive on leaf tissue of the five Bt corn hybrids representing four Bt corn traits, Herculex[®]I, Genuity[®]VT Double ProTM, Triple ProTM, and SmartStaxTM.

2.4 Discussion

Since first being commercialized in 1996, Bt crops have gained an international attention and widely acceptance in the world, especially among corn and cotton producers in the United States (James, 2011; NASS, 2012). Cry1F expressed corn (event TC1507) was registered in 2001 in the United States to control above-ground lepidopteran pests including

Table 2.3. Dominance Level of Cry1F resistance in *Spodoptera frugiperda* computed using data from diet incorporating and leaf tissue bioassays.

Test material and trial	Dominance level*
Stone's dominance "D" value	
Diet incorporating, trial-2	-0.37
Effective dominance " D_{ML} "	
Pioneer 31D59, leaf tissue, trial-1	0.23
DKC 64-04, leaf tissue, trial-1	0.19
DKC 67-88, leaf tissue, trial-1	0.33
DKC 61-21, leaf tissue, trial-1	0.00
N78N-3111, leaf tissue, trial-1	---
Pioneer 31D59, leaf tissue, trial-2	0.12
DKC 63-87, leaf tissue, trial-2	0.08
DKC 62-97, leaf tissue, trial-2	0.00
DKC 61-21, leaf tissue, trial-2	0.00
N78N-3111, leaf tissue, trial-2	---

* Stone's dominance "D" value ranges from -1 to 1: a value of -1 indicates resistance is completely recessive; a value of 0 suggests resistance is additive; and a value of 1 implies resistance is completely dominant. Effective dominance " D_{ML} " ranges between 0 and 1. $D_{ML} = 0$ refers to a completely recessive resistance and $D_{ML} = 1$ means the resistance is completely dominant.

S. frugiperda. In 2003, Cry1F corn was first commercially cultivated in Puerto Rico to control *S. frugiperda*. This insect is the most important lepidopteran corn pest in Puerto Rico (US-EPA, 2007; Storer et al., 2012). Studies have revealed that field resistance to Cry1F corn in *S. frugiperda* occurred in late 2006 (US-EPA, 2007; Matten et al., 2008; Storer et al., 2010; Huang et al., 2011). In the current study, susceptibility of two field populations of *S.*

frugiperda collected from Florida and Puerto Rico to purified Cry1F protein and corn leaf tissue of a commercial Cry1F corn hybrid was evaluated in the laboratory. Limited by the available amount of purified Bt protein, Cry1F susceptibility of *S. frugiperda* could be assayed up to only 100 µg/g in this study. No significant larval mortality of the PR and Cry1F-RR populations was observed even at the highest Cry1F concentrations examined in the bioassays. The LC₅₀ values, therefore, could not be determined with the probit analysis for both populations (Table 2.2). Nevertheless, the results of this study clearly demonstrated that the population from Puerto Rico, compared to the Florida population, was highly resistant to both purified Cry1F protein and Cry1F corn leaf tissue. The Cry1F resistance was recessive or incompletely recessive as measured with both the Stone's dominance "D" value on Cry1F diet and the effective dominance level "D_{ML}" on Cry1F corn leaf tissue. In the calculation of dominance, the FL population was considered homozygous susceptible with no resistance alleles. The resistance could be more recessive than that measured in this study if the assumption was not true. The dominance levels of the Cry1F resistance estimated in this study appeared to be similar as reported in another population of *S. frugiperda* collected from Puerto Rico in 2007 (Storer et al., 2010). It was reported that upon an initial confirmation of the field resistance to Cry1F corn in Puerto Rico, the technology providers immediately stopped the commercial sale of Cry1F corn seeds to growers in this area (Matten et al., 2008; Storer et al., 2010). Although limited by the insect sampling in the current study, our results suggest that the field resistance to Cry1F corn was persistent in Puerto Rico even after several years without planting of Cry1F corn. A recent study also reported that field populations of *S.*

frugiperda collected from two other locations in 2011 were still highly resistant to Cry1F protein in diet (Storer et al., 2012).

In both leaf tissue tests, all five populations of *S. frugiperda* could not survive for 7 days on the Agrisure® Viptera™ 3111 hybrid. The results suggested that Viptera™ 3111 Bt corn could completely overcome the Cry1F resistance and thus should provide a means for managing Cry1F resistance in this important target pest of Bt corn. In a previous study, survival of 14,400 neonates from 150 two-parental family lines of *S. frugiperda* collected from Florida and Louisiana was evaluated on Agrisure® Viptera™ 3111 plants using an F₂ screen (Yang et al., 2013a). Results of that study showed that all larvae were killed within 7 days on Viptera™ 3111 corn leaf tissue. Although both the current and previous studies were not designed to evaluate the high dose assumption, the results of these studies suggest that Viptera™ 3111 corn is highly effective and likely produces a “high-dose” against *S. frugiperda*.

The Cry1F-susceptible population, FL, and the two F₁ populations of two crosses, FL x PR and Cry1F-RS, were also susceptible to the other three pyramided Bt corn products: Genuity® VT Double Pro™, Triple Pro™, and SmartStax™. The 7-day mortality on these Bt corn products was $\geq 94.5\%$ in both trials (Figure 2.2). The results demonstrated that the Cry1F resistance in *S. frugiperda* was functionally recessive or nearly completely recessive on corn leaf tissue of the three pyramided Bt corn traits as showed in the D_{ML} values in Table 2.3. However, performance of the Cry1F resistant populations (PR and Cry1F-RR) on corn leaf tissue of Genuity® VT Double Pro™, Triple Pro™, and SmartStax™ varied between the two trials. In the first trial, PR larvae appeared to be susceptible to the three pyramided Bt

corn products with a 7-day mortality of 85.9-93.8% (Figure 2.2). In contrast, larvae of the backcrossed and reselected population, Cry1F-RR, survived well on the three pyramided Bt corn products in the second trial with a 7-day mortality of only 34.4 to 67.2% (Figure 2.2). The exact reasons causing the difference are unknown. One of the most likely reasons could be due to a result of the continued selections on Cry1F corn leaf tissue both before and after the backcross. For example, the original population (PR) collected from Puerto Rico might still not be homozygous for the Cry1F resistance and continued selection on Cry1F corn leaf tissue could eliminate the susceptible and probably heterozygous individuals in the population and thus could further elevate the resistance level in the Cry1F-RR population.

Additional studies are still needed to demonstrate if the Cry1F-RR population could survive on whole plants of these pyramided Bt corn products. Nevertheless, the results of the current study suggest that at least some levels of cross-resistance to the three pyramided Bt corn traits exist in Cry1F corn resistant *S. frugiperda*. Both VT Double ProTM and Triple ProTM contain Cry1A.105 and Cry2Ab2, while SmartStaxTM expresses those proteins and Cry1F. Cry1A.105 is a chimeric gene comprised of domains I and II which are identical with the respective domains from Cry1Ab and Cry1Ac and domain III of Cry1F (Biosafety Clearing-House, 2009). Thus it should not be surprising that some levels of cross-resistance could exist between Cry1F and Cry1A.105 because of the association in the gene structures of the two proteins. Studies have shown that *S. frugiperda* was somewhat tolerant to the single gene Cry1Ab corn hybrids (US-EPA, 2001b; Chilcutt et al., 2007; Hardke et al., 2011; Huang et al., 2011). In addition, Cry1Ac usually shares similar binding sites with Cry1Ab in the insect midgut membranes (Ballester et al., 1999; Ferré and Van Rie, 2002; Hua et al.,

2001; Tan, 2010) and thus Cry1Ab resistance is often found to be cross-resistant to Cry1Ac in many insect species (Tabashnik et al., 1994; Ferré and Van Rie, 2002; Rang et al., 2004; Siqueira et al., 2004; Wu et al., 2009; Pereira et al., 2010; Tan, 2010; Crespo et al., 2011; Zhang et al., 2013). Cry2Ab2 is a different protein compared to Cry1A and studies have shown that a Cry1A resistant insect is usually not cross-resistant to Cry2Ab2 (Wu et al., 2009; Brévault et al., 2009; Sivasupramaniam et al., 2008). Thus, the survival of the Cry1F-RR population on the three pyramided Bt corn products, Genuity®VT Double Pro™, VT Triple Pro™, and SmartStax™, could be due to a combination factor of cross-resistance and the expression level of Cry2Ab2 protein that may be not high enough by it alone to kill the Cry1F resistant larvae in a 7-day period of bioassays. The possible cross-resistance between single-gene and pyramided Bt corn in *S. frugiperda* suggest that careful selection of different Bt genes is essential in use of gene pyramiding strategy for resistance management.

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CHAPTER 3. LARVAL SURVIVAL AND PLANT INJURY OF CRY1F-SUSCEPTIBLE, -RESISTANT, AND -HETEROZYGOUS FALL ARMYWORM (LEPIDOPTERA: NOCTUIDAE) ON NON-BT AND BT CORN CONTAINING SINGLE OR PYRAMIDED GENES

3.1 Introduction

Event TC1507 *Bacillus thuringiensis* (Bt) corn (e.g. Herculex[®]I), *Zea mays* L., was first registered in 2001 in the United States and later cultivated in Puerto Rico in 2003 for controlling above-ground stalk borers and some noctuid moths including the fall armyworm, *Spodoptera frugiperda* (J.E. Smith), which is the most important corn pest in Puerto Rico (US-EPA, 2007; Storer et al., 2010). Unfortunately, field resistance of *S. frugiperda* to TC1507 was first observed in Puerto Rico in 2006 (US-EPA, 2007; Matten et al., 2008; Storer et al., 2010). Besides the intensive use of TC1507 products in Puerto Rico, several other factors might also contribute to the resistance (US-EPA, 2007; Storer et al., 2010; Huang et al., 2011). Modeling has shown that target insect pests could develop resistance more rapidly to single Bt protein than to multiple toxins (Roush, 1998; Zhao et al., 2003). To delay resistance development, a gene-pyramiding strategy has been utilized to develop transgenic plants that express multiple Bt proteins for targeting a pest species (US-EPA, 2010; Ghimire et al., 2011; Matten et al., 2012). The first commercialized pyramided Bt corn traits for managing lepidopteran pests in the United States included Genuity[®]VT Double Pro[™] (hereafter called VT-2P), Genuity[®]VT Triple Pro[™] (VT-3P), Genuity[®] SmartStax[™] (SmartStax), and Agrisure[®] Viptera[™] 3111 (VIP3) (Difonzo and Collen, 2012). Compared to the first generation single-gene Bt corn, the pyramided Bt corn products are more effective for controlling some noctuid moth species including *S. frugiperda* (Burkness et al., 2010, Yang et al., 2013a; 2013b), and thus *S. frugiperda* has been listed as a target for all pyramided Bt corn traits that have been commercialized for managing above-ground lepidopteran corn pests (US-EPA, 2009; 2010; Monsanto, 2012; Syngenta, 2012).

During 2011, a field population of *S. frugiperda* was established from larvae collected from corn fields in Puerto Rico. A previous study showed that the Puerto Rico population was highly resistant to both purified Cry1F protein and Cry1F corn leaf tissue (Niu et al., 2013). Leaf tissue bioassays also showed that the Cry1F-resistant population collected from Puerto Rico exhibited a significant level of cross-resistance to several pyramided Bt corn traits that are currently used in the United States and several other countries (Niu et al., 2013). The objective of this study was to determine if the currently used pyramided Bt corn traits are effective against the Cry1F-resistant *S. frugiperda*. Information generated from this study is useful in developing effective strategies to manage Cry1F resistance in *S. frugiperda*.

3.2 Materials and Methods

3.2.1 Insect Sources

Three populations, RR, SS-FL, and SS-LA, of *S. frugiperda* collected from Puerto Rico, Florida, and Louisiana, respectively, were used as the insect sources in this study. RR was originated from >300 feral larvae collected from a corn field in south Puerto Rico in 2011. Laboratory bioassays have shown that progeny of the original RR were highly resistant to purified Cry1F protein and Cry1F corn leaf tissue (Niu et al., 2013). SS-FL was initiated from 96 larvae sampled from non-Bt corn fields in Hendry County in south Florida in 2011, which was documented to be highly susceptible to the Cry1F protein (Niu et al., 2013). SS-LA was established from cotton and corn fields in 2008 in Louisiana (Hardke et al., 2011). Since then, SS-LA had been maintained in the laboratory without exposure to Bt proteins or any other insecticides. Diet incorporated bioassays also showed SS-LA was susceptible to Cry1F protein (FH unpublished data).

In the laboratory, larvae of *S. frugiperda* were reared individually on a meridic diet (WARD'S Stonefly Heliothis diet, Rochester, NY) in 30-ml plastic cups (Fill-Rite, Newark, NJ) until pupal stage. The larvae were held in 30-well trays at room conditions until pupation

(Niu et al., 2013). Pupae were placed in 3.8-liter paper containers (Huhtamaki Foodservice, De Soto, KS) containing ~100 g of vermiculite (Sun Gro, Pine Bluff, AR). A cotton/paper ball saturated with 10% sugar solution was held in a 100-ml cup and placed in each container. The containers were then placed in growth chambers maintained at 28 °C, >90% RH, and a photoperiod of 14:10 (L: D) h for adult emergence, mating, and oviposition.

Before the RR strain was used for this study, it was selected on Cry1F corn (Pioneer 31D59) leaf tissue for two generations as described in Niu et al. (2013). The RR strain was then backcrossed with SS-FL and reselected on Cry1F corn leaf tissue in the F₂ generations (Niu et al., 2013). In addition, two F₁ hybrid genotypes, RS-FL and RS-LA, were also developed for this study. RS-FL was generated by crossing SS-FL with the backcrossed and reselected RR, while RS-LA was developed by crossing SS-LA with RR. During 2011-2013, a total of three independent trials were conducted in the greenhouse. SS-FL, the backcrossed and reselected RR, and RS-FL were utilized in trial 1 that was conducted in 2011. In 2013, SS-LA, RR, and RS-LA were used in the trials 2 and 3 because SS-FL was not available when these two trials were conducted.

3.2.2 Source of Corn Plants

Larval survival and plant injury of *S. frugiperda* were evaluated on five non-Bt and eight Bt corn hybrids in the three trials (Table 3.1). The eight Bt corn hybrids represent six Bt corn traits including two single-gene Bt corn hybrids containing Herculex[®] I (hereafter called HX1) and YieldGard[®] (YG) traits, respectively, and six pyramided Bt corn hybrids representing four traits: VT-2P, VT-3P, SmartStax, and VIP3. HX1 contains a single Bt gene, Cry1F, for controlling above-ground lepidopteran species including *S. frugiperda*. YG expresses one Bt gene, Cry1Ab, mainly targeting above-ground stalk borers. VT-2P produces two Cry proteins, Cry1A.105 and Cry2Ab2, which are active against above-ground lepidopteran species. VT-3P contains the same two Cry proteins in VT-2P plus Cry3Bb1.

Table 3.1. Corn hybrids and traits evaluated in the three greenhouse trials

Corn hybrid	Bt trait	Event	Bt genes	Major target pests ^a	Used in trials
DKC 61-22	Non-Bt corn, closely related to DKC 61-21	---	---	--	1
DKC 67-86	Non-Bt corn, closely related to DKC 67-88	---	---	--	1
DKC 63-45	Non-Bt corn, closely related to DKC 63-87	---	---	--	2, 3
N78N-GT	Non-Bt corn, closely related to N78N-3111	---	---	--	2, 3
Pioneer 31P40	Non-Bt corn, closely related to Pioneer 31D59	---	---	--	2, 3
Pioneer 31D59	Herculex [®] I	TC1507	Cry1F	Stalk borers, FAW	1, 2, 3
DKC 69-70	YieldGard [®]	MON810	Cry1Ab	Stalk borers	1, 2, 3
DKC 64-04	Genuity [®] VT Double Pro [™]	MON89034	Cry1A.105, Cry2Ab2	Stalk borers, CEW, FAW	1
DKC 63-87	Genuity [®] VT Double Pro [™]	MON89034	Cry1A.105, Cry2Ab2	Stalk borers, FAW, CEW	2, 3

(Table 3.1 continued)

Corn hybrid	Bt trait	Event	Bt genes	Major target pests ^a	Used in Trials
DKC 67-88	Genuity [®] VT Triple Pro [™]	MON89034+ MON88017	Cry1A.105, Cry2Ab2, Cry3Bb1	Stalk borers, FAW, CEW, rootworms	1
DKC 62-97	Genuity [®] VT Triple Pro [™]	MON89034+ MON88017	Cry1A.105, Cry2Ab2, Cry3Bb1	Stalk borers, FAW, CEW, rootworms	2, 3
DKC 61-21	Genuity [®] SmartStax [®]	MON89034+ TC1507+MON 88017+DAS- 59112-7	Cry1A.105, Cry2Ab, Cry1F, Cry3Bb1, Cry34/35Ab	Stalk borers, FAW, CEW, rootworms	1,2, 3
N78N-3111	Agrisure [®] Viptera [™] 3111	Bt11+MIR162+ MIR604	Vip3A, Cry1Ab, mCry3A	Stalk borers, FAW, CEW, rootworms	1,2 & 3

^a Major stalk borer species in the United States include European corn borer, *Ostrinia nubilalis* (Hübner) and southwestern corn borer *Diatraea grandiosella* Dyar. Major corn rootworm species in the United States include western corn rootworm, *Diabrotica virgifera virgifera* LeConte, northern corn rootworm, *Diabrotica barberi* Smith and Lawrence, and Mexican corn rootworm, *Diabrotica virgifera zea* Krysan and Smith. FAW: fall armyworm, *Spodoptera frugiperda* (J.E. Smith), CEW: corn earworm, *Helicoverpa zea* (Boddie).

SmartStax produces six Bt proteins including the three Bt proteins of VT-3P plus Cry1F and Cry34/35Ab1 (Monsanto, 2012). Cry3Bb1 and Cry34/35Ab1 are active against the below-ground corn rootworms, *Diabrotica* spp (Coleoptera: Chrysomelidae) but offer no activity against lepidopteran species. VIP3 expresses three Bt proteins including Vip3A and Cry1Ab for controlling lepidopteran species and mCry3A for managing rootworms (DiFonzo and Collen, 2012). The five non-Bt corn hybrids were genetically closely related to one or two of the eight Bt corn hybrids. In each trial, seven (trial 1) or nine (trials 1 and 2) of the 13 corn hybrids were evaluated in the greenhouse (Table 3.1).

Two corn seeds of a hybrid were planted in 18.9-liter pots containing ~5 kg of a standard potting soil mixture. Pots were placed within a Louisiana State University Agricultural Center greenhouse located in Baton Rouge, LA. The plants were irrigated and fertilized for optimum growth during the tests as described in Wangila et al. (2012). Expression/not expression of Bt proteins in plants was confirmed using ELISA-based assays (EnviroLogix, Quantiplate™ kits, Portland, ME).

3.2.3 Insect Infestation

Three (trial 1) or five (trials 2 and 3) neonates of an insect genotype of *S. frugiperda* mentioned above were manually placed into the whorl of a plant at V6-V8 (trial 1) or V8-V10 (trials 2 and 3) plant stages. Treatment combinations of corn hybrid and insect genotype in each trial were replicated four times in a randomized complete block design with 4 plants (or 2 pots) in each replication. To minimize larval movement from plant to plant, there was an approximately 1-meter alley between blocks and a distance that did not allow the plants to touch each other between treatment combinations within a block. Corn leaf injury ratings were made twice using the Davis' 1 (no injury or

few pinholes) to 9 (most leaves with long lesions) scales (Davis et al., 1992). The first sampling of leaf injury for all trials was taken at the 7th d after larval infestation, while the second data were sampled at the 15th d for the trial 1 when the trial was terminated. Numbers of live larvae of *S. frugiperda* in each plant in trial 1 was also recorded at the 2nd data samplings. Observations during trial 1 showed that some of heavily damaged plants did not contain live larvae after 15 d, suggesting that some larvae already matured and moved out from the plants for pupation. To increase the accuracy of larval survivorship, larval growth and development of *S. frugiperda* on non-Bt plants in trials 2 and 3 were monitored carefully. Based on the monitoring, the second sampling of leaf injury along with the number of live larvae in trials 2 and 3 was checked after 12 d of larval release when most larvae on the non-Bt corn plants were in the 4th and 5th instars.

3.2.4 Data Analysis

Because of the cannibalistic behavior of *S. frugiperda*, especially in the late larval stages (Raffa, 1987), larval survivorship in this study was measured as percentage of the plants containing live larvae at the termination of each trial (after 15 d for trial 1 and after 12 d for trials 2 and 3). Data of the greenhouse tests showed that performance between the two susceptible strains (SS-FL and SS-LA) as well as between the two heterozygous genotypes (RS-FL and RS-LA) was similar across all corn hybrids in the three trials (Tables 3.2-3.4). To facilitate data analysis, both SS-FL and SS-LA were treated as a same susceptible genotype (SS), while both RS-FL and RS-LA were considered as a same heterozygous genotype (RS) during data analysis and result presentations (Tables 3.2-3.4). Also the performance of an insect genotypes was similar among non-Bt corn hybrids in each trial (data not shown); and thus data on larval survival and leaf injury

Table 3.2. Leaf injury ratings (mean \pm sem) of non-Bt and Bt corn plants containing single or multiple Bt genes recorded after 7 d infested with neonates of Cry1F-susceptible (SS), -heterozygous (RS), and -resistant (RR) genotypes of *Spodoptera frugiperda*

Corn traits ^a	Insect	Trial-1 ^b	Trial-2 ^b	Trial-3 ^b	Combined ^b
Non-Bt	SS	6.4 \pm 0.2c	5.4 \pm 0.1b	5.6 \pm 0.2 b	5.7 \pm 0.1 c
	RS	6.2 \pm 0.3c	5.4 \pm 0.1b	6.0 \pm 0.1 b	5.8 \pm 0.1 c
	RR	6.0 \pm 0.2c	5.3 \pm 0.1b	5.6 \pm 0.2 b	5.6 \pm 0.1 c
HX1	SS	1.9 \pm 0.2 b	1.1 \pm 0.1a	1.3 \pm 0.1 a	1.4 \pm 0.1 b
	RS	1.4 \pm 0.2 ab	1.3 \pm 0.2a	1.4 \pm 0.3 a	1.4 \pm 0.1 b
	RR	5.8 \pm 0.3 c	5.5 \pm 0.1b	6.0 \pm 0.3 b	5.7 \pm 0.2 c
YG	SS	---	5.0 \pm 0.5b	5.2 \pm 0.2 b	5.1 \pm 0.2 c
	RS	---	5.0 \pm 0.0b	5.1 \pm 0.1 b	5.0 \pm 0.0 c
	RR	---	4.8 \pm 0.3b	5.7 \pm 0.1 b	5.2 \pm 0.2 c
VT-2P	SS	1.8 \pm 0.2 ab	1.0 \pm 0.0 a	1.2 \pm 0.1 a	1.3 \pm 0.1 ab
	RS	1.1 \pm 0.1 a	1.0 \pm 0.0 a	1.1 \pm 0.1 a	1.0 \pm 0.0 ab
	RR	1.8 \pm 0.1 b	1.1 \pm 0.1a	1.0 \pm 0.0 a	1.3 \pm 0.1 ab
VT-3P	SS	2.0 \pm 0.1 b	1.0 \pm 0.0 a	1.1 \pm 0.1 a	1.4 \pm 0.1 ab
	RS	1.3 \pm 0.2 ab	1.0 \pm 0.0 a	1.0 \pm 0.0 a	1.1 \pm 0.1 ab
	RR	2.4 \pm 0.4 b	1.0 \pm 0.0 a	1.3 \pm 0.1 a	1.6 \pm 0.2 b
SmartStax	SS	1.4 \pm 0.1 ab	1.0 \pm 0.0a	1.2 \pm 0.1 a	1.2 \pm 0.1 ab
	RS	1.1 \pm 0.1 ab	1.0 \pm 0.0a	1.0 \pm 0.0 a	1.0 \pm 0.0 ab
	RR	2.1 \pm 0.2 b	1.0 \pm 0.0a	1.1 \pm 0.1 a	1.4 \pm 0.2 ab

(Table 3.2 continued)

Corn traits ^a	Insect	Trial-1 ^b	Trial-2 ^b	Trial-3 ^b	Combined ^b
VIP3	SS	1.3 ± 0.2 ab	1.0 ± 0.0a	1.2 ± 0.2 a	1.2 ± 0.1 ab
	RS	1.1 ± 0.1 a	1.0 ± 0.0a	1.0 ± 0.0 a	1.0 ± 0.0 a
	RR	1.3 ± 0.1 ab	1.0 ± 0.0a	1.0 ± 0.0 a	1.1 ± 0.0 ab
Analysis of variance	Effect of insect	$F_{2,63} = 64.50$	$F_{2,84} = 64.66$	$F_{2,84} = 34.26$	$F_{2,268} = 93.54$
		$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
	Effect of corn	$F_{5,63} = 281.52$	$F_{6,84} = 1533.96$	$F_{6,84} = 621.56$	$F_{6,268} = 1243.72$
		$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
	Effect of insect x corn	$F_{10,63} = 15.81$	$F_{12,84} = 67.17$	$F_{12,84} = 29.28$	$F_{12,268} = 57.44$
		$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$

^a Non-Bt = Non-Bt corn plants; HX1 = Herculex[®] I; YG = YieldGard[®] Corn Borer ; VT-2P = Genuity[®] VT Double Pro[™]; VT-3P = Genuity[®] VT Triple Pro[™]; SmartSta = Genuity[®] SmartStax[™]; and VIP3 = Agrisure[®] Viptera[™] 3111.

^b Mean values followed by a same letter in a column are not significantly different ($P > 0.05$; Tukey's honestly significant difference tests).

Table 3.3. Leaf injury ratings (mean \pm sem) of non-Bt and Bt corn plants containing single or multiple Bt genes recorded at the trial terminations after 15 d (for trial 1) or 12 d (for trials 2 and 3) infested with neonates of Cry1F-susceptible (SS), -heterozygous (RS), and -resistant (RR) genotypes of *Spodoptera frugiperda*

Corn trait ^a	Insect	Trial ^b	Trial 2 ^b	Trial 3 ^b	Combined ^b
Non-Bt	SS	8.9 \pm 0.1 b	6.8 \pm 0.1 c	7.2 \pm 0.2 b	7.5 \pm 0.2 c
	RS	8.8 \pm 0.1 b	7.2 \pm 0.1 c	7.6 \pm 0.2 b	7.7 \pm 0.2 c
	RR	9.0 \pm 0.0 b	7.0 \pm 0.2 c	7.3 \pm 0.3 b	7.6 \pm 0.2 c
HX1	SS	2.3 \pm 0.5 a	1.0 \pm 0.0 a	1.1 \pm 0.1 a	1.4 \pm 0.2 a
	RS	1.6 \pm 0.3 a	1.0 \pm 0.0 a	1.1 \pm 0.1 a	1.3 \pm 0.1 a
	RR	8.4 \pm 0.2 b	6.8 \pm 0.1 c	7.6 \pm 0.4 b	7.6 \pm 0.2 c
YG	SS	---	4.9 \pm 0.4 b	6.5 \pm 0.4 b	5.7 \pm 0.4 b
	RS	---	5.2 \pm 0.6 b	6.4 \pm 0.1 b	5.8 \pm 0.4 b
	RR	---	6.3 \pm 0.5 c	7.6 \pm 0.2 b	6.9 \pm 0.4 bc
VT-2P	SS	1.2 \pm 0.2 a	1.0 \pm 0.0 a	1.0 \pm 0.0 a	1.1 \pm 0.1 a
	RS	1.4 \pm 0.4 a	1.0 \pm 0.0 a	1.0 \pm 0.0 a	1.1 \pm 0.1 a
	RR	2.3 \pm 1.0 a	1.0 \pm 0.0 a	1.0 \pm 0.0 a	1.4 \pm 0.3 a
VT-3P	SS	1.0 \pm 0.0 a	1.0 \pm 0.0 a	1.0 \pm 0.0 a	1.0 \pm 0.0 a
	RS	1.0 \pm 0.0 a	1.0 \pm 0.0 a	1.0 \pm 0.0 a	1.0 \pm 0.0 a
	RR	2.1 \pm 0.7 a	1.0 \pm 0.0 a	1.0 \pm 0.0 a	1.4 \pm 0.3 a

(Table 3.3 continued)

Corn trait ^a	Insect	Trial ^b	Trial 2 ^b	Trial 3 ^b	Combined ^b
SmartStax	SS	1.0 ± 0.0 a	1.0 ± 0.0 a	1.0 ± 0.0 a	1.0 ± 0.0 a
	RS	1.0 ± 0.0 a	1.1 ± 0.1 a	1.0 ± 0.0 a	1.0 ± 0.0 a
	RR	1.3 ± 0.3 a	1.0 ± 0.0 a	1.0 ± 0.0 a	1.1 ± 0.1 a
VIP3	SS	1.0 ± 0.0 a	1.0 ± 0.0 a	1.0 ± 0.0 a	1.0 ± 0.0 a
	RS	1.0 ± 0.0 a	1.0 ± 0.0 a	1.0 ± 0.0 a	1.0 ± 0.0 a
	RR	1.4 ± 0.4 a	1.0 ± 0.0 a	1.0 ± 0.0 a	1.1 ± 0.1 a
Analysis of various	Effect of insect	$F_{2,63} = 30.61$	$F_{2,84} = 104.88$	$F_{2,84} = 81.20$	$F_{2,268} = 114.53$
		$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
	Effect of corn	$F_{5,63} = 201.36$	$F_{6,84} = 1534.72$	$F_{6,84} = 1301.19$	$F_{6,268} = 1308.69$
		$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
	Effect of insect x corn	$F_{10,63} = 7.87$	$F_{12,84} = 76.96$	$F_{12,84} = 63.76$	$F_{12,268} = 59.61$
		$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$

^a Non-Bt = Non-Bt corn plants; HX1 = Herculex[®] I; YG = YieldGard[®] Corn Borer ; VT-2P = Genuity[®] VT Double Pro[™]; VT-3P = Genuity[®] VT Triple Pro[™]; SmartSta = Genuity[®] SmartStax[™]; and VIP3 = Agrisure[®] Viptera[™] 3111.

^b Mean values followed by a same letter in a column are not significantly different ($P > 0.05$; Tukey's honestly significant difference tests)

Table 3.4. Percent plants (mean \pm sem) containing live larvae of non-Bt and Bt corn plants containing single or multiple Bt genes recorded at the trial terminations after 15 d (for trial 1) or 12 d (for trials 2 and 3) infested with neonates of Cry1F-susceptible (SS), -heterozygous (RS), and -resistant (RR) genotypes of *Spodoptera frugiperda*

Corn trait ^a	Insect	Trial 1 ^b	Trial 2 ^b	Trial 3 ^b	Combined ^b
Non-Bt	SS	56.3 \pm 9.1 b	97.9 \pm 2.1 b	93.8 \pm 3.3 b	85.9 \pm 4.0 b
	RS	50.0 \pm 8.2 b	95.8 \pm 2.8 b	91.7 \pm 4.7 b	82.8 \pm 4.4 b
	RR	68.8 \pm 9.1b	97.9 \pm 2.1 b	87.5 \pm 5.8 b	86.7 \pm 3.7 b
Herculex I	SS	0.0 \pm 0.0 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a
	RS	0.0 \pm 0.0 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a
	RR	43.8 \pm 12.0 b	100 \pm 0.0 b	75.0 \pm 14.4 b	72.9 \pm 8.9 b
YG	SS	---	81.3 \pm 12.0 b	93.8 \pm 6.3 b	87.5 \pm 6.7 b
	RS	---	91.7 \pm 8.3 b	100 \pm 0.0 b	95.8 \pm 4.2 b
	RR	---	93.8 \pm 6.2 b	100 \pm 0.0 b	96.9 \pm 3.1 b
VT-2P	SS	0.0 \pm 0.0 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a
	RS	0.0 \pm 0.0 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a
	RR	0.0 \pm 0.0 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a
VT-3P	SS	0.0 \pm 0.0 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a
	RS	0.0 \pm 0.0 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a
	RR	0.0 \pm 0.0 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a
SmartStax	SS	0.0 \pm 0.0 a	0.0 \pm 0.0a	0.0 \pm 0.0 a	0.0 \pm 0.0 a
	RS	0.0 \pm 0.0 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a
	RR	0.0 \pm 0.0 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a
VIP	SS	0.0 \pm 0.0 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a
	RS	0.0 \pm 0.0 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a
	RR	0.0 \pm 0.0 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a

(Table 3.4 continued)

Corn trait ^a	Insect	Trial 1 ^b	Trial 2 ^b	Trial 3 ^b	Combined ^b
Analysis of variance	Effect of insect	$F_{2,63} = 28.20$ $P < 0.0001$	$F_{2,84} = 28.36$ $P < 0.0001$	$F_{2,84} = 7.22$ $P = 0.0013$	$F_{2,268} = 20.29$ $P < 0.0001$
	Effect of corn	$F_{5,63} = 82.07$ $P < 0.0001$	$F_{6,84} = 408.67$ $P < 0.0001$	$F_{6,84} = 2109.6$ $P < 0.0001$	$F_{6,268} = 401.46$ $P < 0.0001$
	Effect of insect x corn	$F_{10,63} = 3.20$ $P = 0.0025$	$F_{12,84} = 22.31$ $P < 0.0001$	$F_{12,84} = 7.27$ $P < 0.0001$	$F_{12,268} = 16.44$ $P < 0.0001$

^a Non-Bt = Non-Bt corn plants; HX1 = Herculex[®] I; YG = YieldGard[®] Corn Borer ; VT-2P = Genuity[®] VT Double Pro[™]; VT-3P = Genuity[®] VT Triple Pro[™]; SmartSta = Genuity[®] SmartStax[™]; and VIP3 = Agrisure[®] Viptera[™] 3111.

^b Mean values followed by a same letter in a column are not significantly different ($P > 0.05$; Tukey's honestly significant difference tests).

ratings recorded from the non-Bt corn hybrids (two hybrids in trial 1 and three in trials 2 and 3) were combined for data analysis. Data of leaf injury ratings were transformed to the $\log(x+1)$ scale, while percentages of plants with live larvae were transformed using arcsine of $(x^{0.5})$ to normalize treatment variances (Zar, 1984). The transformed data were first analyzed with a two-way analysis of variance (ANOVA) for each trial (SAS Institute, 2010) with corn hybrid and insect genotype as the two main factors. In addition, because the overall results were also consistent across the trials, data for each variable were combined across the three trials. The leaf injury ratings recorded at the trial terminations were treated as one variable in the combined data because both observation times measured nearly the maximum leaf injury of the insect. Correspondingly, data on the percent plants containing live larvae in the three trials were combined in the same way. The combined data were analyzed using mixed models with trial as a random factor (SAS Institute, 2010). Treatment means for each trial and the combined data were separated using Tukey's honestly significant difference tests at $\alpha = 0.05$ level.

3.3 Results

3.3.1 Leaf Injury Ratings of Non-Bt and Bt Corn Containing Single or Pyramided Genes caused by Cry1F-susceptible, -resistant and –heterozygous Genotypes of *S. frugiperda*

Leaf injury ratings caused by *S. frugiperda* were consistent across trials and observation times. The effects of corn hybrid, insect genotype, and their interaction on leaf injury ratings at both sampling times were all significant for individual trials and for combined analysis (Tables 3.2 and 3.3). There were no significant ($P < 0.05$) differences in the leaf injury ratings among non-Bt corn traits infested with the three insect genotypes. All three insect genotypes caused heavy leaf injuries to non-Bt corn plants with an average leaf injury rating of 5.7 after 7 d (Table 3.2) and 7.6 after 12-15 d (Table 3.3).

On Bt corn plants, both SS and RS caused little damage to HX1 with a leaf injury rating of 1.4-1.9 after 7 d and 1.0 - 2.3 after 12-15 d and the differences between the two insect genotypes were not significant ($P > 0.05$) in all three trials as well as in the combined analysis (Tables 3.2 and 3.3). However, leaf injury rating of HX1 by the Cry1F-resistant RR strain was high, averaging 5.7 after 7 d and 7.6 after 12-15 d, which were the same levels as the overall leaf injury ratings observed on the non-Bt corn plants. Leaf injury rating of YG plants was also high and not significantly different among the three insect genotypes, ranging from 4.8-5.7 after 7 d and 4.9-7.6 after 12 d in the two trials (Tables 3.2 and 3.3). Based on the combined analysis, the leaf injury ratings of YG were not different after 7 d compared to those of non-Bt corn plant and they were significantly ($P < 0.05$), although only a little less than those of the non-Bt corn after 12-15 d. In contrast, no or little leaf damage was observed on plants of the four pyramided Bt corn traits infested with the three insect genotypes. For the three trials, RR larvae caused an average leaf injury rating of 1.1-1.6 after 7 d and 1.0-1.4 after 12-15 d across the four pyramided Bt corn traits. The amount of injury caused by the RR strain was not significantly ($P < 0.05$) different compared to that caused by SS (1.2-1.4 after 7 d and 1.0-1.1 after 12-15 d) or RS (1.0-1.1) (Tables 3.2 and 3.3).

3.3.2 Larval Survival of Cry1F-susceptible, -resistant and –heterozygous genotypes of *S. frugiperda* on Non-Bt and Bt Corn Containing Single or Pyramided genes

Larval survivorship rates of the three genotypes of *S. frugiperda* on a corn hybrid were highly correlated with the leaf injury ratings of the plant. The overall percentage of plants containing live larvae observed in the trial 1 at 15 d was less than those recorded in the trials 2 and 3 which were observed at 12-d after larval release. Based on our observation in trial 1, this difference was likely due to the difference in number of larvae infested per plant and because some mature larvae that had pupated before the data were taken in trial 1. However, the overall survival of the three insect genotypes on each corn trait was consistent across all trials. As observed in the leaf injury ratings, the effect of corn trait, insect genotype,

and their interaction on larval survival was all significant across the three trials and in the combined analysis (Table 3.4). Live larvae were observed on 50.0-97.9% of the non-Bt plants after 12-15 d and the differences among the three insect genotypes were not significant ($P > 0.05$) across the three trials as well as for the combined analysis (Table 3.4).

On Bt corn plants, no live larvae were observed from HX1 plants infested with either SS or RS, while across the three trials, 43.8-100% HX1 plants contained live larvae if they were inoculated with RR larvae. Survival on HX1 plants infested with RR larvae were not different than the larval survival recorded on non-Bt corn plants for each of the three trials as well as for the combined data (Table 3.4). In trials 2 and 3 in which YG was evaluated, an average of 87.5-96.9% YG plants that were inoculated with the three insect genotypes contained live larvae after 12 d, which was not ($P > 0.05$) different than the larval survivorship rates observed on the non-Bt corn plants (Table 3.4). In contrast, no live larvae were found after 12-15 d from plants of the four pyramided Bt corn traits inoculated with any of the insect genotypes (Table 3.4).

3.4 Discussion

The overall survival and damage of the three insect genotypes of *S. frugiperda* on each corn product was consistent across the three trials. The greenhouse study showed that the RR strain of *S. frugiperda*, which was highly resistant to purified Cry1F protein (Niu et al. 2013), was also highly resistant to whole plants of Cry1F Bt corn. Field resistance of *S. frugiperda* to Cry1F corn was initially confirmed in Puerto Rico in 2006 and the technology providers immediately stopped the commercial sale of Cry1F corn seeds to growers in this area (Matten et al., 2008; Storer et al., 2010). A recent study by Storer et al. (2012) also showed that field populations of *S. frugiperda* collected from two other locations in Puerto Rico in 2011 were highly resistant to Cry1F protein in diet. The results of the current study

further confirmed that the field resistance of *S. frugiperda* to Cry1F corn was persistent in Puerto Rico even after several years without planting of Cry1F corn (Niu et al., 2013).

Data of this study also showed that leaf injury rating and survivorship of RR larvae on Cry1F corn plants was nearly the same as observed on non-Bt corn plants, suggesting that the RR strain had a complete resistance (Tabashnik and Carrière, 2007) to Cry1F corn. In most other cases, resistance to Bt plants have been reported to be incomplete, in which resistant populations on Bt plants usually has a less fitness compared to on non-Bt plants (Liu et al., 1999; US-EPA, 2002; Bird and Akhurst, 2004; Carrière et al., 2006; Huang et al., 2007; Pereira et al., 2008). Several factors have been discussed to be major contributors for the field resistance of *S. frugiperda* to Cry1F corn in Puerto Rico (Storer et al., 2010; Huang et al., 2011). Results of the current study suggest that the complete resistance feature could be another major factor that had contributed to the rapid development of Cry1F resistance in the field populations of *S. frugiperda* in Puerto Rico. In all three trials, larvae of the heterozygous genotype (RS-FL or RS-LA), just like the susceptible genotypes (SS-FL or SS-LA), could not survive on Cry1F corn plants, suggesting that the resistance in the RR strain was recessive. The recessive inheritance of the Cry1F resistance observed in the RR strain in the current study was similar to a previously reported population collected from Puerto Rico in 2006 (Storer et al., 2010). In addition, data of this study also showed that larvae of the three insect genotypes of *S. frugiperda* survived well and caused heavy leaf injury on YG corn plants. The results demonstrated that the single-gene Cry1Ab corn product (YG) was not effective against *S. frugiperda*.

Data from our greenhouse tests showed that the highly Cry1F-resistant *S. frugiperda*, just like its susceptible and heterozygous counterparts, could not survive on the plants of the four pyramided Bt corn products, and they caused only little or no leaf injury to the plants.

The results demonstrated that these pyramided Bt corn traits were effective for controlling the Cry1F-resistant populations of *S. frugiperda*.

A previous study with leaf tissue bioassays showed that the Cry1F-resistant strain (RR) of *S. frugiperda* also exhibited a significant level of cross-resistance to three of the four pyramided Bt corn traits tested in the current study (Niu et al., 2013). In a 7-d leaf tissue bioassay, mortality of the Cry1F-susceptible strain (SS-FL) on leaf tissue of VT-2P, VT-3P, and SmatStax was 100%, while 65.6, 32.8, and 50.8% of RR larvae survived on leaf tissue of the three pyramided Bt corn traits, respectively (Niu et al., 2013). Results of the current study showed that the cross-resistance levels reported in the 7-d leaf tissue bioassay (Niu et al., 2013) was not sufficient enough to allow the Cry1F-resistant *S. frugiperda* to survive on whole plants of the pyramided Bt corn hybrids. A similar conclusion was also made based on an F₂ screen of two-parent families of *S. frugiperda* established from field populations sampled in Florida and Louisiana (Yang et al., 2013b).

In recent years, unexpected survival of *S. frugiperda* on Bt corn hybrids has been reported in several occasions in the U.S. south region (FH, unpublished data). Results of our recent monitoring indicate that there is a serious threat of Cry1F resistance in *S. frugiperda* in the U.S. south region. For example, an F₂ screen conducted in 2011 showed that resistance allele frequency to Cry1F corn in *S. frugiperda* was estimated to be 0.058 in two Louisiana populations and 0.252 in a Florida population (FH unpublished data), which are considerably greater than the values reported in other corn lepidopteran species in the U.S. (see reviews in Tabashnik et al., 2009; Huang et al., 2011). In addition, diet incorporated bioassays also showed that field populations of *S. frugiperda* collected from non-Bt corn fields in several locations in Louisiana and Florida in 2012 exhibited a significant level of resistance to purified Cry1F protein (FH unpublished data). Our results suggest that these pyramided Bt corn technologies can be used for managing the Cry1F resistance in *S. frugiperda*. However,

“pyramided” Bt corn may not be considered as “pyramided” anymore if resistance to one Bt protein (e.g. Cry1F) exists. Thus, IRM strategies for such conditions still need to be investigated.

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CHAPTER 4. SUMMARY AND CONCLUSIONS

The fall armyworm, *Spodoptera frugiperda*, is a major corn pest in both the South and North America. Except for Cry1F corn, the first generation Bt corn expressing a single Cry gene is not very effective against *S. frugiperda*. In 2006 after only three years of commercial use of Cry1F corn in Puerto Rico, field populations of *S. frugiperda* in Puerto Rico became highly resistant to Cry1F corn (Storer et al. 2010). It was reported that upon an initial confirmation of the field resistance to Cry1F corn in Puerto Rico, the technology providers immediately stopped the commercial sale of Cry1F corn seeds to growers in this area (Storer et al. 2010). During 2010-2011 crop seasons, transgenic corn technologies (e.g. Genuity[®] SmartStax[™], Agrisure[®] Viptera[™] 3111) expressing multiple dissimilar Bt proteins that target lepidopteran pests were first commercially planted in the United States. The use of pyramided Bt corn hybrids is expected to delay resistance development in target insect populations. With the recent availability of the more effective pyramided Bt corn, *S. frugiperda* becomes a target species of the 2nd generation (pyramided) Bt corn. The objectives of this study were 1) to determine the susceptibility of two field populations of *S. frugiperda* collected from Florida and Puerto Rico to purified Cry1F protein and corn leaf tissue containing single and pyramided Bt genes and 2) to evaluate larval survival and plant injury of Cry1F-susceptible, -resistant, and -heterozygous genotypes of *S. frugiperda* on whole plants of transgenic corn containing single and pyramided Bt genes.

In the objective 1 of this study, larval survival of Cry1F-susceptible (FL), -resistant (PR and Cry1F-RR), and -heterozygous (FL x PR and Cry1F-RS) populations of *S. frugiperda* to purified Cry1F protein and corn leaf tissue of seven Bt corn hybrids and five non-Bt corn hybrids was evaluated in the laboratory. The seven Bt corn hybrids represent five Bt corn traits: Herculex[®]I, which expresses a single Bt protein (Cry1F), and Genuity[®]VT Double Pro[™], VT Triple Pro[™], SmartStax[™], and Agrisure[®] Viptera[™] 3111, which contain

\geq two pyramided Bt genes. The original FL and PR populations were collected from corn fields in 2011 in Florida and Puerto Rico, respectively. Susceptibility of *S. frugiperda* was evaluated using two approaches: 1) a diet incorporating bioassay with purified Cry1F protein and 2) testing on leaf tissue of Bt and non-Bt corn hybrids. There were two independent trials for each test approach. For the diet incorporation bioassays, the first trial used the original two populations (FL and PR) that were established from larvae collected from fields without further selection in the laboratory. In the first trial with leaf tissue bioassays, larval mortality was evaluated for three insect populations including FL, PR, and an F₁ population (FL x PR) that was generated by crossing FL and PR. In the second trial, susceptibility of *S. frugiperda* was evaluated for all three populations including FL, Cry1F-RR (a documented Cry1F-resistant population), and Cry1F-RS (F₁ cross of Cry1F-RR and FL) in both diet incorporation and leaf tissue bioassays.

Diet-incorporation bioassays showed that FL was susceptible to Cry1F protein with a LC₅₀ value of 0.13-0.23 $\mu\text{g/g}$, while PR was highly resistant to Cry1F protein (>137 -fold). FL was also susceptible to all seven Bt corn hybrids with a 7-day mortality of $>95\%$, while PR and a backcrossed and reselected population, Cry1F-RR, were highly resistant to Cry1F corn leaf tissue. The resistance was recessive or incompletely recessive in the diet-incorporated bioassays and leaf tissue tests. All five populations of *S. frugiperda* could not survive on VipteraTM 3111, suggesting this Bt corn trait can completely overcome the resistance and thus should provide a means of managing Cry1F resistance in *S. frugiperda*. However, Cry1F-RR exhibited a significant cross-resistance to the leaf tissue of the other three pyramided Bt corn traits. The possible cross-resistance between single-gene and pyramided Bt corn products suggest that careful selection of Bt genes is essential in use of gene pyramiding strategy for resistance management.

To accomplish the 2nd objective of the study, three greenhouse trials were conducted to evaluate larval survival and leaf injury of Cry1F-susceptible, -resistant, and -heterozygous genotypes of *S. frugiperda* on whole plants of five non-Bt and eight Bt corn hybrids including all the seven Bt corn hybrids used in the objective one plus a YieldGard (Cy1Ab) hybrid. In each trial, 3-5 neonates of a genotype of *S. frugiperda* were manually placed into the whorl of a plant at vegetative plant stages (V6-V10). Larvae of the three insect genotypes on non-Bt corn hybrids survived well and caused serious plant damage. Cry1Ab corn was ineffective against all three insect genotypes. On Cry1F corn plants, resistant larvae survived on 72.9% plants after 12-15 d and caused a leaf injury rating (Davis' 1 to 9 scales) of 5.7 after 7 d and 7.6 after 12-15 d. Both the larval survivorship and leaf injury rates of the resistant larvae on Cry1F corn plants were not significantly different from those observed on non-Bt corn hybrids. In contrast, no live larvae and little or no leaf injury were observed on the Cry1F corn plants that were infested with susceptible or heterozygous genotypes, or on the pyramided Bt plants infested with the three insect genotypes. The results demonstrated that the Cry1F-resistant *S. frugiperda* was highly resistant to whole plants of Cry1F corn and the resistance was recessive in the whole plant tests. Corn hybrids containing anyone of the four pyramided Bt traits are effective for managing the Cry1F resistance in *S. frugiperda*.

In recent years, unexpected survival of *S. frugiperda* on Bt corn hybrids has been mentioned in several occasions in the U.S. south region. Results of the current study showed that all corn hybrids containing one of the four pyramided Bt traits were very effective in controlling the Cry1F-resistant *S. frugiperda*. The results suggest that these pyramided Bt corn technologies can be used as an effective tool for managing the Cry1F resistance in *S. frugiperda*. However, the “pyramided” Bt corn may not be considered as “pyramided” anymore if resistance of a target species to one Bt protein (e.g. Cry1F) in the plant occurs and the corresponding IRM strategies for such conditions still need to be investigated.

APPENDIX: LETTERS OF PERMISSION

A.1. Letter of permission from the Florida Entomologist to reprint Chapter 2

October 10, 2013

Ms. Ying Niu

Department of Entomology

Louisiana State University

Agricultural Center

Baton Rouge, LA 70803-1710, USA.

Dear Ms Ying:

The purpose of this letter is to inform you that– as far as the Florida Entomological Society is concerned –you are at liberty to include in your thesis the following report already published in the Florida Entomologist: “Susceptibility of field populations of the fall armyworm (Lepidoptera: Noctuidae) from Florida and Puerto Rico to purified Cry1F and corn leaf tissue containing single and pyramided Bt genes" will be published in the Florida Entomologist (vol. 96(3): 701-713)”.

The contents of the Florida Entomologist are not copyrighted, and each report remains as the intellectual property of the authors. Thus you can make any use of your report that you and your co-authors agree.

Your report is important contribution to the use of host plant resistance as a major approach to the protection of crops – especially field crops, but eventually to horticultural crops, also.

Best wishes for success in securing your graduate degree from a prestigious institution.

Sincerely,

Waldemar Klassen

Waldemar Klassen

Professor Emeritus

Editor, Florida Entomologist

A.2. Letter of permission from the Crop Protection to reprint Chapter 3

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Apr 12, 2014

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VITA

Ying Niu is the only child of Mr. Ken Niu and Mrs. Yinglan Zhu. She was born and raised in the city of Chongqing in China. She attended the Chongqing No.1 High School and graduated in 2006. She received bachelor degree majoring in plant protection from Southwest University, Chongqing, China in 2010. She joined Louisiana State University in the fall, 2011 for pursuing master degree in the Department of Entomology. Her thesis research with Dr. Fangneng Huang has focused on the evaluation of fall armyworm resistance to *Bacillus thuringiensis*. She has currently completed the requirements for the degree of master science and expects to receive her master's degree in the May of 2014. Ms. Ying Niu plans to continue her graduate study for Ph.D degree in Entomology.