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Characterization of Cry1F Resistance in Fall Armyworm, *Spodoptera frugiperda* (J.E. Smith) Obtained from Puerto Rico and Florida

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CHARACTERIZATION OF CRY1F RESISTANCE IN FALL ARMYWORM, *SPODOPTERA*
FRUGIPERDA (J. E. SMITH) OBTAINED FROM PUERTO RICO AND FLORIDA

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
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Vikash Dangal
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TABLE OF CONTENTS

| | |
|---|-----|
| ACKNOWLEDGMENTS | ii |
| LIST OF TABLES | v |
| LIST OF FIGURES | vi |
| ABSTRACT..... | vii |
| CHAPTER 1. INTRODUCTION | 1 |
| 1.1. Corn production in USA | 1 |
| 1.2. Major insect pests of corn and their management | 1 |
| 1.3. Transgenic Bt crops | 2 |
| 1.4. Bt resistance | 2 |
| 1.5. Resistance management for Bt crops | 4 |
| 1.6. Fall armyworm..... | 6 |
| 1.7. Inheritance of Bt resistance..... | 7 |
| 1.8. Fitness costs of Bt resistance | 10 |
| 1.9. Objectives | 13 |
| 1.10. References..... | 14 |
| CHAPTER 2. ANALYSIS OF INHERITANCE OF CRY1F RESISTANCE IN TWO POPULATIONS OF FALL ARMYWORM, <i>SPODOPTERA FRUGIPERDA</i> COLLECTED FROM PUERTO RICO AND FLORIDA | 22 |
| 2.1 Introduction..... | 22 |
| 2.2. Materials and Methods..... | 25 |
| 2.2.1. Source of Cry1F protein..... | 25 |
| 2.2.2. Sources of Cry1F-susceptible and –resistant fall armyworm | 25 |
| 2.2.3 Genetic crosses..... | 26 |
| 2.2.4 Bioassay | 28 |
| 2.2.5 Data analysis | 29 |
| 2.2 Results..... | 30 |
| 2.3.1. Overall response of different fall armyworm populations to Cry1F toxin incorporated into the diet | 30 |
| 2.3.2 Sex linkage of Cry1F resistance in fall armyworm | 35 |
| 2.3.3. Dominance levels of Cry1F resistance in fall armyworm | 36 |
| 2.3.3 Test for fitting the Mendelian monogenic model | 38 |
| 2.4 Discussion | 38 |
| 2.5 References..... | 43 |
| CHAPTER 3. ASSESSMENT OF FITNESS COST OF CRY1F RESISTANCE IN TWO POPULATIONS OF FALL ARMYWORM COLLECTED FROM PUERTO RICO AND FLORIDA | 47 |
| 3.1. Introduction..... | 47 |
| 3.2. Materials and Methods..... | 49 |

| | |
|--|--------|
| 3.2.1. Sources of insects..... | 49 |
| 3.2.2. Corn plants..... | 51 |
| 3.2.3. Growth and development of fall armyworm on non-Bt diet..... | 51 |
| 3.2.4. Growth and development of fall armyworm in combined rearing of non-Bt corn leaf tissue and non-Bt diet..... | 51 |
| 3.2.5 Data analysis | 52 |
| 3.3. Results..... | 53 |
| 3.3.1. Pupal mass of fall armyworm reared on non-treated diet | 53 |
| 3.3.2 Neonate-to-adult survivorship of fall armyworm reared on non-Bt diet | 53 |
| 3.3.3. Neonate-to-adult emergence time of fall armyworm reared on non-Bt diet..... | 55 |
| 3.3.4 Sex ratio of fall armyworm reared on non-Bt diet..... | 55 |
| 3.3.5 Larval mass of fall armyworm at the 10 th day reared on non-Bt corn leaf tissue | 56 |
| 3.3.6. Pupal mass of fall armyworm in the combined rearing of non-Bt corn leaf tissue and non-Bt diet..... | 56 |
| 3.3.7 Neonate-to-adult survivorship of fall armyworm in the combined rearing of non-Bt corn leaf tissue and non-Bt diet | 56 |
| 3.3.8. Neonate-to-adult developmental time of fall armyworm in combined rearing of non-Bt corn leaf tissue and non-Bt diet | 58 |
| 3.3.9. Sex ratio of fall armyworm in combined rearing of non-Bt corn leaf tissue and non-Bt diet | 59 |
| 3.4. Discussion | 59 |
| 3.5 References..... | 61 |
| CHAPTER 4. SUMMARY AND CONCLUSION | 65 |
| VITA..... | 68 |

LIST OF TABLES

| | |
|---|----|
| Table 2.1. Response of different genotypes of fall armyworm to Cry1F toxin in diet..... | 33 |
| Table 2.2. Effective dominance (D_{ML}), of Cry1F resistance of fall armyworm obtained from Puerto Rico and Florida on Cry1F treated diet..... | 37 |
| Table 2.3. Tests for fitting the Mendelian monogenic model for Cry1F resistance in fall armyworm..... | 39 |
| Table 3.1. Pupal mass (mean \pm SEM), neonate-to-adult survivorship (% mean \pm SEM), adult emergence time, and sex ratio (mean \pm SEM) of seven genotypes of fall armyworm on non-treated diet..... | 54 |
| Table 3.2. Body mass (mean \pm SEM), neonate-to-adult survivorship (% mean \pm SEM), adult emergence time (mean \pm SEM), and sex ratio (mean \pm SEM) of seven genotypes of fall armyworm on non-Bt corn leaf tissues transferred to non-treated diet at 10th day..... | 57 |

LIST OF FIGURES

Figure 2.1. Illustration of populations obtained from various genetic crosses for determining inheritance of Cry1F resistance in the fall armyworm: (A) crosses of Cry1F-susceptible (Bt-SS) and the Puerto Rico Cry1F-resistant (RR-PR) populations; (B) crosses of Bt-SS and the Florida Cry1F resistant population (RR-FL); (C) crosses between RR-PR and RR-FL.....27

Figure 2.2. Dose response of different populations of fall armyworm to Cry1F diet. RR-PR: Cry1F-resistant population obtained from Puerto Rico; $F_{1a_1} = F_1$ obtained from crossing males of RR-PR with females of Bt-SS; $F_{1a_2} = F_1$ obtained from crossing females of RR-PR with males of Bt-SS; $F_{2a_1} = F_2$ obtained from sib-mating F_{1a_1} ; $F_{2a_2} = F_2$ obtained from sib-mating F_{1a_2} ; BCR1-PR = the population obtained from backcross of F_{1a_1} with Bt-SS; and BCR2-PR = the population obtained from backcross of F_{1a_2} with Bt-SS.....31

Figure 2.3. Dose response of different populations of fall armyworm to Cry1F diet. Bt-SS = Cry1F susceptible population; RR-FL= Cry1F-resistant population obtained from Florida; $F_{1b_1} = F_1$ obtained from crossing males of RR-FL with females of Bt-SS; $F_{1b_2} = F_1$ obtained from crossing females of RR-FL with males of Bt-SS; $F_{2b_1} = F_2$ obtained from sib-mating F_{1b_1} ; $F_{2b_2} = F_2$ obtained from sib-mating F_{1b_2} ; BCR1-FL = the population obtained from backcross of F_{1b_1} with Bt-SS; and BCR2-FL= the population obtained from backcross of F_{1b_2} with Bt-SS.....32

ABSTRACT

The fall armyworm (*Spodoptera frugiperda* (J. E. Smith)) is a major pest targeted by transgenic corn expressing *Bacillus thuringiensis* (Bt) proteins in both North and South America. The objective of this study was to characterize the inheritance and fitness costs of the Cry1F resistance in two fall armyworm populations collected from Puerto Rico (RR-PR) and Florida (RR-FL). To determine the inheritance of the resistance, besides RR-PR, RR-FL, and a Cry1F-susceptible population (Bt-SS), 14 other populations were developed by reciprocal crosses, F₁ by F₁ crosses, backcrosses, and crosses between RR-PR and RR-FL. Diet-incorporated bioassays were conducted to determine the Cry1F susceptibility for all 17 populations. To assess the fitness costs of the resistance, seven insect populations were assayed on a non-toxic diet as well as on a combined rearing of non-Bt corn leaf tissue and non-Bt diet. The seven populations were RR-PR, RR-FL, Bt-SS, and four F₁ populations that were developed from the reciprocal crosses between Bt-SS and the two resistant populations. Biological parameters measured in the fitness tests were neonate-to-adult survivorship, neonate-to-adult development time, 10-day larval mass on non-Bt corn leaf tissue, pupal mass, and sex ratios. The results showed that there might be a different genetic basis for the Cry1F resistance between the Puerto Rico and Florida populations. The Cry1F resistance in RR-PR was likely inherited in >1 recessive or incompletely recessive genes and the genes associated the resistance were sex-linked to the males of the insect. In contrast, the resistance in RR-FL was dominant and more likely controlled by autosomal genes. Cry1F resistance in both resistant populations was associated with considerable fitness costs, especially for the Florida population. The fitness costs in the Cry1F-resistant fall armyworm were revealed in reduced growth, increased mortality, and delayed development. Data generated from this

study should be valuable in understanding the mechanisms of Cry1F resistance in fall armyworm and developing effective strategies for resistance management.

CHAPTER 1. INTRODUCTION

1.1. Corn production in USA

Field corn (*Zea mays* L.) is one of the major crops that is grown globally mainly for grains and silage. It is also the most widely planted field crop in the U. S. with a total of 38.6 million hectares in 2013 with a production value of \$62.71 billion (NASS, 2013). Of the total corn planted in the U.S. in 2013, 76% was the transgenic corn expressing *Bacillus thuringiensis* (Bt) proteins (NASS, 2013). Corn is also a major crop in the southern states of the U.S. In Louisiana, a total of 303,600 hectares of corn were planted in 2013 with a production value of \$627.2 million (NASS, 2013).

1.2. Major insect pests of corn and their management

There are various arthropod pests that act as a major constraint in corn production. Generally, insects that belong to the order Lepidoptera are common above-ground pests feeding on leaves, stalks, and ears, whereas coleopteran species are usually below-ground pests feeding on roots. Lepidopteran species such as the European corn borer (*Ostrina nubilalis* (Hubner)), sugarcane borer (*Diatraea saccharalis* (F.)), and southwestern corn borer (*Diatraea grandiosella* (Dyar)) are major stalk boring insects in the U.S. They bore tunnels inside the stalk of corn and damage the plant. The sugarcane borer is a major pest in mid-south, while European corn borer and southwestern corn borer are common in north central and mid-western region (Ostlie et al., 1997; Huang et al., 2011). In Louisiana, the majority of stalk damage in corn is done by sugarcane borer (Huang and Leonard, 2008). In addition, fall armyworm (*Spodoptera frugiperda* (L.)) and corn earworm (*Helicoverpa zea* (Boddie)) are the major leaf feeding and ear feeding insects in the U.S. (Siebert et al., 2012). Coleopteran species such as root worm, white grub and wire worms are common below ground pests feeding on roots.

1.3. Transgenic Bt crops

Bt is a soil dwelling bacterium that was first discovered by a Japanese scientist in 1901 (Gill et al., 1992). It is an aerobic bacterium that produces endospores (Madigan and Martinko, 2005). When there is lack of nutrients, it produces crystalline insecticidal δ -endotoxins commonly known as “Cry” proteins. Since 1955, commercial formulations containing Cry proteins have been used as microbial insecticides for insect pest management (NPTN 2000). Bt insecticides are safe to non-target organisms and can be easily integrated with other control measures in integrated pest management (IPM). For these reasons, Bt toxins have been widely used for controlling agricultural and health insect pests, which accounted for 90-95 % of the world’s biological microbial insecticide market (Gill et al., 1992).

The advances in biotechnology have made transfer of Bt genes into crop plants possible. Transgenic Bt crops produce Bt proteins within the plant tissues and kill insects while they consume the plant tissues (Vaeck et al., 1987; Grassler and Fraley, 1989). Since Bt crops provide protection throughout the season against the target insects, their use has been pervasive in the U.S. and several other countries in the world since its commercialization in 1996, especially among corn and cotton producers (James, 2013).

1.4. Bt resistance

Resistance to a given Bt toxin for an insect population is the heritable reduction in its susceptibility to the toxin (Tabashnik, 1994a). Susceptibility is measured by determining the response of offspring of insects sampled from the field to the toxin in laboratory bioassays. Increases in toxin concentration required to kill the 50% of the insects (also known as LC_{50}) are usually calculated based on lab bioassays (Tabashnik, 1994a). The National Research Council (1986) defines insecticide resistance as the heritable capacity of an insect to tolerate the doses of

toxin which would kill majority of the insects in a normal population of that species. A major resistant allele to a Bt plant is said to be present in an insect population, if homozygous resistant insects can complete its life-cycle and lay viable eggs on a Bt crop (Andow, 2008). Major Bt resistance alleles have been detected in several target species of Bt crops (Downes et al., 2009; Liu et al., 2001; Huang et al., 2011; Meihls et al; 2008; Tabashnik et al., 2008), but the identification of major resistance alleles in a population doesn't imply control failure in the field (Huang et al., 2011). Up to date, field resistance to Bt corn or Bt cotton that has led to significantly reduced efficacy or control failure have been documented in at least five cases in the world.

The first case is the resistance of fall armyworm to TC1507 Bt corn in Puerto Rico in 2006 (Matten et al., 2008; Storer et al., 2010). TC1507 corn expressing the Cry1F protein was registered in the U.S. in 2001 with a purpose of controlling various lepidopteran pests including the fall armyworm (Siebert et al., 2008). Initially in 1996, it was grown in experimental plots in Puerto Rico. In 2003, TC1507 was first commercially planted in silage and dairy farms in Puerto Rico. Fall armyworm is the most important corn pest and the primary target of Bt corn in Puerto Rico. In the continuous corn production scheme in Puerto Rico, fall armyworm can complete many generations in one year (Storer et al., 2010). Reduced yield performance of corn due to the damage of fall armyworm was reported in Puerto Rico in 2006, which led to further investigations. Bioassay results showed that the insect populations sampled from Bt corn fields were less sensitive to Cry1F protein in comparison with the colonies collected from the non-Bt corn regions. The unexpected field survival and damage of fall armyworm were confirmed to be due to resistance development to the Cry1F protein in the plants (Storer et al., 2010). The resistance observed was found to be autosomally inherited and highly recessive. Since then, at

least four other cases of field resistance to Bt crops have been documented including the resistance of African stem borer (*Busseola fusca*) to Cry1Ab corn in South Africa (Van Rensburg, 2007), resistance of pink bollworm (*Pectinophora gossypiella*) to Cry1Ac cotton in India (Dhuru and Gujar, 2011), resistance of western corn rootworm (*Diabrotica virgifera virgifera*) to Cry3Bb1 corn in the U.S.A. (Gassmann et al., 2011), and resistance of fall armyworm to Cry1F corn in the southwest coast region of the U.S. mainland (Huang et al., 2014).

1.5. Resistance management for Bt crops

The widespread exposure of insects to Bt toxin is regarded as one of the largest selection for resistance globally (Tabashnik, 1994b; Gould, 1998; Shelton et al., 2002; Ferré and Van Rie, 2002). Because resistance can be delayed but cannot be avoided, insecticide resistance management (IRM) strategies have to be developed and incorporated into IPM plans for preserving the Bt crop technology (Bates, 2005). When transgenic Bt crops were released initially, resistant management strategies used were largely based on the knowledge garnered from the works being done in conventional pesticides. On the other hand, Bt crops are considered by many to be ‘special case’ because insects are exposed to Bt toxins throughout the season (Tabashnik, 1994b).

Since resistance of insects to Bt proteins in the plants is a major threat for the sustainability of transgenic Bt corn technology as an effective pest management tool, an IRM strategy (the “high dose/refuge” strategy) has been employed for planting Bt corn in the U.S. and Canada (US EPA, 2001; Baute, 2004). This strategy requires planting a portion of non Bt corn as refuge along with Bt corn in an area. The Bt corn should express sufficient high dose of Bt proteins to kill heterozygous insects for Bt resistance (Roush, 1997a; Roush, 1998). The non Bt corn acts as

refuge where the susceptible individuals are hosted and these susceptible refuge insects should randomly mate with the rare resistant homozygotes that are not killed by the Bt corn. Therefore, the majority of offspring that possess resistance alleles will be heterozygous. These heterozygous individuals will then be killed by the high dose proteins expressed in the Bt corn plants so that resistance allele frequencies in the field population will be maintained at a low level for a long period of time (Ostle et al., 1997; US EPA, 2001; Baute, 2004; Huang et al. 2011). Initially, insects with homozygous resistant alleles are very rare in the field that the rate of resistance development is primarily controlled by the number of heterozygous resistant insects (Roush, 1997a; Roush, 1997b). The key assumptions of the high dose/refuge strategy include 1) a very low initial resistant allele frequency (e.g. <0.001), 2) recessive inheritance of resistance, and 3) random mating between resistant and susceptible insects. Although Bt crops may express a high dose of Bt toxins for some target pests, cases of non-recessive inheritance of resistance have been documented in some insect species (Frutos et al., 1999; Akhurst et al., 2003; Burd et al., 2003; Ghimire et al., 2011; Wangila et al., 2012). Initially, the non-Bt refuge plants must be planted structurally. Recently, with the availability of the second generation pyramided Bt corn products, a seed mixture method is also used in the U.S. The use of the seed mixture approach is expected to help random mating between susceptible and resistant insects (Roush, 1997a), but studies have shown that this strategy could be less effective due to larval interplant movement (Mallet et al., 1992; Roush, 1997a; Roush, 1997b; Roush, 1998; Tang et al., 2001).

Up to date, the transgenic Bt corn products can be categorized into two generations (Huang et al., 2011). The first generation of Bt corn produces only a single Bt protein for a target species, while the second generation contains two or more pyramided Bt genes for a target. Compared to the use of single-gene Bt corn, it is believed that the pyramided Bt corn products can delay

resistance evolution in the pest populations. Best results from gene pyramiding are achieved if there is absence of cross-resistance between toxins (Roush, 1997a). Pyramided transgenic crops that target above-ground lepidopteran insect pests were first commercialized in 2010 in the U.S. (Mansanto, 2010). Common pyramided Bt corn products currently planted in the U.S. include Genuity® VT Double Pro™, Triple Pro™, SmartStax™, and Agrisure® Viptera™ 3111. IRM strategies discussed above can certainly help in delaying evolution of resistance to Bt toxins, but integrating them with cultural and biological control should further enhance the suppression of the local pest populations or magnify the efficacy of refuge (Carrière et al., 2003).

1.6. Fall armyworm

Fall armyworm is a major pest of various field crops including corn in the U.S. (Buntin, 1986). It is native to tropics and sub-tropics of western hemisphere and is a serious pest in southern U.S. (Sparks, 1979). This pest migrates towards north during spring in the U.S. whereas overwinters in the southern part of Texas and Florida (Capinera, 2000). Fall armyworm doesn't diapause thus being susceptible to freezing temperature (Luginbill, 1928). Fall armyworm completes its life cycle in about 30 days in summer, 60 days in spring and autumn and 80 to 90 days in winter. The number of generations per year of fall armyworm in the U.S. varies from one to seven generation depending on the location. It can complete four generations a year in Louisiana (Capinera, 2000). Female moth of fall armyworm lays dome shaped eggs *en masse* with 100 to 200 eggs per mass with total number of eggs about 1500-2000 per female and the egg masses are deposited normally on the bottom surface of the leaves. However, if the pest density is high, it can lay eggs on the upper surface of the foliage too (Sparks, 1979; Capinera, 2000). Fall armyworm has generally six instars during larval stages before pupation occurs in soil by creating a loose cocoon webbing together soil and silk (Sparks, 1979; Capinera, 2000).

Mating is accompanied by the release of sex pheromones from female moths that attract adult male moths (Sparks, 1979).

Fall armyworm is a polyphagous insect with > 80 host plant species from 23 families. Corn, cotton, sorghum, rice and Bermuda grass are its major favorable hosts (Buntin, 1986; Capinera, 2000; Meagher and Gallo-Meagher, 2003). Based on the host preference, there are two strains of fall armyworm: corn strain and rice strain. The differences between the two strains cannot be distinguished morphologically (Pashley, 1988) but differences can be seen by molecular study (Lu et al., 1994; McMichael and Prowell, 1999; Nagoshi et al., 2007). Differences among the strains can also be assessed based on their physiology (Prowell, 1988; Quisenberry and Whitford, 1988; Prowell et al., 2004).

1.7. Inheritance of Bt resistance

It is important to know the genetic basis of Bt resistance in order to develop effective IRM strategies (Bourguet, 2004; Tabashnik and Carrière, 2007). One of the key assumptions of the “high dose/refuge” IRM strategy is that the inheritance of the resistant traits must be recessive (Tabashnik., 1994b). Dominance relationships are measured in different ways where the common method is to compare the dose mortality curves for susceptible homozygous, resistant homozygous and heterozygous populations (Bourgeut et al., 2000). By employing this method, inheritance of Bt resistance in the diamondback moth (*Plutella xylostella*), was found to be varying from almost completely recessive to partially recessive (Ferré et al., 2002). In the cabbage looper (*Trichoplusia ni*), inheritance of resistance to Bt *kustaki* was found to be autosomal and partially recessive (Janmaat et al., 2004). In contrast to this, inheritance of resistance in laboratory population of the European corn borer to a commercial microbial Bt insecticide, Dipel ES, to be governed by incompletely dominant autosomal gene (Huang et al.,

1999). Similarly in another study, the inheritance of resistance of tobacco budworm (*Heliothis virescens*) to a recombinant *Pseudomonas* that expresses 130-kDA- δ -endotoxin protein was found to be autosomal and incompletely dominant (Sims et al., 1991). These exceptions suggest that there should be species-specific knowledge of inheritance of resistance in order to devise appropriate IRM strategies (Janmaat et al., 2004). The inheritance of Cry1F resistance has been evaluated in European corn borer which displayed 3000-fold resistance to Cry1F. Diet incorporated bioassays of reciprocal parental crosses showed that the inheritance of the resistance was autosomal and recessive. It was also found through bioassays of the backcrosses of F₁ with the parental strains that the inheritance of the resistance was due to single locus or a set of tightly linked loci (Pereira et al., 2008). In greenhouse experiments conducted in that study, it was found that some Cry1F resistant larvae survived the high dose of Cry1F corn plants although the F₁ populations had fitness near to zero. The results obtained in this study suggested that the “high dose/refuge” strategy was likely appropriate for Bt corn hybrids expressing the Cry1F protein for controlling European corn borer (Pereira et al., 2008). There is also much debate on the role of monogenic or polygenic traits in the development of resistance (Mckenzie, 1996). Most of the studies show that the field evolved resistance to synthetic insecticides involves monogenic traits (Mallet, 1989; Roush et al., 1987). That is why, in IRM strategies, it is commonly assumed that the resistance is due to a single gene with a susceptible allele and a resistant allele (Tabashnik, 1986). The study of inheritance of Cry1F resistance in laboratory selected European corn borer was consistent with monogenic model of resistance (Pereira et al., 2008). However, exceptions have been seen for the resistance to 130-kDA- δ -endotoxin in a laboratory colony of the tobacco budworm (Sims et al., 1991). In a study of inheritance of Cry1Ab resistance in European corn borer, it was found that the inheritance was autosomal but it

was controlled by more than one locus (Alves et al., 2006). In another study where inheritance of Cry1Ab resistance in the sugarcane borer was examined, the effective dominance level was found to be dependent on the dose of Cry1Ab protein. In Cry1Ab treated diet, the resistance was found to be incompletely recessive whereas it was more dominant at low doses (i.e 0.062 μ g/g) and became completely recessive at high dose (i.e 64 μ g/g) (Wu et al., 2009). In leaf tissue bioassays and intact corn plant tests, resistance was found to be incompletely recessive (Wu et al., 2009). It was also found that the inheritance of Cry1Ab resistance in the sugarcane borer was inherited as a single or a few tightly linked gene and the genes were autosomal (Wu et al., 2009). Similarly, inheritance of Cry1Ac resistance in field obtained strain of the pink bollworm was also studied where the results indicated a recessive autosomal inheritance. Resistance in the pink bollworm was recessive at higher concentrations but dominant at higher concentrations (Liu et al., 2001). Survival and growth of progeny from backcross suggested that the resistance of pink bollworm to Cry1Ac toxin was controlled by one or a few major loci (Tabashnik et al., 2002). After the reports of poor field performance of Cry1F corn against fall armyworm in Puerto Rico, laboratory bioassays showed that the Puerto Rico population of fall armyworm collected from the Bt corn field was less sensitive to Cry1F protein compared to the populations obtained from other places. Inheritance studies suggested that the resistance was autosomal and highly recessive (Storer et al., 2010). Recently, Vélez et al. (2013) studied the inheritance of the Cry1F resistance in another population of fall armyworm collected from Puerto Rico and found that the resistance was also controlled by an autosomal and recessive gene.

During 2011, a total of 72 two-parent family lines were developed from single-pair mating of field populations of fall armyworm collected from a corn field in south Florida and screened for resistance to a Cry1F corn hybrid using an F₂ screen method (Huang et al., 2014). Forty-six out

of the 72 family lines were identified to possess at least one major resistance allele to Cry1F corn plants. The resistant families of the fall armyworm showed high resistance to both purified Cry1F protein and whole plants of Cry1F corn. Field populations from non-Bt corn in 2012–2013 in Florida exhibited 18.8-fold to >85.4-fold resistance to purified Cry1F protein and those collected from unexpectedly damaged Bt corn plants at several locations in Florida and North Carolina had >85.4-fold resistance. In addition, reduced efficacy and control failure of Cry1F corn against natural populations of fall armyworm were documented in field trials using Cry1F-based and pyramided Bt corn products in south Florida (Huang et al., 2014). The results provide compelling evidence that field resistance in the fall armyworm to Cry1F corn in the southeastern region of the U.S. has occurred. The factors that led to the field resistance of fall armyworm to Cry1F corn in Florida and North Carolina are still unknown. Local selection pressure due to the planting of Bt corn in the area appears not to be a major factor driving the development of field resistance. Although it is unclear if local selection caused by the use of Bt microbial insecticides is a contributing factor, the more possible reason for the field resistance appears to be the migration of resistant populations from Puerto Rico through other Caribbean islands to FL (Huang et al., 2014). The objective 1 of this project was designed to conduct a series of comparative studies to characterize the Cry1F resistance in fall armyworm between the populations collected from Florida and Puerto Rico. Data generated from this study should be useful in developing effective IRM strategies and answering an important question -if the Cry1F resistance in fall armyworm in the U.S. Mainland is due to migrations from Puerto Rico.

1.8. Fitness costs of Bt resistance

Fitness costs of Bt resistance happen if fitness of resistant insects is lower compared to the susceptible insects in the absence of Bt proteins. Fitness cost is a major factor that influences the

evolution of resistance (Tabashnik et al., 2007; Tabashnik et al., 2008). Fitness costs are often associated with resistance and can be used in IRM. Resistance to Bt protein associated with fitness costs, if the selection is stopped, causes reduction in resistance in population consisting of multifarious genotypes (Tabashnik et al., 1994). For non-recessive fitness costs, development of resistance in insect populations in field can be delayed or even be reversed if there is absence of selection pressure for long period time (Tabashnik et al., 2005). Therefore, a good knowledge on fitness of insecticide resistance is important in developing effective IRM strategies for the sustainable use of Bt crop technologies.

Fitness costs are usually detected in two methods. First is by comparing any of the fitness components such as survival or weight between Bt- resistant and susceptible strains. This method is applied only when any of the fitness components is lower in resistant insects than in susceptible in absence of selection pressures. Another method checks the stability of Bt resistance in the heterozygous population (cage studies). This method is used to check if the resistance allele frequency is reduced over time in the absence of selection (Tabashnik et al., 1998; Gassmann et al., 2009). Whether we use any of the methods, genotypes used in the experiment should have a common genetic background otherwise there might be differences due to strain origin and associated epistatic interactions rather than the value of relative fitness at the resistance locus (Mckenzie, 1996).

Earlier studies conducted on fitness costs associated with resistance to insecticidal agents were mainly focused in dominance and ecological variation. Studies of fitness costs associated with Bt resistance have been done in Lepidoptera (Groeters et al., 1993; Oppert et al., 2000; Carrière et al., 2001a, 2001b; Janmaat and Mayers, 2003; Pereira et al., 2009; Wu et al., 2009; Crespo et al., 2010; Vélez et al., 2013; Zhang et al., 2014), Coleoptera (Muggleton, 1983;

Agentine et al., 1989; Trisyono and Whalon, 1997; Alyokhin and Ferro, 1999), and Diptera (Ferrari and Georghiou, 2981; Roush and Plapp, 1982). In order to evaluate how fitness costs help resistance management, dominance of fitness costs should be analyzed using various fitness components (Mckenzie, 1996; Crespo et al., 2010).

In one of the earlier studies of fitness costs of Bt resistance in the diamondback moth, it was found that the fitness in the absence of Bt toxins was lower for resistant moths compared to susceptible moths. The survival of the resistant strain was lower by 25% in comparison with the susceptible strain (Groeters et al., 1994). The study also showed that there was significant effect of resistance where egg hatchability and fecundity were reduced by 10% in the resistant populations compared to their susceptible counterpart (Groeters et al., 1994).

Several studies evaluated the dominance of fitness costs of Bt resistance and the results showed that the fitness costs are recessive (Gassmann et al., 2009). Although most fitness costs are recessive, non-recessive costs help in delaying resistance as they can strongly select against resistance (Gassmann et al., 2009). In Cry1F-resistant European corn borer, weak fitness cost was detected and fitness in F_1 individuals was similar or slightly greater than the susceptible parents suggesting that the inheritance of fitness costs was recessive (Pereira et al., 2009). Fitness costs in the European corn borer was also found for Cry1Ab resistance where the resistant insects displayed reduced weight of pupae and increased developmental time in comparison to the susceptible insects and F_1 hybrids obtained from the reciprocal crosses. The results showed that inheritance of fitness costs of Cry1Ab resistance in European corn borer was recessive to incompletely recessive. Compared to the Bt-susceptible strain of European corn borer, higher proportions of failed mating with a decreased fertility were also observed in the Bt-resistant individuals (Crespo et al., 2010). However, Bt resistance may be not associated with

fitness costs in some cases. For example, larval growth and development of Cry1Ab-resistance in the sugarcane borer on non-Bt treated diet and non-Bt corn plants were similar to the susceptible strain (Wu et al., 2009, Zhang et al., 2014). Similarly, fitness costs were also not associated with the Cry3Bb1 resistance in western corn rootworm (Ostwald et al., 2012). Actually, emergence of resistance lines was earlier than the susceptible lines in both Bt plants and its isoline non-Bt plants, implying that there was increase in larval development because of the selection for Cry3Bb1 resistance (Ostwald et al., 2012).

Two studies on fitness costs of Cry1F resistance have been done in fall armyworm. One was conducted by comparing the life history traits and population growth rates between the Cry1F-resistant population collected from Puerto Rico and -susceptible populations of fall armyworm and their F₁ hybrids (Vélez et al., 2013). No fitness costs were evident in either resistant parents or the F₁ hybrids. However, the results indicated that there was presence of a hybrid vigor in the heterozygous progeny obtained from the reciprocal crosses (Vélez et al., 2013). Another study was done to assess the fitness costs of a Cry1F-resistant population of fall armyworm also originated from Puerto Rico and the results also showed that no fitness costs were associated with the resistance (Jakka et al., 2014). The objective 2 of this study was designed to assess if fitness costs were associated with the Cry1F resistance in the populations of armyworm collected from Puerto Rico and Florida.

1.9. Objectives

- Analysis of inheritance of Cry1F resistance in two populations of fall armyworm collected from Florida and Puerto Rico; and
- Assessment of fitness costs of Cry1F resistance in two populations of fall armyworm collected from Florida and Puerto Rico

1.10. References

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CHAPTER 2. ANALYSIS OF INHERITANCE OF CRY1F RESISTANCE IN TWO POPULATIONS OF FALL ARMYWORM, *SPODOPTERA FRUGIPERDA* COLLECTED FROM PUERTO RICO AND FLORIDA

2.1 Introduction

Bacillus thuringiensis (Bt) is a soil inhabiting bacterium which produces crystalline (Cry) proteins that bind to the midgut receptors and cause toxicity and death of the insects (Whalon and Wingered, 2003). Since 1996, transgenic plants that express the Bt insecticidal proteins have been commercialized for controlling different pests of agricultural importance. Bt crops are regarded safe to human health and environment compared to the broad-spectrum insecticides (Shelton et al., 2002; Mendelsohn et al., 2003). Field corn (*Zea mays* L.) is one of the crops that have been genetically modified to produce Bt toxins in order to protect it from injury of some herbivorous insect pests (Koziel et al., 1993; Moellenbeck., 2001). In 2013, out of 38.5 million hectares of corn planted in the U.S., 76% of which was Bt corn (NASS, 2013). Fall armyworm (*Spodoptera frugiperda* (J. E. Smith)) is a major pest of corn in both North and South America (Buntin, 1986). It is native to tropics and sub-tropics of western hemisphere and is a serious pest in the southern region of the U.S. (Sparks, 1979). This pest migrates towards north during spring in the U.S. whereas overwinters in the southern part of Texas and Florida (Capinera, 2000). Fall armyworm doesn't diapause therefore being susceptible to freezing temperature (Luginbill, 1928).

In order to control various lepidopteron pests including fall armyworm, transgenic corn event TC1507 expressing the Cry1F protein was registered in the U.S. in 2001 (Siebert et al., 2008). It was commercially planted in Puerto Rico beginning in 2013. However, unprecedented amount of damage of the TC1507 corn and yield losses caused by the fall armyworm were reported in 2006 in Puerto Rico. The field control failure of the TC1507 was documented later to be due the

development of resistance in fall armyworm to the Cry1F protein in the plants (Storer et al., 2010). The resistance of the fall armyworm to the Cry1F corn identified in the Puerto Rico was the first case of field resistance against transgenic Bt crop technology (Huang et al., 2011). The resistance detected in fall armyworm in Puerto Rico was found to be autosomal and recessive (Storer et al., 2010). Other field resistance cases to Bt crops were the resistance of African stem borer (*Busseola fusca*) to Cry1Ab corn in South Africa (Van Rensburg, 2007), resistance of pink bollworm (*Pectinophora gossypiella*) to Cry1Ac cotton in India (Dhuru and Gujar, 2011), resistance of western root cutworm (*Diabrotica virgifera virgifera*) to Cry3Bb1 corn in the U.S. (Gassmann et al., 2011), and resistance of fall armyworm to Cry1F corn in the southeastern coast region of the U.S. Mainland (Huang et al., 2014).

The current approach to delay the evolution of resistance to Bt corn is termed as the “high dose /refuge” strategy (Ostlie et al., 1997; US EPA, 2001). This strategy requires a portion of non Bt corn as refuge to be planted along with Bt corn. The Bt corn should produce a sufficiently high dose of Bt toxins to kill the heterozygous individuals for resistance whereas the refuge should produce relatively abundant susceptible insects. The susceptible populations from the non-Bt refuge plants will mate with the rare resistant survivors from the Bt corn (Tabashnik et al., 2009). This strategy can be effective only when the inheritance of resistance is functionally recessive. Otherwise if the resistance is dominant, Bt crops will not be able to kill the resistant heterozygotes. Thus if the resistance is dominant, the effectiveness of “high dose /refuge” strategy will be highly diminished (Huang et al., 2009). Therefore it is essential to know the genetic basis of resistance in developing effective insect resistance management (IRM) strategies for the sustainable use of Bt crop technologies (Wu, 2014).

During 2011, a total of 72 two-parent family lines were developed from single-pairing of field populations of fall armyworm collected from a corn field in south Florida and F₂ progeny of each family were screened for resistance to Cry1F corn plants using an F₂ screening method (Huang et al., 2014). Many of the Florida families of fall armyworm were identified to possess major resistance alleles to TC1507 corn plants. The resistant lines of the fall armyworm collected from Florida were highly resistant to both the purified Cry1F protein and whole plants of Cry1F corn (Huang et al., 2014). In addition, another well-documented Cry1F-resistant population of fall armyworm population was collected from Puerto Rico (Niu et al., 2013, 2014). Both Cry1F-resistant populations of fall armyworm had been maintained in the Corn and Small Grains Insect Research Laboratory at the Louisiana State University Agricultural Center in Baton Rouge, LA. It was highly believed that the Cry1F resistance in the fall armyworm populations observed in Florida was caused by migration of resistant populations from Puerto Rico through other Caribbean islands (Huang et al., 2014). The availability of two Cry1F-resistant populations of fall armyworm derived from different geographical regions provided the opportunity to analyze if the genetic bases of the resistance were the same between the populations originated from Florida and Puerto Rico. The objective of this study was to determine the inheritance of the Cry1F resistance in the two populations collected from Florida and Puerto Rico. Information generated from the study should be useful in resistance monitoring and developing effective IRM strategies for the sustainable use of Bt corn technology as an pest management tool. In addition, data obtained from the study should also have values in analyzing if the field resistance in Florida was due to migration of resistant populations from Puerto Rico.

2.2. Materials and Methods

2.2.1. Source of Cry1F protein

Purified (99.9 %) Cry1F protein used in the study was obtained from Case Western Reserve University, Ohio.

2.2.2. Sources of Cry1F-susceptible and –resistant fall armyworm

A Cry1F-susceptible strain of fall armyworm collected from Louisiana (Bt-SS) was maintained in the Corn and Small Grain Insect Research Laboratory in the Department of Entomology, Louisiana State University Agricultural Center in Baton Rouge, LA. The Bt-SS was originally collected from cotton fields near Winnsboro, Louisiana during 2005, and supplemented from field corn in the same area during 2006 and 2008. 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 \approx 100g of vermiculite (Sun Gro, Pine Bluff, AR) for adult emergence, mating, and oviposition. The Bt-SS strain has never been exposed to Bt toxins or any other insecticides in the laboratory. Bioassays have shown that the Bt-SS was susceptible to purified Cry1F protein in diet (Huang et al., 2014).

In this study, two Cry1F-resistant populations of fall armyworm were used to examine the inheritance of the resistance. One (RR-PR) was originated from >300 larvae collected from corn fields in southern Puerto Rico during 2011. The field-collected population had been selected on Cry1F corn (Pioneer 31D59) leaf tissue for several generations before it was used in the current study. The reselected population has been confirmed to be highly resistant to both purified Cry1F

protein (>769-fold) and whole plants of Cry1F corn (Niu et al., 2013, 2014). The second resistant population (RR-FL) was developed using an F₂ screen of a two-parent family line collected from corn field in south Florida in 2011. RR-FL has been confirmed to possess major resistance alleles to allow the insect to survive and complete normal larval development on commercial Cry1F corn plants (Huang et al., 2014). RR-FL also has shown highly resistant to the purified Cry1F protein (>270-fold) in diet bioassays (Huang et al., 2014).

2.2.3 Genetic crosses

Pupae of each of the three parental populations of fall armyworm (Bt-SS, RR-PR, and RR-FL) were first separated based on the sex. In order to evaluate the inheritance of the resistance, a total of fourteen additional populations were developed by four types of crosses: 1) reciprocal crosses between Bt-SS and the two resistant populations, 2) F₁ by F₁ crosses, 2) backcrosses of F₁ to Bt-SS, and 4) reciprocal crosses between the two resistant populations.

There were four F₁ hybrid populations that were developed from the reciprocal crosses between Bt-SS and the two resistant populations and they were denoted as, F₁ (a₁)-PR_mSS_f obtained from crossing males of RR-PR with females of Bt-SS; F₁ (a₂)-PR_fSS_m obtained from crossing females of RR-PR with males of Bt-SS; F₁ (b₁)-FL_mSS_f obtained from crossing males of RR-FL with females of Bt-SS; and F₁ (b₂)-FL_fSS_m obtained from crossing females of RR-FL with males of Bt-SS. The four F₁ populations were also sib-mated within each population to obtain four F₂ populations: F₂ (a₁) which was produced from the sib-mating of F₁ (a₁); F₂ (a₂) which was generated from the sib-mating of F₁ (a₂); F₂ (b₁) which was established from the sib-mating of F₁ (b₁); and F₂ (b₂) which was obtained from the sib-mating of F₁ (b₂) (Fig 2.1).

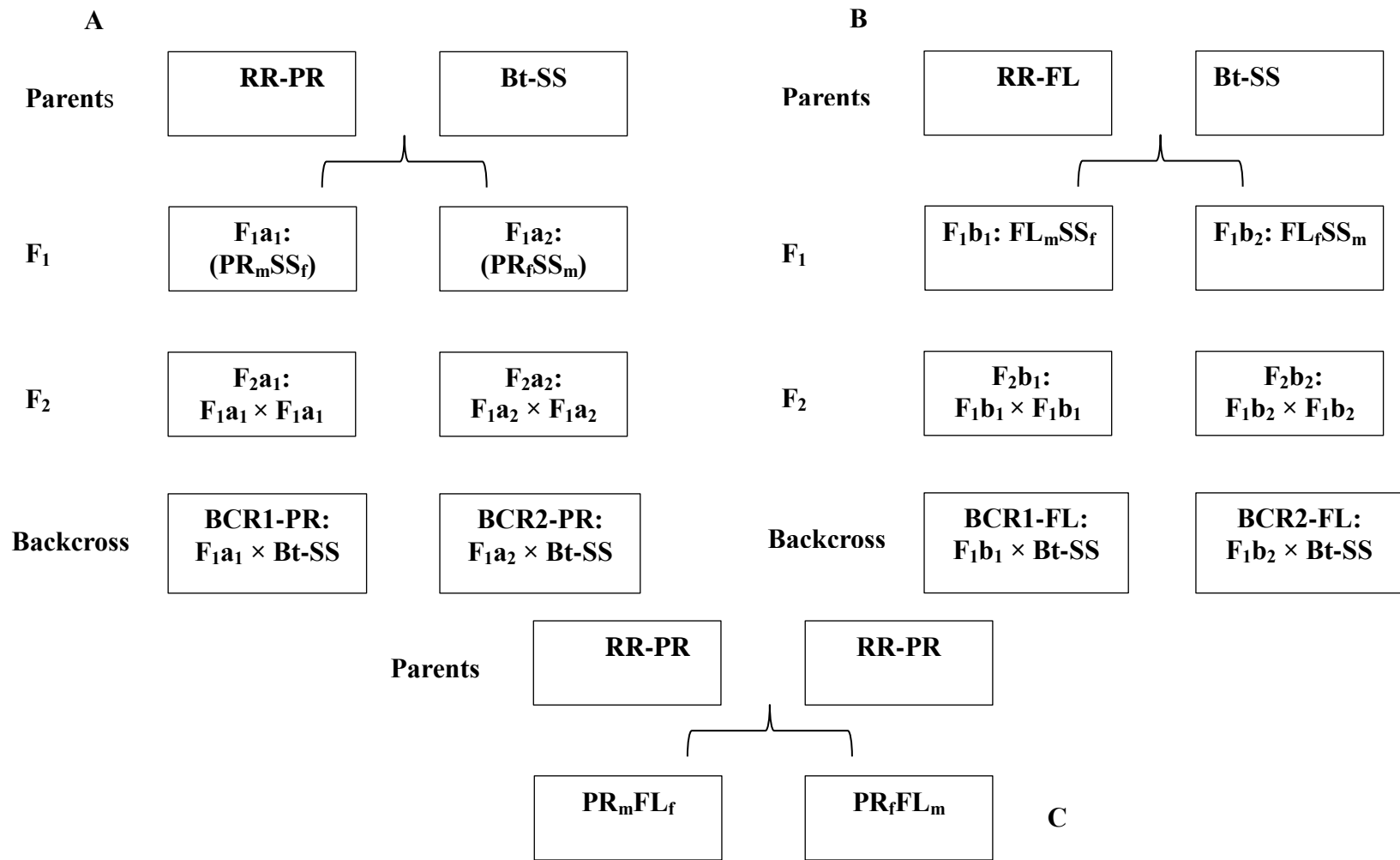


Figure 2.1. Illustration of populations obtained from various genetic crosses for determining inheritance of Cry1F resistance in the fall armyworm: (A) crosses of Cry1F-susceptible (Bt-SS) and the Puerto Rico Cry1F-resistant (RR-PR) populations; (B) crosses of Bt-SS and the Florida Cry1F resistant population (RR-FL); (C) crosses between RR-PR and RR-FL.

Each F₁ populations of RR-PR and RR-FL were crossed with Bt-SS to get four backcross populations as BCR1-PR, BCR2-PR, BCR1-FL and BCR2-FL. In addition, two populations were developed by reciprocal crosses between the two resistant populations, which were referred as PR_mFL_f generated from crossing of the RR-PR males with females RR-FL, and PR_fFL_m obtained by crossing RR-PR females with RR-FL males. Cry1F susceptibility of the three original susceptible and resistant populations along with the 14 cross and backcross populations (a total of 17 populations) were determined using a dose- response bioassay method as described below.

2.2.4 Bioassay

A diet-incorporated bioassay (Niu et al, 2013) was used to determine the response of the 17 populations of fall armyworm mentioned above to purified Cry1F protein. The number of Cry1F concentrations used in the bioassays varied from 6 to 8 with a concentration range of 0.0316 to 10, 31.6, or 100µg/g. Limited by the amount of Cry1F protein available, high concentrations (e.g. 31.6, 100 µg/g) were included in the bioassays for only those populations that were expected to have a high level of resistance. Each bioassay also included a non-treated control. The bioassays were conducted in the 128-cell trays (Bio-Ba-128, C-D International, Pitman, NJ). To prepare the concentrations, purified Cry1F protein was first dissolved in distilled water. A certain amount of the meridic diet (WARD'S Stonefly Heliiothis diet, Rochester, NY) was then added to the solutions containing appropriate concentrations of the Cry1F protein. Diet treated with the same amount of distilled water only was used as the control. Approximately 0.7mg of the Bt-treated or the control diet was placed in each cell of the bioassay trays and the diet was then pressed down to the cell with a 1-cm diameter wood stick to make an even surface of the diet. One neonate (<24-h old) was then transferred on the surface of the diet. There were four

replications for each combination of insect population and Cry1F concentration with 16-32 neonates in each replication. After properly covering the cells with vented lids (C-D International, Pitman, NJ), these bioassay trays were kept in environmental chambers with a temperature of 28 °C, 50% relative humidity and a photoperiod of 16:8(L:D). Number of real dead larvae and number of surviving larvae that were severely stunted and didn't reach the 2nd instar were recorded on the 7th d after inoculation. The real dead larvae as well as the runt larvae that didn't reach the 2nd instar after 7 days were considered as dead larvae in calculating the mortality.

2.2.5 Data analysis

Larval mortality at each Cry1F concentration in the dose response bioassay were calculated and corrected based on the control mortality using the methods of (Abbott, 1925), followed by probit analysis (SAS Institute 2010). The lethal concentration required for 50 % mortality (LC₅₀) as well as their corresponding 95% confidence intervals (CI) were calculated for each insect population mentioned above. In some cases, the LC₅₀ value of an insect population was considered to be greater than the highest Cry1F concentration used in the bioassay if its larval mortality was < 50% at the highest concentration. Resistance ratios for a population were calculated using the LC₅₀ value of a population divided by the LC₅₀ of the Bt-SS.

Maternal effect of Cry1F resistance in fall armyworm was assessed by comparing the LC₅₀s and slopes of the dose-response curves of the two F₁ populations obtained from reciprocal crosses between Bt-SS and each of the resistant populations (Roush and Daly, 1990). A significant difference in the dose-mortality curve of the reciprocal crosses suggests that the resistance was sex linked, otherwise the resistance was considered as autosomal. The dominance

level of resistance was measured as the effective dominance “ D_{ML} ” (Roush and McKenzie, 1987; Bourguet et. al., 2000), which calculates the dominance level by the formula:

$$D_{ML} = (ML_{RS} - ML_{SS}) / (ML_{RR} - ML_{SS})$$

Where, ML_{RS} , ML_{RR} and ML_{SS} denote the mortality levels for heterozygous, resistant and susceptible insect populations, respectively. The value of D_{ML} ranges from 0 to 1, where 0 refers completely recessive and 1 means completely dominant. In this study, D_{ML} for RR-PR and RR-FL was calculated based on the larval mortality at 10 $\mu\text{g/g}$, which was the highest Cry1F concentration used in the bioassays for all parental and F_1 populations.

To determine the number of genes that controlled the Cry1F resistance in fall armyworm, Chi-square tests (χ^2) were used to test if the observed mortality data in the F_2 and backcrossed populations fitted the single gene Mendelian model (Lande, 1981; Tabashnik, 1991). The formula for the Chi-square test is as follow:

$$\chi^2 = \Sigma (\mathbf{O} - \mathbf{E})^2 / \mathbf{np} (\mathbf{1-p})$$

where O is the observed number of dead larvae and E is the expected number of dead larvae, n is the total number of neonates assayed and p is the expected mortality. Expected number of dead larvae was calculated using the mortality of the corresponding parental and F_1 hybrid populations.

2.2 Results

2.3.1. Overall response of different fall armyworm populations to Cry1F toxin incorporated into the diet

Probit analysis showed that the LC_{50} value of Cry1F toxin for the Bt-susceptible population of fall armyworm (Bt-SS) was 0.65 $\mu\text{g/g}$ with a 95% CI of 0.45-1.01 $\mu\text{g/g}$ (Fig 2.2, Fig 2.3) (Table 2.1). Both RR-PR and RR-FL were highly resistant to the purified Cry1F protein incorporated into the diet. Larval mortality for RR-PR and RR-FL at 100 $\mu\text{g/g}$, the highest

concentration tested, was only 30.9 and 32.8 %, respectively, suggesting that the LC_{50} value of the two populations should be much greater than 100 $\mu\text{g/g}$. Thus the resistance ratio of RR-PR and RR-FL, relative to Bt-SS, was estimated to be much greater than 153.8-fold.

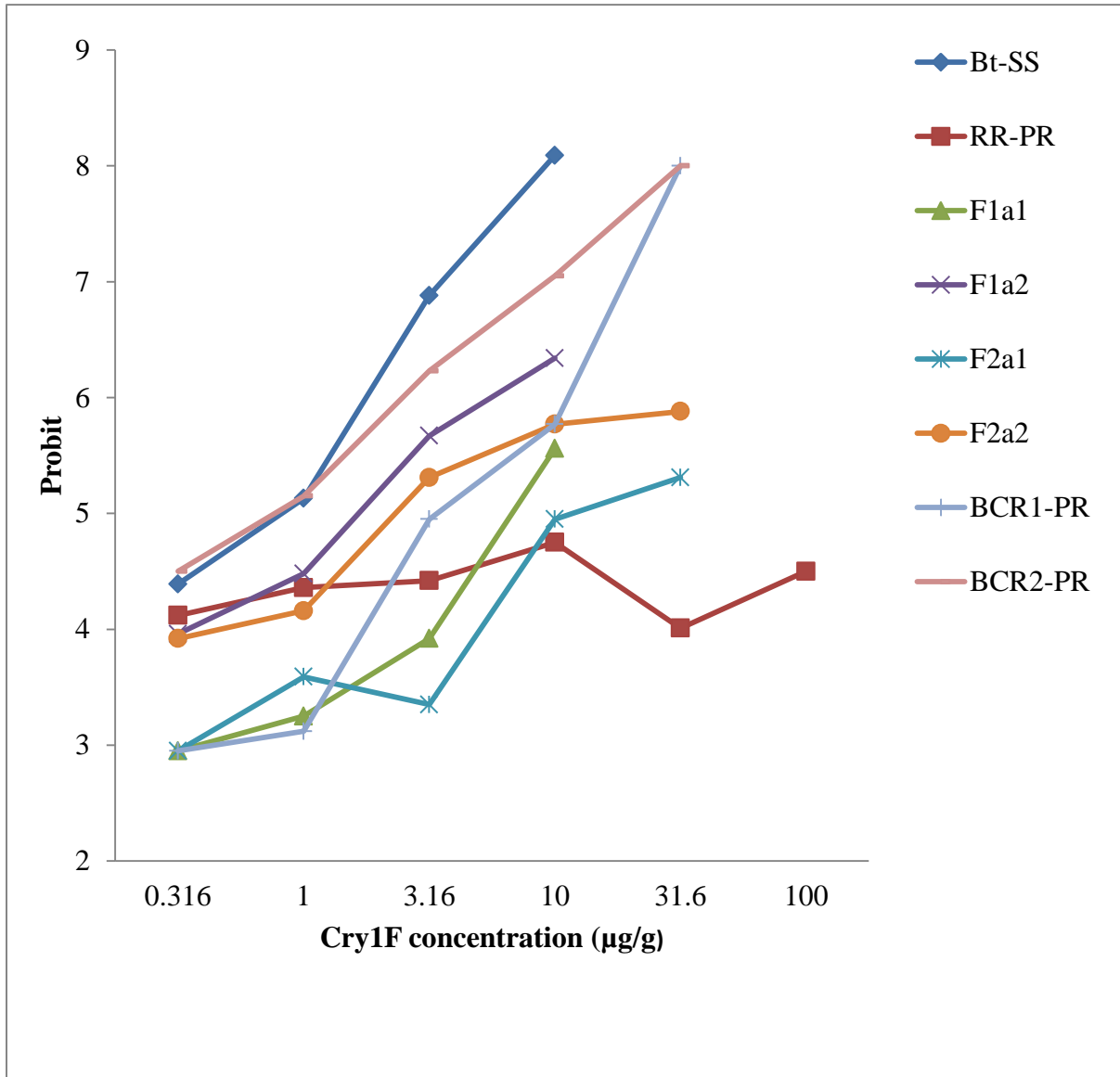


Figure 2.2. Dose response of different populations of fall armyworm to Cry1F diet. RR-PR: Cry1F-resistant population obtained from Puerto Rico; F_{1a1} = F_1 obtained from crossing males of RR-PR with females of Bt-SS; F_{1a2} = F_1 obtained from crossing females of RR-PR with males of Bt-SS; F_{2a1} = F_2 obtained from sib-mating F_{1a1} ; F_{2a2} = F_2 obtained from sib-mating F_{1a2} ; BCR1-PR = the population obtained from backcross of F_{1a1} with Bt-SS; and BCR2-PR = the population obtained from backcross of F_{1a2} with Bt-SS.

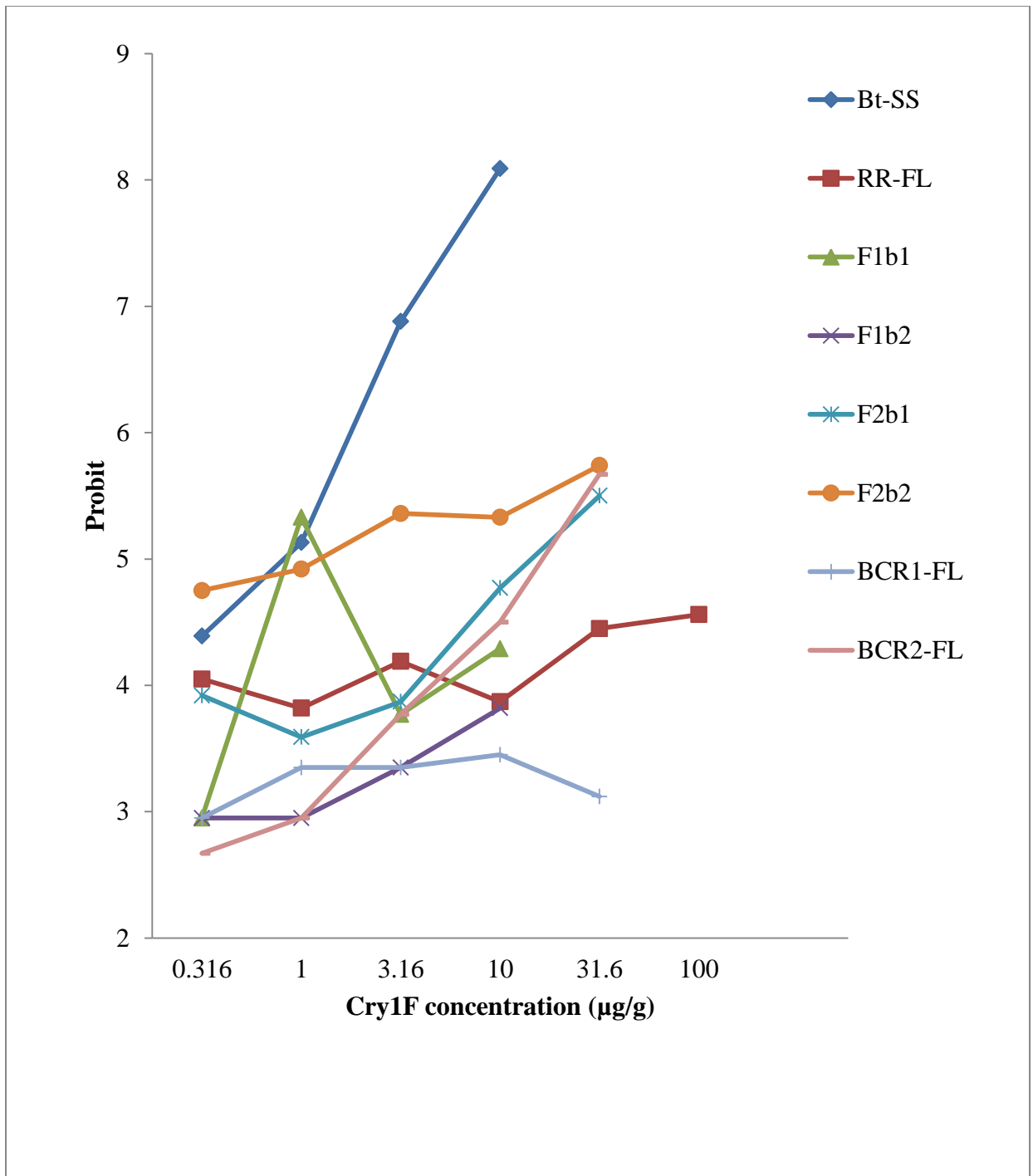


Figure 2.3. Dose response of different populations of fall armyworm to Cry1F diet. Bt-SS = Cry1F susceptible population; RR-FL= Cry1F-resistant population obtained from Florida; F₁b₁= F₁ obtained from crossing males of RR-FL with females of Bt-SS; F₁b₂= F₁ obtained from crossing females of RR-FL with males of Bt-SS; F₂b₁= F₂ obtained from sib-mating F₁b₁; F₂b₂= F₂ obtained from sib-mating F₁b₂; BCR1-FL = the population obtained from backcross of F₁b₁ with Bt-SS; and BCR2-FL= the population obtained from backcross of F₁b₂ with Bt-SS.

Table 2.1. Response of different populations of fall armyworm to Cry1F toxin in diet

| Insect Population ¹ | Highest Cry1F concentration tested ($\mu\text{g/g}$) | % mortality at the highest concentration (mean \pm SEM) | Probit analysis | | | |
|--|--|---|-----------------|-----------------|---------------------------------------|-------------------------------|
| | | | n | slope | LC ₅₀ (95%CI) ⁴ | Resistance Ratio ² |
| Bt-SS | 10 | 100 \pm 0 | 893 | 1.41 \pm 0.16 | 0.65 (0.45 - 1.01) | 1 |
| RR-PR | 100 | 30.9 \pm 5.0 | 2693 | NC ³ | >> 100 | >> 153.8 |
| F _{1a1} : PR _m SS _f | 10 | 43.8 \pm 8.7 | 890 | NC | > 10 | > 15.3 |
| F _{1a2} : PR _f SS _m | 10 | 90.6 \pm 5.9 | 890 | 0.95 \pm 0.2 | 2.21 (0.91 - 11.14) | 3.4 |
| F _{2a1} | 31.6 | 62.5 \pm 4.9 | 502 | 0.99 \pm 0.21 | 21.15 (9.12 - 105.39) | 32.53 |
| F _{2a2} | 31.6 | 81.2 \pm 2.5 | 510 | 0.66 \pm 0.12 | 2.45 (1.01 - 7.79) | 3.76 |
| BCR1-PR | 31.6 | 100 \pm 0 | 509 | 1.45 \pm 0.47 | 3.37 (0.88 - 20.99) | 5.18 |
| BCR2-PR | 31.6 | 100 \pm 0 | 503 | 1.27 \pm 0.13 | 0.6 (0.4 - 0.9) | 0.92 |
| RR-FL | 100 | 32.8 \pm 4.6 | 1890 | NC | >> 100 | >> 153.8 |
| F _{1b1} : FL _m SS _f | 10 | 24.2 \pm 8.0 | 888 | NC | > 10 | > 15.3 |
| F _{1b2} : FL _f SS _m | 10 | 12.5 \pm 6.2 | 890 | NC | > 10 | > 15.3 |
| F _{2b1} | 31.6 | 68.5 \pm 8.9 | 501 | 1.25 \pm 0.29 | 26.16 (12.57 - 115.74) | 40.24 |
| F _{2b2} | 31.6 | 76.9 \pm 4.6 | 487 | 0.72 \pm 0.10 | 5.62 (2.96 - 13.54) | 8.64 |
| BCR1-FL | 31.6 | 3.1 \pm 3.1 | 488 | NC | > 31.6 | > 48.3 |
| BCR2-FL | 31.6 | 75.4 \pm 3.3 | 475 | 1.49 \pm 0.32 | 17.59 (9.81 - 46.15) | 27.06 |
| PR _m FL _f | 100 | 34.3 \pm 1.8 | 1024 | NC | >> 100 | >> 153.8 |
| PR _f FL _m | 100 | 34.3 \pm 1.8 | 1024 | NC | >> 100 | >> 153.8 |

¹Bt-SS = Cry1F susceptible population; RR-PR = Cry1F-resistant population obtained from Puerto Rico; F_{1a1}: PR_mSS_f was obtained from crossing males of RR-PR with females of Bt-SS; F_{1a2}: PR_fSS_m was obtained from crossing females of RR-PR with males of Bt-SS; F_{2a1} = F₂ obtained from sib-mating of F_{1a1}: PR_mSS_f; F_{2a2} = F₂ obtained from sib-mating of F_{1a2}: PR_fSS_m; BCR1-PR was obtained from backcross of F_{1a1}: PR_mSS_f with Bt-SS; BCR2-PR was obtained from backcross of F_{1a2}: PR_fSS_m with Bt-SS; RR-FL = Cry1F-resistant strain obtained from Florida; F_{1b1}: FL_mSS_f was obtained from crossing males of RR-FL with females of Bt-SS; F_{1b2}: FL_fSS_m

was obtained from crossing females of RR-FL with males of Bt-SS; $F_2b_1 = F_2$ obtained from sib-mating of $F_1b_1: FL_mSS_f$; $F_2b_2 = F_2$ obtained from sib-mating of $F_1b_2: FL_fSS_m$; BCR1-FL was obtained from backcross of $F_1b_1: FL_mSS_f$ with Bt-SS; BCR2-FL was obtained from backcross of $F_1b_2: FL_fSS_m$ with Bt-SS; PR_mFL_f was obtained from crossing males of RR-PR and females of RR-FL; PR_fFL_m was obtained from crossing females of RR-PR and males of RR-FL.

²Resistance ratio was calculated using the LC_{50} of a given population divided by the LC_{50} of Bt-SS.

³NC means 'not calculated'; slope could not be determined because of insufficient dose response.

⁴'>' = more than while '>>' = much more than.

LC₅₀s of the F₁ hybrid population F_{1a1}-PR_mSS_f was 2.21 µg/g, while it couldn't be calculated with the probit analysis for the three other F₁ populations because their mortality at the highest concentration tested (10 µg/g) was <50% (Table 2.1). LC₅₀s of the four F₂ populations generated from the sib-mating of the four F₁ hybrids were varied, ranging from 2.45 µg/g for F_{2a2} to 21.16 µg/g for F_{2b1}. The LC₅₀ values of the two backcross populations associated with RR-FL were >31.6 µg/g for BCR1-FL and 17.59 µg/g for BCR2-FL, which were considerably greater than the LC₅₀s (0.6 for BCR1-PR and 3.37 µg/g for BCR2-PR) of the two backcross populations associated with RR-PR. The two populations (PR_mFL_f and PR_fFL_m) generated from the reciprocal crosses between RR-PR and RR-FL were also highly resistant to Cry1F and the mortality at 100 µg/g, the highest concentration tested, was <50% for both populations.

2.3.2 Sex linkage of Cry1F resistance in fall armyworm

For RR-PR, the dose-response was considerably different between the two F₁ populations of the reciprocal crosses. F_{1a1}-PR_mSS_f, which was generated from the cross of RR-PR males with Bt-SS females, was resistant to the Cry1F protein, while its counterpart F_{1a2}-PR_fSS_m appeared to be susceptible to the Bt toxin. In the dose-response bioassays, F_{1a1}-PR_mSS_f at the Cry1F concentration of 10 µg/g (the highest concentration tested) showed a mortality level of only 43.8%, indicating a resistance ratio of > 15.3-fold. However, F_{1a2}-PR_fSS_m exhibited a LC₅₀ of 2.21 µg/g, which was not significantly greater than the LC₅₀ of Bt-SS based on their overlapped 95% CIs. In addition, the F₂ (F_{2a1}) and backcross (and BCR1-PR) populations that had genetic background from RR-PR males also showed a much greater resistance level than their counterpart populations that had genetic background from the RR-PR females. For example, F_{2a1} had a LC₅₀ of 21.15 µg/g, while it for F_{2a2} was only 2.45 µg/g. The 8.6-fold difference between the two F₂ populations was significant based on their non-overlapped 95% CIs. There was also a

5.6-fold difference in the LC_{50} s between two backcross populations, BCR1-PR and BCR2-PR, although the 95% CIs of the LC_{50} s were slightly overlapped. The results strongly suggested that the Cry1F resistance in RR-PR was not autosomal but somewhat linked to the males of the insect.

For RR-FL, both F_1 reciprocal populations showed a significant level (>15-fold) of resistance. Mortality of F_1b_1 -FL_mSS_f and F_1b_2 -FL_fSS_m at the highest concentration tested (10 μ g/g) was low <25% and was not significantly different from each other ($t = 0.48$, $df = 6$, $P = 0.6471$). Significant difference in the LC_{50} values was also not found in the F_2 populations with genetic background between RR-FL males and RR-FL-females. However, there might have been some differences in the Cry1F susceptibility between the two backcross populations. Thus, results of the current study didn't provide clear evidence that sex linkage was associated with the Cry1F resistance in RR-FL. Based on the data available; the resistance in RR-FL was more likely autosomal not sex-linked.

2.3.3. Dominance levels of Cry1F resistance in fall armyworm

As mentioned above, because the Cry1F resistance in one of the two resistant populations was not autosomal, for each resistant population two D_{ML} values were calculated based on the mortalities of the two F_1 populations obtained from the reciprocal crosses. Based on the mortality measured at the Cry1F concentration of 10 μ g/g, the Cry1F resistance in the Puerto Rico population, RR-PR, was more recessive than the Florida population, RR-FL. D_{ML} of the RR-PR calculated based on the mortality of the two F_1 populations at 10 μ g/g was 0.11 and 0.26, respectively, suggesting that the resistance was recessive or incompletely recessive. In contrast, the corresponding D_{ML} for the RR-PR was 0.86 and 1, respectively, indicating a completely or near completely dominant inheritance of the resistance (Table 2.2).

Table 2.2. Effective dominance (D_{ML}), of Cry1F resistance in fall armyworm populations collected from Puerto Rico (RR-PR) and Florida (RR-FL) tested on Cry1F treated diet.*

| Population | F ₁ hybrid | Effective dominance (D_{ML}) | Results |
|------------|--|-------------------------------------|---------------------------|
| RR-PR | F _{1a1} : PR _m SS _f | 0.26 | Incompletely recessive |
| | F _{1a2} : PR _f SS _m | 0.11 | Near completely recessive |
| RR-FL | F _{1b1} : FL _m SS _f | 0.86 | Near completely dominant |
| | F _{1b2} : FL _f SS _m | 1 | Completely dominant |

*Mortality used for calculating the D_{ML} s was measured at the Cry1F concentration of 10 µg/g, which was the highest concentration tested for all populations. F_{1a1}: PR_mSS_f obtained from crossing males of RR-PR with females of Cry1F susceptible population Bt-SS; F_{1a2}: PR_fSS_m obtained from crossing females of RR-PR with males of Bt-SS; F_{1b1}: FL_mSS_f obtained from crossing males of RR-FL with females of Bt-SS; F_{1b2}: FL_fSS_m obtained from crossing females of RR-FL with males of Bt-SS

2.3.3 Test for fitting the Mendelian monogenic model

Similarly as described in the calculation of the effective dominance levels, tests for fitting the Mendelian monogenic model for each resistant population was conducted separately based on the insect sources from the two reciprocal crosses. For the Puerto Rico resistant population, the χ^2 tests showed that both of the F₂ and backcross populations had a genetic background from the males of the Cry1F-resistant population, RR-PR, did not fit ($P < 0.05$) the monogenic model (Table 2.3). Although mortality of the F₂a₂ fit ($P > 0.05$) the single gene model, this F₂ population came from the crosses between Bt-SS and the females of RR-PR. As the analysis described above, the Cry1F resistance in RR-PR was sex-linked with the males but not with the females. Thus, the data generated from the F₂ and backcross populations strongly suggest that it was possible that the Cry1F resistance in RR-PR could be associated with >1 genes.

For the RR-FL, mortality of both the F₂ and backcross populations that had a genetic background of males of RR-FL fit the Mendelian monogenic model well with a P value of 0.7772 or greater in the χ^2 tests (Table 2.3). However, the two corresponding populations associated with the females of RR-FL did not fit ($P < 0.05$) the single gene model. Thus, a clear conclusion about the number of genes associated with the Cry1F resistance in RR-FL couldn't be reached based on the available data of this study (Table 2.3).

2.4 Discussion

The reciprocal crosses between the two Cry1F-resistant populations were designed to determine if both RR-PR and RR-FL shared a same genetic basis of resistance. Because of the dominant resistance character of RR-FL, the dose mortality of the two cross

Table 2.3 Tests for fitting the Mendelian monogenic model for the Cry1F resistance in fall armyworm collected from Puerto Rico (RR-PR) and Florida (RR-FL).*

| Population | n | No. dead larvae | | χ^2 test | | Population | n | No. dead larvae | | χ^2 test | |
|------------------|----|-----------------|------|---------------|---------|------------------|----|-----------------|------|---------------|---------|
| | | Obs. | Exp. | χ^2 | P-value | | | Obs. | Exp. | χ^2 | P-value |
| F _{2a1} | 62 | 29.1 | 41.0 | 10.10 | 0.0014 | F _{2a2} | 64 | 49.5 | 42.4 | 3.50 | 0.0613 |
| BCR1-PR | 64 | 50 | 56.4 | 6.10 | 0.0135 | BCR2-PR | 64 | 62.8 | 56.4 | 6.10 | 0.0135 |
| | | | | | | | | | | | |
| F _{2b1} | 64 | 16.5 | 16.3 | 0.003 | 0.9563 | F _{2b2} | 57 | 35.5 | 14.5 | 39.9 | 0.0000 |
| BCR1-FL | 57 | 30.8 | 29.3 | 0.08 | 0.7772 | BCR2-FL | 59 | 17.7 | 30.3 | 10.7 | 0.0010 |

*Mortality used for calculating the D_{MLS} was measured at the Cry1F concentration of 10 µg/g, which was the highest concentration tested for all populations. n = number of insect individuals tested. F_{2a1} and F_{2a2} are F₂ populations obtained from sib-mating the reciprocal crosses of F₁ populations of Cry1F-resistant Puerto Rico population (RR-PR) and Cry1F-susceptible population (Bt-SS); F_{2b1} and F_{2b2} are F₂ populations obtained from sib-mating the reciprocal crosses of F₁ populations of Cry1F-resistant Florida population (RR-FL) and Cry1F-susceptible population (Bt-SS); BCR1-PR and BCR2-PR are backcross populations of reciprocal F₁ hybrids of RR-PR and Bt-SS; BCR1-FL and BCR2-FL are backcross populations of reciprocal F₁ hybrids and Bt-SS. Obs = observed and Exp = expected.

populations obtained from the current study was still not sufficient to make a clear conclusion. However, based on the data from bioassays with the other 15 populations, there likely had a different genetic basis for the Cry1F resistance between the Puerto Rico and Florida populations. The Cry1F resistance in the Puerto Rico population, RR-PR, was likely inherited in more than one recessive to incompletely recessive genes and the genes contributing to the resistance were sex-linked to the males of the insect. In contrast, for the population collected from Florida, RR-FL, the resistance was dominant and more likely controlled by autosomal genes. The results from the current study were somewhat different compared to the published data. Inheritance of Cry1F resistance in fall armyworm has been evaluated in two other Puerto Rico populations. Both studies reported that the Cry1F resistances in the fall armyworm were autosomal and highly recessive (Storer et al., 2010; Vélez et al., 2013). Bioassay results from backcross of F₁ with resistant parents in the study of Vélez et al. (2013) also indicated that the resistance in that population was controlled by a single gene. The varied results suggest that there could be diverse genetic bases that can be involved in the Cry1F resistance in fall armyworm.

Results of the current study showed that inheritance of the Cry1F resistance in the Puerto Rico population is likely functionally recessive on Bt corn plants, while it could be dominant in the Florida resistant population. In the field at the early stage of resistance development, most individuals carrying resistance alleles in an insect population are heterozygous. The dominant inheritance of Cry1F resistance found in the Florida population of fall armyworm violates the requirement of a recessive resistance for the currently adopted “high dose/refuge” resistance management strategy. The dominant character of a resistance suggests that the resistant-heterozygotes wouldn't be killed by Bt corn plants and thus the resistance alleles carried by the heterozygotes will pass into their offspring, which could result in development of resistance

rapidly in the field. Therefore, results of this study suggest that it may be necessary to use different Bt corn technologies that have different mode of actions or different management tactics for managing the fall armyworm where resistance has occurred.

Inheritance of Bt resistance have been studied in several insects targeted by Bt formulations or transgenic Bt crops. Inheritance of resistance in the diamondback moth (*Plutella xylostella*) against Bt subspecies *kurstaki* was found to be varying from almost completely recessive to partially recessive (Ferré et al., 2002), while Bt resistance in the cabbage looper (*Trichoplusia ni*) was autosomal and partially recessive (Janmaat et al., 2004). In contrast, inheritance of resistance to a commercial Bt insecticide, Dipel ES, was found to be governed by incompletely dominant autosomal genes in a laboratory-selected population of the European corn borer (*Ostrina nubilalis*) (Huang et al., 1999). Similarly, the inheritance of resistance of the tobacco budworm (*Heliothis virescens*) to a recombinant *Pseudomonas* expressing 130-kDA- δ -endotoxin of Bt was found to be autosomal and incompletely dominant (Sims et al., 1991). The inheritance of Cry1Ab resistance on commercial Cry1Ab corn hybrids has also been evaluated in European corn borer. The results showed that the resistance was controlled by a single (or a set of tightly linked) autosomal and recessive locus (Pereira et al., 2008). In another study of inheritance of resistance of Cry1Ab in European corn borer, it was found that although the inheritance was autosomal, progeny of backcrosses suggested that the resistance was controlled by more than one locus (Alves et al., 2006). In the study where inheritance of Cry1Ab resistance in sugarcane borer (*Diatraea saccharalis*) was examined, the results suggested the resistance was controlled by a single autosomal gene, but the effective dominance level was highly dose dependent (Wu et al., 2009). Similar results were found in the Cry1Ac resistance in the pink bollworm (Liu et al., 2001). There is also much debate on the role of monogenic or polygenic traits in the

development of resistance (Mckenzie, 1996). Most of the studies show that the field evolved resistance to synthetic insecticides involves monogenic traits (Mallet, 1989., Roush et al., 1987). Similarly, as described above the published data also have shown that high levels of Bt resistance is usually controlled by a single autosomal recessive locus. However, results of the current study with the two fall armyworm populations that were highly resistant to both purified Cry1F protein and Cry1F corn plants appeared to be inherited differently compared the most published cases. The varied results suggest that there must be species-toxin-specific knowledge of inheritance of resistance for implementing effective resistance management strategies (Janmaat et al., 2004).

A recent study documented that field resistance in fall armyworm to Cry1F corn (TC 3507) has already occurred in the southeastern region of the U.S. (Huang et al. 2014). Surprisingly high (0.293) Cry1F resistance allele frequency has been detected in a population collected from non-Bt corn in south Florida. High levels of Cry1F resistance have been observed in several field populations collected from both Bt and non-Bt corn fields. In addition, reduced efficacy and control failure of Cry1F corn due to resistance have been observed in commercial fields and in trial fields in the area. The Cry1F-resistance in the fall armyworm in the U.S. southeastern region is the first documented case of field resistance in a lepidopteran target species to transgenic Bt corn in the U.S. Mainland (Huang et al., 2014). The reasons that resulted in the field resistance of fall armyworm to Cry1F corn in the U.S. Mainland are still unknown. It is highly believed that the resistance could be due to migration of resistant populations of fall armyworm from Puerto Rico through other Caribbean islands to the U.S. southeastern region. However, the results of the current study indicated that the genetic bases of the Cry1F resistance appeared to be different between the two populations originated from Puerto Rico and Florida. The observed differences in the resistance inheritance between the two populations could be due to different genetic bases

of resistance or different sources of resistance alleles. The later could indicate that the Cry1F resistance in the Florida population was caused by location selections rather than migration of the resistant populations. Further studies are warranted to reveal the reasons that led to the field resistance of fall armyworm in the southeastern region of the U.S. Mainland.

2.5 References

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CHAPTER 3. ASSESSMENT OF FITNESS COST OF CRY1F RESISTANCE IN TWO POPULATIONS OF FALL ARMYWORM COLLECTED FROM PUERTO RICO AND FLORIDA

3.1. Introduction

Field corn (*Zea mays* L.) is one of the major crops that have been genetically modified with *Bacillus thuringiensis* (Bt) genes for the expression of Bt proteins targeting herbivorous insect pests (Koziel et al., 1993; Moellenbeck et al., 2001). Corn is also a major crop in the U.S. with a total acreage of 38.5 million hectares planted in 2013, out of which 76 % was Bt corn (NASS, 2013). Fall armyworm (*Spodoptera frugiperda* (J.E. Smith)) is a common pest in field corn in both the North and South America (Buntin et al., 2004). It also is a major pest of many other crops in the tropical regions. In subtropical regions, fall armyworm is highly destructive as a late season pest in late-planted crops. It doesn't diapause in winter and acts as a sporadic pest in temperate regions (Mitchell, 1979; Johnson, 1987). Traditional chemical control tactics are unable to produce satisfactory results against the fall armyworm (Siebert et al., 2008).

Transgenic corn containing the event TC1507 expressing the Cry1F protein was registered in the U.S. in 2001 for controlling various pests including the fall armyworm (Siebert et al., 2008). Beginning in 1996, corn products expressing the Cry1F protein was grown in experimental plots in Puerto Rico. In 2003, it was first commercially planted in the territory for silage and dairy farms (Storer et al., 2010). However, soon after the commercialization, unprecedented damage of Cry1F corn plants was reported in 2006 in Puerto Rico (Blanco et al., 2010; Storer et al., 2010). The unprecedented damage by fall armyworms and reduced yield led to further investigation. Bioassay results showed that the insect populations sampled from Cry1F corn fields were less sensitive to Cry1F protein in comparison with the colonies collected from other non-Bt corn regions (Storer et al., 2010). The unexpected field survival and damage of fall armyworm were

then confirmed to be due to resistance development to Cry1F protein in the plants. The inheritance of resistance was found to be autosomal and highly recessive (Storer et al., 2010). A strategy named the “high dose-refuge strategy” has been used in the U.S. to delay the resistance development in the target pests of Bt crops (Gould et al., 1998; Hutchison et al., 2010). The principles underlying this strategy is that the non-Bt plants act as refuge to susceptible insects and the rare resistant insects surviving from the Bt plants can mate with the susceptible individuals from the non-Bt refuge plants (Ostlie et al., 1997). The dose of Bt proteins expressed in Bt plants should be high enough to kill most of the individuals produced from mating of susceptible and resistant parents (Tabashnik, 1994; Gould, 1998., Georghiou and Taylor, 1977; Tabashnik and Croft, 1982; Tabashnik et al., 2004). Theoretically, for this strategy to be successful, inheritance of resistance should be recessive, resistance allele frequency should be very low, and susceptible individuals produced from non-Bt refuges must be abundant and are able to mate the rare resistant homozygotes from the Bt plants (Tabashnik et al., 1994; Gould, 1998; Tabashnik et al., 2004; Tabashnik ., 2008; Tabashnik et al., 2009).

Fitness costs are regarded as one of the major factors influencing the evolution of resistance (Tabashnik et al., 2007; Tabashnik et al., 2008; Carrière et al., 2001; Carriere et al., 2010; Grassman et al., 2009). Fitness cost of Bt resistance occurs only when insect genotypes conferring at least one allele of resistance has lower fitness than those individuals without any allele for Bt resistance in the absence of Bt proteins (Gassmann et al., 2009). In many cases where resistance to Bt has been detected, there has been rapid decline in resistance level after the selection pressure has been removed (Gassmann et al., 2009). In addition, mathematic modeling also suggests that fitness costs could play a key role in delaying resistance by selecting against resistant genotypes in the refuges where the Bt toxin is not present (Tabashnik et al., 2003;

Gassmann et al., 2009). Studies have also shown that the crop used in the refuge affects both the magnitude and the dominance of fitness costs (Bird and Akhurst, 2006). Most studies have found that dominance of fitness costs of Bt resistance is recessive (Gassmann et al., 2009). Fitness costs are detected either by comparing any of the fitness components such as survival or weight between Bt- resistant and susceptible strains or by checking the stability of Bt resistance in heterozygous populations. Irrespective of the methods, strains used in detecting the fitness costs of Bt resistance should have common genetic background otherwise there might be differences due to strain origin and associated epistatic interactions rather than the value of relative fitness at the resistance locus (Mckenzie, 1996).

In this study, relative fitness of two Cry1F resistant populations obtained from Puerto Rico and Florida along with a susceptible population collected from Texas and their F₁ hybrids in non-Bt diet and non-Bt corn leaf tissue were evaluated to examine if the resistance is associated with any fitness costs in the two populations. Data obtained from this study should be useful in understanding the resistance mechanisms and developing effective strategies for managing the Cry1F resistance in fall armyworm.

3.2. Materials and Methods

3.2.1. Sources of insects

Three insect populations, a susceptible population (Bt-SS) of fall armyworm collected from Texas and two homozygous resistant populations obtained from Puerto Rico (RR-PR) and Florida (RR-FL) were used as the original insect sources in this study. To ensure a similar genetic background among the three populations, both RR-PR and RR-FL were backcrossed with the Bt-SS to generate the F₁ (RS) generations and then F₁ populations were backcrossed with SS one more time. The second backcrossed populations were then sib-mated within each

crossed population to generate the corresponding F₂ populations. Progeny of F₂ generations were reselected for Cry1F resistance on Cry1F corn leaf tissue for three more generations before they were used in the study. The methods used in the re-selection of Cry1F resistance were similar as described in (Niu et al., 2013). 2-4 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 crossed population, a total of 1000-1500 neonates were selected on Cry1F corn leaf tissue. After 7 days, the survivors were transferred into a meridic diet (Ward's Stonefly Heliiothis diet, Rochester, NY) in 30-ml plastic cups (Fill-Rite, Newark, NJ) until the pupal stage. After 7 days, approximately 120-150 survivors of each population were transferred into 30-ml plastic cups (Fill-Rite, Newark, NJ) containing a meridic diet (Ward's Stonefly Heliiothis diet, Rochester, NY). In the selection, only the survivors with a relatively big body size ($\geq 3^{\text{rd}}$ instars) were transferred and used to develop the next generation (Niu et al., 2013). The larval-rearing cups were held in 30-well trays (Bio-Serv, Frenchtown, NJ) and placed under the room conditions until pupation. In addition, four F₁ hybrid populations were developed from reciprocal crosses of Bt-SS with the backcross-and-reselected RR-FL and RR-PR. The two F₁ populations generated from the reciprocal cross between Bt-SS and RR-RR were denoted as PR_mSS_f (cross between males of RR-PR and females of Bt-SS) and PR_fSS_m (cross between females of RR-PR and males of Bt-SS) while the two populations developed between Bt-SS and RR-FL were referred as FL_mSS_f and FL_fSS_m, respectively. Fitness of the seven insect populations was examined in two assay methods: a non-Bt diet and a combined rearing of non-Bt corn leaf tissue and non-Bt diet.

3.2.2. Corn plants

A non-Bt corn hybrid, Pioneer 31P40 (Pioneer Hi-Bred, Johnston, Iowa), was planted in 5 gallon plastic pots filled with approximately 5 kg of a standard potting mixture (Perfect Mix, Expert Gardener products, St. Louis, MO) as described in Wu et al. (2007). The pots were held in a greenhouse at the Louisiana State University Agricultural Center in Baton Rouge, Louisiana. Two plants per pot were maintained with regular irrigation and fertilization.

3.2.3. Growth and development of fall armyworm on non-Bt diet

To determine if fitness costs were associated with the Cry1F resistance in the fall armyworm, growth and development of the seven populations of fall armyworm were first examined on a non-Bt meridic diet. In the diet assay, approximately 1 g of non-Bt diet (WARD'S Stonefly Heliopsis diet, Rochester, NY) was placed into each cell of the 128-cell bioassay trays (Bio-Ba-128, C-D International Inc. Pitman, NJ). One neonate (<24 h old) was placed on the diet surface in each cell. The bioassay trays were held in an environmental chamber maintained at 28⁰C temperature, ~50% R. H, and a photoperiod of 16h: 8h (L: D). There were four blocks for each insect population with 32 larvae in each block ($n = 4 \times 32 = 128$) where an individual chamber was treated as a block. After 5 days, larvae were transferred into 30-ml-cups (1 larva/cup) (SOLO, Chicago, IL) containing approximately 8 g of diet and were allowed to develop to the pupal stage. After the first pupa was observed, all cups were observed daily until all insects pupated or died. Larval survival, pupation time, pupation success, sex ratio, pupal weight, and adult emergence were recorded for each individual.

3.2.4. Growth and development of fall armyworm in combined rearing of non-Bt corn leaf tissue and non-Bt diet

The combined rearing involved the use of non-Bt corn leaf tissue and non-Bt diet as the food sources of the insect. In the combined assay, leaf tissue of non-Bt corn plants were first removed

from the greenhouse grown V7-V9 stage plants. Two-three pieces of leaf tissue (approx. 3-4 cm) were placed in each well of the 32-well C-D International trays (Bio-Ba-32, C-D International, Pitman, NJ). One neonate (<24 h) of an insect population was placed in each well. After properly covering the them, bioassay trays containing leaf tissues and neonates were kept in growth chambers maintained at temperature of 28⁰C, ~50% relative humidity (RH), and a photoperiod of 16h: 8h (L: D). Leaf tissue was replaced every three days. After 10 days, live larvae recovered from the leaf tissue were then transferred into 30-ml-cups (1 larva/cup) (SOLO, Chicago, IL) containing approximately 8 g of diet and continued the rearing until pupation. There were four blocks for each insect population with 32 larvae in each blocks ($n = 4 \times 32 = 128$). Number of live larvae and weight of individual larva was measured before transferring them onto the diet. As described in the diet bioassay, after the first pupa was seen, observation was done daily until all insects pupated or died. Larval survival, pupation time, pupation success, sex ratio, pupal weight, and adult emergence were recorded for each individual.

3.2.5 Data analysis

Larval development time, larval/pupal body mass and, adult emergence time were transformed to log ($x + 1$) scale, while neonate-to-adult survivorship and sex ratio were transformed to square root values. Transformation was done to normalize treatment variances before statistical analysis. Transformed data for each parameter were analyzed using one-way ANOVA and differences among insect populations were separated using the Tukey test at $\alpha = 0.05$ level (SAS Institute, 2010). Comparisons in all biological parameters measured in this study were conducted among the seven insect populations in each of the two assay methods (i.e. on non-treated diet and on combined rearing). Untransformed means and their respective standard errors of mean are presented in the tables.

3.3. Results

3.3.1. Pupal mass of fall armyworm reared on non-treated diet

Effect of insect population on pupal mass of fall armyworm grown on non-Bt diet was significant for both male and female pupa ($F = 26.83$; $df = 6, 18$; $P < 0.0001$ for male and $F = 14.92$; $df = 6, 18$; $P < 0.0001$ for female) (Table 3.1). Pupal masses of Bt-SS males and females were somewhat greater than those of the corresponding sex for both Cry1F-resistant populations. The differences were significant ($P < 0.05$) for females of both resistant populations and males of RR-FL. Both resistant populations had similar pupal weight in females, while male of RR-PR appeared to be greater than that of RR-FL. Some levels of variations were observed among the four F_1 hybrid populations. For the crosses between RR-PR and Bt-SS, pupal mass of PR_mSS_f was similar ($P > 0.05$) to that of their resistant parents, while it for PR_fSS_m was similar ($P > 0.05$) to that of Bt-SS. For the crosses between PP-FL and Bt-SS, the pupal masses were not significantly different ($P > 0.05$) between the two reciprocal crosses for both males and females and the values were between those of Bt-SS and RR-FL.

3.3.2 Neonate-to-adult survivorship of fall armyworm reared on non-Bt diet

The effect of insect population on neonate-to-adult survivorship fed on non-Bt diet was significant ($F = 12.04$; $df = 6, 18$; $P < 0.0001$). The overall pattern of the survivorships among populations was similar between the two crosses (Bt-SS x RR-PR and Bt-SS x RR-FL). The survivorship rates of the two resistant populations, RR-PR and RR-FL, were similar ($P > 0.05$) but were significantly ($P < 0.05$) less than that of Bt-SS (Table 3.1). The survivorship of the four F_1 hybrid populations was similar compared to Bt-SS, but it was, in general, significantly greater than that of the resistant populations.

Table 3.1. Pupal mass (mean \pm SEM), neonate-to-adult survivorship (% mean \pm SEM), neonate-to-adult-emergence time, and sex ratio (mean \pm SEM) of seven populations of fall armyworm on non-treated diet

| population ¹ | Pupal mass(mg/pupa) ² | | Neonate-to-adult survivorship (%) ² | Neonate-to-adult emergence time (days) ² | | sex ratio ² |
|---------------------------------|--|--|--|---|--|---|
| | Male (♂) | Female (♀) | | Male (♂) | Female (♀) | |
| Bt-SS | 231.0 \pm 2.2 ab | 237.2 \pm 5.8 a | 91.4 \pm 1.49 a | 21.8 \pm 0.2 cd | 20.4 \pm 0.2 d | 0.98 \pm 0.09 a |
| RR-PR | 223.5 \pm 4.4 bc | 215.6 \pm 3.3 bc | 73.4 \pm 6.05 bc | 25.1 \pm 0.3 a | 24.0 \pm 0.4 a | 1.45 \pm 0.39 a |
| PR _m SS _f | 214.4 \pm 1.0 c | 215.9 \pm 2.8 bc | 88.2 \pm 1.4 ab | 23.0 \pm 0.9 bcd | 21.2 \pm 0.5 cd | 0.8 \pm 0.11 a |
| PR _f SS _m | 237.0 \pm 2.1 a | 235.0 \pm 2.8 a | 95.3 \pm 2.01 a | 21.7 \pm 0.09 d | 20.6 \pm 0.1 d | 0.85 \pm 0.09 a |
| RR-FL | 199.2 \pm 2.0 d | 203.6 \pm 4.0 c | 61.7 \pm 4.3 c | 24.7 \pm 0.7 ab | 24.2 \pm 0.7 ab | 1.05 \pm 0.1 a |
| FL _m SS _f | 220.9 \pm 3.7 bc | 224.0 \pm 3.1 ab | 85.9 \pm 2.7 ab | 23.7 \pm 0.4 abc | 23.0 \pm 0.7 abc | 1.02 \pm 0.13 a |
| FL _f SS _m | 215.2 \pm 3.4 c | 213.2 \pm 5.0 bc | 89.06 \pm 3.2 ab | 22.8 \pm 0.7 bcd | 21.8 \pm 0.4 bcd | 1.09 \pm 0.18 a |
| Analysis of variance | F _{6, 18} = 26.83 P < 0.0001 | F _{6, 18} = 14.92 P < 0.0001 | F _{6, 18} = 12.04 P < 0.0001 | F _{6, 18} = 9.79 P < 0.0001 | F _{6, 18} = 11.85 P < 0.0001 | F _{6, 18} = 1.21 P = 0.3393 |

¹Populations of fall armyworm: Bt-SS = Cry1F-susceptible population obtained from Texas; RR-PR = Cry1F resistant population obtained from Puerto Rico; RR-FL = Cry1F-resistant population obtained from Florida; PR_mSS_f = F₁ hybrid of RR-PR males and Bt-SS females; PR_fSS_m = F₁ hybrid of RR-PR females and Bt-SS males; FL_mSS_f = F₁ hybrid of RR-FL males and Bt-SS females; FL_fSS_m = F₁ hybrid of RR-FL females and Bt-SS males.

²Mean values within a column followed by a same letter are not significantly different (P > 0.05; by Tukey's Honest Significance Difference Test).

3.3.3. Neonate-to-adult emergence time of fall armyworm reared on non-Bt diet

Effect of insect population on neonate-to-adult emergence time of fall armyworm reared on non-Bt diet was significant for both males and females ($F = 9.79$; $df = 6, 18$; $P < 0.0001$ for male and $F = 11.85$; $df = 6, 18$; $P < 0.0001$ for female) (Table 3.1). Similarly as observed in the neonate-to-adult survivorship, the overall pattern of the neonate-to-adult developmental time among populations was similar between the two crosses (Bt-SS x RR-PR and Bt-SS x RR-FL). The overall time of neonate-to-adult emergence was 23.3 days for males and 22.1 days for females (Table 3.1). Compared to Bt-SS, both the males and females of RR-PR and RR-FL required significantly ($P < 0.05$) longer time to become adults. There were no significant differences in the neonate-to-adult developmental time among the four F_1 hybrid populations. The developmental time of the two F_1 populations of the crosses between Bt-SS and RR-PR was similar to that of Bt-SS for both males and females but it was significantly shorter than that of RR-PR. For the two F_1 populations generated from the crosses of Bt-SS and RR-FL, the difference in the developmental time was significant ($P < 0.05$) between FL_rSS_m and RR-FL, but it was not significant ($P > 0.05$) between FL_mSS_f and its parent populations.

3.3.4 Sex ratio of fall armyworm reared on non-Bt diet

The effect of the populations on sex ratios was not significant ($F = 1.21$; $df = 6, 18$; $P = 0.3393$). Some variations in the sex ratios were observed among the seven populations, but the differences were not significant ($P > 0.05$) with an overall ratio of 1.03: 1 (male: female) (Table 3.1).

3.3.5 Larval mass of fall armyworm at the 10th day reared on non-Bt corn leaf tissue

The effect of population on larval mass after 10 days on non-Bt corn leaf tissue was significant ($F = 9.34$; $df = 6, 18$; $P < 0.0001$). A larva of Bt-SS at 10th day feeding non-Bt corn leaf tissue weighed 235.6 mg, which was considerably ($P < 0.05$) greater than that of all other six populations (Table 3.2). Larval mass of the two resistant populations at 10 days feeding non-Bt corn leaf tissue was only 91.7 mg/larva (RR-PR) and 78.1 mg/larva (RR-FL), a reduction of 61.1 and 66.9%, respectively. In general, larval mass of the four F₁ hybrid populations was similar, ranging from 120.6 mg/larva for PR_mSS_f to 174.9 mg/larva for FL_mSS_f, which were significantly greater than that of their resistant parental populations except for PR_mSS_f.

3.3.6. Pupal mass of fall armyworm in the combined rearing of non-Bt corn leaf tissue and non-Bt diet

The effect of population on pupal mass of fall armyworm in the combined rearing was significant for both the sexes ($F = 3.59$; $df = 6, 18$; $P = 0.0161$ for male and $F = 4.16$; $df = 6, 18$; $P = 0.0066$ for female) (Table 3.2). For both sexes, pupal mass of both resistant populations was numerically less than that of Bt-SS, but the differences were not significant. For both sexes, there were no significant ($P > 0.05$) differences in the pupal masses within the parents and F₁ populations generated from the crosses between Bt-SS and each of the two resistant populations. Pupal mass of RR-PR and the associated two F₁ populations was also similar ($P > 0.05$) to that of RR-FL and the related two F₁ populations for both sexes.

3.3.7 Neonate-to-adult survivorship of fall armyworm in the combined rearing of non-Bt corn leaf tissue and non-Bt diet

Again, the effect of population on the neonate-to-adult survivorship rate was significant ($F = 4.93$; $df = 6, 18$; $P = 0.0027$). An average of neonate-to-adult survivorship rate of Bt-SS was 88.2%, while it was 69.5% for RR-PR and 60.9% for RR-FL (Table 3.2). The difference between

Table 3.2. Body mass (mean \pm SEM), neonate-to-adult survivorship (% mean \pm SEM), neonate-to-adult emergence time (mean \pm SEM), and sex ratio (mean \pm SEM) of seven populations of fall armyworm on non-Bt corn leaf tissues transferred to non-treated diet at 10th day

| Genotype ¹ | Body mass(mg/individual) | | | Neonate-to-adult Survivorship ² (%) | Neonate-to-adult emergence time (days) ² | | sex ratio ² |
|---------------------------------|--|---|---|--|---|--|---|
| | Larval mass at 10th day on leaf ² | Pupal mass ² | | | Male (σ) | Female (ρ) | |
| | | Male (σ) | Female (ρ) | | | | |
| Bt-SS | 235.6 \pm 7.8 a | 195.2 \pm 8.8 ab | 201.7 \pm 7.7 ab | 88.2 \pm 2.6 a | 21.2 \pm 0.3 c | 20.7 \pm 0.1 b | 0.89 \pm 0.15 a |
| RR-PR | 91.7 \pm 8.1 de | 192.9 \pm 6.5 ab | 189.8 \pm 7.1 b | 69.5 \pm 5.1 ab | 25.0 \pm 0.6 ab | 24.7 \pm 0.6 a | 1.3 \pm 0.17 a |
| PR _m SS _f | 120.6 \pm 9.8 cd | 222.7 \pm 3.5 a | 217.8 \pm 2.8 a | 70.3 \pm 7.04 ab | 22.5 \pm 0.2 c | 21.1 \pm 0.2 b | 1.02 \pm 0.16 a |
| PR _f SS _m | 145.9 \pm 7.3 b | 192.9 \pm 6.5 ab | 201.2 \pm 2.8 ab | 92.9 \pm 2.3 a | 23.3 \pm 0.5 bc | 22.5 \pm 0.4 b | 1.0 \pm 0.13 a |
| RR-FL | 78.1 \pm 5.3 e | 188.9 \pm 10.2 b | 201.6 \pm 4.6 ab | 60.9 \pm 7.4 b | 27.4 \pm 1.4 a | 25.3 \pm 0.9 a | 1.5 \pm 0.51 a |
| FL _m SS _f | 174.9 \pm 10.8 b | 213.3 \pm 1.9 ab | 213.7 \pm 1.3 a | 84.3 \pm 4.5 a | 22.2 \pm 0.4 c | 20.9 \pm 0.3 b | 0.89 \pm 0.1 a |
| FL _f SS _m | 150.0 \pm 8.8 bc | 213.1 \pm 3.8 ab | 212.6 \pm 1.1 a | 78.1 \pm 3.8 ab | 22.1 \pm 0.3 c | 21.4 \pm 0.3 b | 0.94 \pm 0.1 a |
| Analysis of variance | F _{6, 18} = 9.34 P < 0.0001 | F _{6, 18} = 3.59 P = 0.0161 | F _{6, 18} = 4.16 P = 0.0066 | F _{6, 18} = 4.93 P = 0.0027 | F _{6, 18} = 17.77 P < 0.0001 | F _{6, 18} = 16.69 P < 0.0001 | F _{6, 18} = 1.12 P = 0.3900 |

¹populations of fall armyworm: Bt-SS = Cry1F-susceptible population obtained from Texas; RR-PR = Cry1F resistant population obtained from Puerto Rico; RR-FL = Cry1F-resistant population obtained from Florida; PR_mSS_f = F₁ hybrid of RR-PR males and Bt-SS females; PR_fSS_m = F₁ hybrid of RR-PR females and Bt-SS males; FL_mSS_f = F₁ hybrid of RR-FL males and Bt-SS females; FL_fSS_m = F₁ hybrid of RR-FL females and Bt-SS males.

Bt-SS and the two resistant populations was significant ($P < 0.05$) for Bt-FL but not significant ($P > 0.05$) for RR-PR. The survivorship of the two F_1 populations generated from the crosses between Bt-SS and RR-PR was similar and not significantly different compared to their susceptible or resistant parental populations. The survivorship (84.3%) of FL_mSS_f was also not significantly different compared to Bt-SS and FL_fSS_m (78.1%), but it was significantly greater than the survivorship of RR-FL. There were also no significant ($P > 0.05$) differences in the survivorship rates between the two resistant populations and among the four F_1 hybrid populations.

3.3.8. Neonate-to-adult developmental time of fall armyworm in combined rearing of non-Bt corn leaf tissue and non-Bt diet

The effect of insect population on the neonate-to-adult developmental time of fall armyworm in the combined rearing was significant for both sexes ($F = 17.77$; $df = 6, 18$; $P < 0.0001$ for male and $F = 16.69$; $df = 6, 18$; $P < 0.0001$ for female) (Table 3.2). Overall female of fall armyworm developed to adults from neonates approximately one day earlier than its male counterpart (Table 3.2). A neonate of Bt-SS required an average of 21.1 days (male) and 20.7 days (female) to become an adult. In contrast, the neonate-to-adult developmental time was 24.7 days for male RR-PR, 25.3 days for female RR-PR, 27.4 days for RR-FL, and 25.3 days for female RR-FL. The differences between the two resistant populations were not significant for both sexes. There were no significant ($P > 0.05$) differences in the developmental time among the four F_1 populations. In general, the developmental time of F_1 hybrid populations was also similar ($P > 0.05$) to that of their susceptible parental population but significantly ($P < 0.05$) shorter than that of their resistant parental populations.

3.3.9. Sex ratio of fall armyworm in combined rearing of non-Bt corn leaf tissue and non-Bt diet

As observed in the diet bioassay, the effect of insect population on sex ratio in the combined rearing was also not significant ($F = 1.12$; $df = 6, 18$; $P = 0.3900$) with an overall ratio of 1.07:1(male: female) (Table 3.2).

3.4. Discussion

Results of the current study showed that the Cry1F resistance in the fall armyworm was associated with considerable fitness costs, especially for RR-FL. The fitness costs in the Cry1F-resistant fall armyworm were revealed in reduced growth, increased mortality, and delayed development. In the assay with non-Bt diet, both resistant populations exhibited fitness costs in three of the four biological parameters measured. Compared to the susceptible population (Bt-SS), the Puerto Rico Cry1F-resistant population (RR-PR) showed an average of 6.1% reduced pupal mass, 19.7% greater neonate-to-adult mortality, and 3.5 days delay from neonate to adult. The fitness costs for RR-FL were even more significant. In the diet assay, RR-FL demonstrated an average of 14.0% reduction in pupal mass, 32.5% higher insect mortality, and 3.4-days delay in neonate-to-adult developmental time. Except the pupal mass, results of the combined rearing of non-Bt corn leaf tissue and non-Bt diet were generally consistent with those observed in the non-Bt diet assay with only few variations. Compared to Bt-SS population, RR-PR showed an average of 61.1% reduced larval mass at 10 day feeding non-Bt corn leaf tissue, 21.2% higher neonate-to-adult mortality, and 3.9 days delay in neonate-to-adult developmental time and those value for RR-FL were 66.9%, 31.0%, and 5.4 days, respectively.

Recently, assessments of fitness costs of Cry1F resistance in fall armyworm have been recently performed in two studies. Vélez et al. (2013) compared the life-history traits and population growth rates of a susceptible parental strain, a homozygous Cry1F-resistant strain

originated from Puerto Rico, and their F₁ hybrids feeding on non-Bt diet. In another study, Jakka et al. (2014), compared larval survival, larval and pupal weights and development time, adult longevity, fertility and fecundity as well as sex ratio between Cry1F-resistant and -susceptible strains of fall armyworm reared on different hosts including meridic diet, leaf tissue of corn and soybean, as well as reproductive tissue of cotton. The Cry1F-resistant strain evaluated by Jakka et al. (2014) was also collected from Puerto Rico. Contrary to the results of the current study, both of the published studies that the Cry1F resistance in the fall armyworm was not associated with fitness costs (Vélez et al., 2013; Jakka et al., 2014). The varied results between the previous and current study indicate that a diversified genetic basis may exist for the Cry1F resistance in fall armyworm. In addition, the different results could also be caused by the assay methods or test conditions. Studies have shown that the intensity of fitness costs of Bt resistance in insect populations can be varied depending on the environmental conditions such as host plants, insect pathogens, intraspecific competition, and other factors (Gassmann et al., 2009; Janmaat and Myers, 2011; Kruger et al., 2012).

It was highly expected that the Cry1F resistance observed in the southeast costal region of the U.S. Mainland was caused by migration of resistant populations from Puerto Rico through other Caribbean islands (F.H. personal communication). The results of previous chapter (genetic study) and the fitness tests in the current study more support a dissimilar genetic basis of the Cry1F resistance between the population collected from Florida (RR-FL) and those collected from Puerto Rico e.g. RR-PR and the two evaluated in Vélez et al. (2013) and Jakka et al. (2014). As discussed above, the difference among the populations evaluated could be caused by a diversified genetic basis of the resistance even from a similar source such as the territory of

Puerto Rico or simply just due to location selections. Further studies are warranted to reveal whether the field resistance in the U.S. mainland was due to migrations or location selections.

Disadvantages in life-history traits of homozygous resistant strains (RR) might be sometimes due to the reasons that are independent to resistance (Amin et al., 1984; Boggid et al., 1958; Mcnair et al., 1983; Roush & Croft, 1986; Roush et al., 1982). Even if fitness costs of Bt resistance cause RR individuals to be less fit than susceptible (SS) individuals, it is more critical to see if differences between heterozygous resistant strains (RS) and SS strains exist. This is because, in the field, RS individuals are much more abundant than RR insects during the early stages of resistant development (Roush et al., 1982). Therefore, non-recessive fitness costs of resistance could be an important factor in delaying resistance development in field pest populations (Gassmann et al., 2009). However, published data showed that Bt resistance is often associated with fitness costs, but most of the costs are recessively inherited (Anilkumar et al., 2008; Gassmann et al. 2009). Results of the current studies suggested that the inheritance of the fitness costs of the Cry1F resistance in the fall armyworm was varied from intermediate to completely recessive depending on the assay methods, biological parameters measured, and populations. For example, on the non-Bt diet, fitness costs in the neonate-to-adult survivorship on non-Bt diet were inherited recessively but the fitness costs in the 10-day larval mass were intermediate. The non-recessive fitness costs of the Cry1F resistance in the fall armyworm identified in the current study could be useful in developing resistance management strategies.

3.5 References

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CHAPTER 4. SUMMARY AND CONCLUSION

Transgenic corn hybrids expressing *Bacillus thuringiensis* (Bt) proteins have been widely used to control corn insect pests in the U.S. and several other countries of the world. Resistance development in target pest populations to the Bt proteins in the plants is a major threaten for the sustainability of Bt corn technologies as an effective pest management tool. Fall armyworm (*Spodoptera frugiperda* (J. E. Smith)) is a major pest targeted by Bt corn in both North and South America. In 2003, transgenic corn event expressing the Cry1F protein from *Bt* var. *aizawai* (Event TC1507) was commercially planted to control the fall armyworm in Puerto Rico. However, field resistance to the Event TC1507 corn was documented in the fall armyworm populations in Puerto Rico in 2006. Several years later, unexpected field damage to TC1507 corn by fall armyworm was observed in several occasions in the southeastern coast regions of the U.S. Mainland. The reduced efficacy of TC1507 in the U.S. Mainland was recently documented to be also caused by resistance in the fall armyworm.

In 2011, two populations, RR-PR and RR-FL, of fall armyworm that were highly resistant to both the purified Cry1F protein and Cry1F corn plants were established from field collections in Puerto Rico and Florida, respectively. The objective of this study was to characterize the inheritance and fitness costs of the Cry1F resistance in the two Cry1F-resistant populations of fall armyworm populations.

In the inheritance study, besides RR-PR, RR-FL, and a Cry1F-susceptible population (Bt-SS), 14 other populations were developed by reciprocal crosses, F_1 yby F_1 crosses, backcrosses, and crosses between RR-PR and RR-FL. Diet-incorporated bioassays with purified Cry1F protein were conducted to determine the Cry1F susceptibility for all 17 populations. Maternal effect was analyzed using the dose-response data obtained from the reciprocal crosses between

the resistant and susceptible populations. The dominance level of resistance was measured as the effective dominance “ D_{ML} ”. Mortality of the F_2 and backcross populations was used to fit for the Mendelian monogenic model. The results showed that there might be a different genetic basis for the Cry1F resistance between the Puerto Rico and Florida populations. The Cry1F resistance in RR-PR was likely inherited in >1 recessive (or incompletely recessive) genes and the genes associated the resistance were sex-linked to the males of the insect. In contrast, the resistance in RR-FL was dominant and more likely controlled by autosomal genes.

In the study of fitness costs, seven insect populations were assayed on a non-toxic diet as well as on a combined rearing of non-Bt corn leaf tissue and non-Bt diet. The seven populations were RR-PR, RR-FL, Bt-SS, and four F_1 populations that were developed from the reciprocal crosses between Bt-SS and the two resistant populations. Biological parameters measured in the fitness tests were neonate-to-adult survivorship, neonate-to-adult development time, 10-day larval mass on non-Bt corn leaf tissue, pupal mass, and sex ratios. Cry1F resistance in the fall armyworm in both resistant populations was associated with considerable fitness costs, especially for the Florida population. In the bioassays with non-Bt diet, compared to Bt-SS, the Puerto Rico resistant population showed an average of 6.1% reduced pupal mass, 19.7% greater neonate-to-adult mortality, and 3.5 days delay from neonate to adult. In contrast, RR-FL demonstrated an average of 14.0% reduction in pupal mass, 32.5% higher insect mortality, and 3.4-days delay in neonate-to-adult developmental time. In the combined bioassays of non-Bt diet and non-Bt plant tissue, except the pupal mass, the results were generally consistent with those observed in the non-Bt diet assay. Compared to Bt-SS population, RR-PR showed an average of 61.1% reduced larval mass at 10 day feeding non-Bt corn leaf tissue, 21.2% higher neonate-to-

adult mortality, and 3.9 days delay in neonate-to-adult developmental time and those value for RR-FL were 66.9%, 31.0%, and 5.4 days, respectively.

Knowledge generated from this study should be useful in understanding the resistance mechanisms, and developing effective monitoring and management strategies for the sustainable use of the Bt corn technology. It also should lay a foundation for further studies to reveal if the Cry1F resistance in Puerto Rico and the U.S. Mainland are related.

VITA

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