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Resistance to *Bacillus thuringiensis* in sugarcane borer, *Diatraea saccharalis* (F.)

Xiaoyi Wu

Louisiana State University and Agricultural and Mechanical College, im_wu@hotmail.com

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**RESISTANCE TO *BACILLUS THURINGIENSIS*
IN SUGARCANE BORER, *DIATRAEA SACCHARALIS* (F.)**

A Dissertation

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

in

The Department of Entomology

by
Xiaoyi Wu
B.S., Nanjing Agricultural University, 1994
M.S., Nanjing Agricultural University, 1997
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ABSTRACT

The sugarcane borer, *Diatraea saccharalis* (F.), is a dominant corn stack boring pest and a major target of *Bacillus thuringiensis* (Bt)-corn in many areas of the mid-southern region of the United States. A Cry1Ab-resistant strain of *D. saccharalis*, capable of survival on commercial Bt corn plants, was established using an F₂ screening procedure. Larval survival of Cry1Ab-resistant, -susceptible, and -heterozygous genotypes was evaluated on five non-Bt and seven Bt field corn hybrids at two plant stages. During the vegetative stages, all seven Bt corn hybrids were highly efficacious against the three genotypes, while 8-18% of the heterozygous genotype survived on reproductive stage plants for four Bt corn hybrids. Susceptibilities of Cry1Ab-susceptible and -resistant strains were evaluated for four Bt proteins: Cry1Aa, Cry1Ac, Cry1A.105, and Cry2Ab2. LC₅₀ values of the Cry1Ab-resistant strain were >80-, 45-, 4.1-, and -0.5-fold greater than that of the susceptible strain to the four proteins, respectively. Relative fitness on non-toxic diet, diet treated with low concentrations of Cry1Ab toxin, and on conventional corn plants was compared for five genotypes of *D. saccharalis*. Larvae of Cry1Ab-susceptible and Cry1Ab-resistant strains on both non-toxic diet and non-Bt corn plants developed normally. There were no significant differences between the two strains in all measured biological parameters, suggesting a lack-of-fitness cost of the Cry1Ab resistance in *D. saccharalis*. Larval development, growth, and survival of the Cry1Ab-susceptible strain were significantly affected on diet treated with low concentrations of Cry1Ab toxin, while the effect to the resistant strains was not/or less significant. Using various genetic crosses, inheritance of Cry1Ab resistance in *D. saccharalis* was assessed on Bt corn leaf tissue, intact Bt corn plants, and diet containing Cry1Ab toxin. Cry1Ab resistance in *D. saccharalis* was inherited as a single autosomal gene. The resistance was incompletely or nearly completely recessive on Bt corn leaf tissue and intact Bt corn plants, while the dominance increased as Cry1Ab concentrations

decreased when it was tested on Cry1Ab-treated diet. Results generated from this study will provide valuable information in understanding Bt resistance mechanisms and developing effective strategies for managing resistance of *D. saccharalis* to Bt corn.

CHAPTER 1

INTRODUCTION

As a major feed grain for livestock, food resource for humans, and raw material for ethanol production, corn, *Zea mays* L., is the most widely planted field crop in the United States. In 2007, a total of 93.6 million acres of field corn was planted in the United States (NASS 2007). Field corn also represents substantial acreage and contributes significant crop value to Louisiana agriculture. In 2007, a total of 730,000 acres of field corn was planted in Louisiana (NASS 2007).

Bacillus thuringiensis

Bacillus thuringiensis (Bt) is a widespread soil dwelling bacterium. It was first discovered in 1901 by a Japanese scientist (Gill et al. 1992). The Japanese Bt strain was found to kill the silkworm, *Bombyx mori* L., at a silk production farm. Another strain was found in Germany about 10 years later by Ernst Berliner. Like the other members of genus *Bacillus*, Bt is aerobic and capable of producing endospores (Madigan and Martinko 2005). When nutrients are lacking, the bacterium produces crystal insecticidal δ -endotoxins. The crystal insecticidal proteins are often called “Cry” toxins. These Cry toxins have specific activities against some species within the orders of Lepidoptera, Diptera, and Coleoptera. Since 1955, Bt Cry toxins have been used as important biological insecticides under various trade names, such as AbleTM, Biobit®, Dipel®, and Thuricide® (NPTN 2000).

Compared to traditional chemical insecticides, Bt microbial insecticides exhibit many advantages. For example, Bt insecticides are usually very safe to most non-target organisms including natural enemies of insects (predators and parasites), pollinators, i.e., honeybees, and fish. Bt insecticides can also be well integrated with other control methods in many pest

management (IPM) programs. Bt proteins are essentially nontoxic to people, pets, and wildlife. As a biological control agent, Bt toxins are the most widely used microbial insecticides, accounting for 90-95% of the biological insecticide market in the world (Gill et al. 1992).

Transgenic Bt Plants

Bt microbial insecticides have some disadvantages for insect management. Control efficacy of Bt microbial insecticide applications can be affected by many factors including weather conditions, poor coverage, and instability to UV light. These factors greatly limited the use of Bt microbial insecticides for managing insect pests in many crop systems. The advances in biotechnology have allowed scientists to transfer Bt genes into plant genomes. Transgenic plants directly produce insecticidal Bt proteins within their tissues (Höfte et al. 1986). The first genetically engineered Bt plant, Bt tobacco, was developed in 1985 by a Belgian company, Plant Genetic Systems (Höfte et al. 1986). Since then, many plant species have been bio-engineered to express Bt proteins for controlling insect pests (Huang et al. 1999a). During 1996, three of these Bt crops (i.e., potato, cotton, and corn) became commercially available in the United States and several other countries. Since then, adoption of Bt crops has increased rapidly because of the high efficacy in controlling the target pests. By 2007, the number of countries planting Bt crops increased to 21 with a total of > 36.8 million hectares throughout the world (James 2007). Corn and cotton have been the predominant commercialized Bt crops. Only a very small acreage of Bt potato and Bt rice have been planted in the United States and Iran, respectively. Among the 36.8 million hectares of Bt crops planted in 2007, about 23.3 million hectares (or 63%) were Bt corn planted in 15 countries and about 13.5 million hectares (or 37%) were Bt cotton planted in 9 countries. Since the commercialization of Bt crops, the United States has been the leading country in planting Bt crops, accounting for approximately one-third of the accumulated Bt crop

acreage during the past 12 years. During 2007, the United States alone planted 18.4 million hectares of Bt corn and 2.6 million hectares of Bt cotton, which accounted for approximately 49% and 59% of the total acres, respectively (NASS 2007).

The first generation of Bt corn hybrids produced only a single Bt Cry protein. For example, the most commonly planted Bt corn, YieldGard[®], expresses only the Cry1Ab Bt protein. Newer transgenic Bt corn hybrids can express two or more Cry proteins and are able to control more than one group of insect pests. For example, YieldGard[®] Plus corn produces both the Cry1Ab and Cry3Bb1 proteins which are efficacious against both Lepidopteran stalk-boring pests and Coleopteran corn rootworms, and shows some activity against earworms, and armyworms (US EPA 2005a). Currently, both Bt crops expressing single Bt gene or multi Bt genes are planted in the United States. It is expected that the use of stacked-gene varieties will be increased. Single-Bt-gene varieties such as YieldGard[®] will likely be phased out in the United States and other countries in the near future (Johnson 2007).

Transgenic Bt corn was first planted commercially in the mid-southern region of the United States in 1999 (Castro et al. 2004a). Since then, adoption of this technology has increased rapidly across the region because of increasing yield losses from a complex of corn stalk boring pests including the European corn borer, *Ostrinia nubilalis* (Hübner), southwestern corn borer, *Diatraea grandiosella* Dyar, and sugarcane borer, *Diatraea saccharalis* (F.) (Castro et al. 2004a, Sankula and Blumenthal 2004, Porter et al. 2005, Huang et al. 2006a, Huang and Leonard 2008). Yield loss in non-Bt corn caused by corn stalk boring pests was estimated > 30% in this region (Sankula and Blumenthal 2004). Currently, Bt corn is the primary tool for managing corn stalk borer problems in the mid-southern region, including Louisiana. Bt corn acreage in this region

has reached the maximum level (50%) allowed by the US Environmental Protection Agency (US EPA) for Bt resistance management (Huang and Leonard 2008, Yue et al. 2008).

Diatraea saccharalis

D. saccharalis, is native to the western hemisphere, but not to the United States (Kelsheimer et al. 1950, Capinera 2001, Falco et al. 2001). It was introduced into Louisiana about 1855, and then spread to the warmer portions of other Gulf Coast States. *D. saccharalis* also occurs throughout the Caribbean, Central America, and the warmer portions of South America to northern Argentina (Kelsheimer et al. 1950, Capinera 2001, Falco et al. 2001, Reagan 2001). *D. saccharalis* has a wide range of hosts, infesting plants in the family Gramineae. Though it is primarily a pest of sugarcane, *Saccharum officinarum* L., *D. saccharalis* also feeds on other crops such as corn, rice, *Oryza sativa* L., sorghum, *Sorghum bicolor*, sudangrass, *Sorghum X drummondii*, and many wild or weed grasses including Johnsongrass, *Sorghum halepense*, *Paspalum* sp., *Panicum* spp., *Holcus* sp., and *Adropogon* sp. (Reagan 1981, Ogunwolu et al. 1988, Capinera 2001, Braga et al. 2003). *D. saccharalis* is also a pest of sweet corn in the southern United States such as Florida (Kelsheimer et al. 1950).

In Louisiana, three corn borer species are known to infest corn: *O. nubilalis*, *D. grandiosella*, and *D. saccharalis* (Castro et al. 2004a). As a key sugarcane pest, *D. saccharalis* once was a sporadic pest of field corn in Louisiana, but has recently expanded its geographic range and become the dominant corn stalk borer in the state and other areas of the mid-southern region (Castro et al. 2004a, Porter et al. 2005, Huang and Leonard 2008). In Louisiana, field corn was severely damaged by *D. saccharalis* during 2002-2003. Corn yield loss to non-Bt corn caused by this corn borer pest was estimated to be >30% during the two years in many fields across the state. A recent four-year (2004-2007) field survey in Louisiana showed that *D. saccharalis*

accounted for 82% of the total corn borer populations across the major corn-producing areas of this state (Huang and Leonard 2008). The remaining infestations were determined to be *D. grandiosella* (17%) and *O. nubilalis* (<1%). *D. grandiosella* and *O. nubilalis* occur only in the northern region of the state. *D. saccharalis* has also been a predominant corn borer species in the central gulf coastal area of Texas (Porter et al. 2005). Field samples collected during 2006 and 2007 showed that *D. saccharalis* accounted for >95% of the total corn borer populations in the coastal area of TX (RP and FH, unpublished data). In some corn fields, an average of >10 larvae/plant could be found during the 2007 growing season. *D. saccharalis* damage to field corn was also observed in Mississippi and Arkansas, two other states of the mid-southern region (Davis et al. 1999). Because of the economical importance in corn production in the mid-southern region, *D. saccharalis* was recently officially listed as a target pest of Bt corn in the United States (US EPA 2005a, b).

Field Corn Stalk Borer Management

Prior to use of transgenic Bt corn, management of corn borers in corn depended primarily on application of chemical insecticides. Suggested insecticides included Mustang Max[™], Furadan[®] 4F, Asana[®] XL, Pounce[®], Warrior T[®], Intrepid[®], and Baythroid[®] (Baldwin et al. 2008). Some cultural practices are also recommended for *D. saccharalis* control. Late instars of *D. saccharalis* can overwinter in the stubble of corn, sorghum, and rice in the non-sugarcane growing areas of Louisiana (Huang et al. 2006a). Therefore, destruction of overwintering habitats could reduce overwintering populations. Some *D. saccharalis* - resistant/tolerant corn varieties also have been identified (Hoisington et al. 1996). Since 1999, Bt corn hybrids have been used to manage corn stalk borers across the mid-southern region (Castro et al. 2004a, Huang et al. 2006a). Transgenic Bt corn hybrids have been very effective against *D. saccharalis*, and are currently the most

important tool for controlling *D. saccharalis* on corn in Louisiana, accounting for approximately 50% of the state's total corn acreage (Huang and Leonard 2008, Yue et al. 2008).

Importance of Resistance Management for Bt Corn in Louisiana

The widespread acceptance of transgenic Bt corn, will place a high level of selection pressure on the target insect and may result in development of resistance in these populations (Ostlie et al. 1997; Gould 1998; Tabashnik et al. 1991, 2003). Recent studies have shown that management of *D. saccharalis* resistance to Bt corn is more important than that proposed for *O. nubilalis* and *D. grandiosella* for some areas of the mid-southern region, especially Louisiana (Huang et al. 2007a, b; Huang and Leonard 2008). Two published studies indicated that performance of Cry1Ab corn against *D. saccharalis* varied among different Bt corn cultivars (Castro et al. 2004b, McAllister et al. 2004). Laboratory bioassays also showed that *D. saccharalis* is significantly less susceptible to Cry1Ab compared to *O. nubilalis* and *D. grandiosella* (Huang et al. 2006b). In addition, a major Bt resistance allele has been detected in three Louisiana populations of *D. saccharalis* since 2004 (Huang et al. 2007a, Huang and Leonard 2008). Unlike laboratory-selected Bt-resistant strains of *O. nubilalis* (Huang et al. 1997, Bolin et al. 1999, Chaufaux et al. 2001), the Cry1Ab-resistant strain of *D. saccharalis* is capable of surviving and completing larval development (neonate to pupa stage) on intact commercial Bt corn plants (Huang et al. 2007a, Wu et al. 2007). This resistant *D. saccharalis* strain has shown a significant resistance level to purified trypsin-activated Cry1Ab toxin (Huang et al. 2007b). This resistance allele detected in *D. saccharalis* is the first major resistance allele to Cry1Ab corn documented for any cornstalk borer species worldwide.

Current Resistance Management Strategy for Bt Corn in the United States

Bt resistance in target insect populations is a primary concern for long-term success of the transgenic Bt corn technology as an effective pest management tool. In order to delay resistance

development to Bt corn in target pests, the “high dose/refuge” insecticide resistance management (IRM) strategy has been implemented for planting Bt corn in the United States and Canada (FIFRA Scientific Advisory Panel 1998, US EPA 2001, Baute 2004). The principle of this IRM strategy is not very complicated. This strategy requires planting Bt corn in only a portion of the corn acreage in an area. The Bt corn should express a sufficient high dose to kill heterozygous individuals for Bt resistance. The remaining portion of the corn acreage should be planted with non-Bt corn as refuge for Bt-susceptible corn borers. Thus, the predominant number of susceptible individuals surviving from non-Bt corn refuge plants could randomly mate with those rare resistant homozygotes surviving on Bt corn plants. Therefore, the majority of their offspring carrying resistance alleles will be heterozygotes (carrying only a single resistance allele). These heterozygous individuals should be killed by the high dose expressed Bt corn (Ostle et al. 1997, US EPA 2001, Baute 2004). As a result, resistance allele frequencies in the field insect populations should be maintained at a relatively low level. In the United States, when planting Bt corn to control corn borers, the current “high dose/refuge” IRM plan requires that growers must plant at least 20% non-Bt corn as refuge outside cotton-production regions, but need to plant a minimum of 50% non-Bt corn in regions that produce cotton. The non-Bt refuge should be planted within 800 m of the Bt corn on every farm with a field that contains Bt corn (US EPA 2001).

Key Assumptions of the “High Dose/Refuge” IRM Strategy

There are four key assumptions for the success of this IRM strategy (Tabashnik 1994a, b). First, Bt corn plant must express a “high dose” of Bt proteins so that heterozygous individuals for Bt resistance can be killed. Second, resistance allele frequency in target insect populations should be very low (e.g. <0.001). Third, resistance should be inherited as a completely or

incompletely recessive gene. Finally, there is a random mating between resistant and susceptible populations of the target insect species. There are several practical definitions of “high dose”. A US EPA expert panel for Bt resistance management defined a high dose as a dose that kills >95% of resistant heterozygotes (Gould 1988, Van Rie 1991, Roush 1994). Another definition of “high dose” referred to 25 times the concentration needed to kill 99% of the susceptible individuals (Gould et al. 1994).

The current “high dose/refuge” IRM strategy for Bt corn was developed primarily based on the information generated with *O. nubilalis* and *D. grandiosella*. These two species are the most economically important corn borer pests in the North Central and Midwestern US Corn Belts. Field and laboratory studies have shown all current commercial Bt corn for controlling corn borers express a high dose of Bt toxins for *O. nubilalis* and *D. grandiosella* (US EPA 2001). Intensive screenings for Bt resistance in nearly 20 populations of *O. nubilalis* and *D. grandiosella* collected in the United States have not detected major resistance alleles, suggesting that resistance alleles in these corn borer pests are rare (Andow and Alstad 1998; Andow et al. 1998, 2000; Bourguet et al. 2003; Farinós et al. 2004; Huang et al. 2007c). Random mating assumption should be met by appropriate arrangement of non-Bt corn refuge as required for the IRM strategy (Bourguet et al. 2003). The genetic basis of corn borer resistance to Bt corn is unknown because highly resistant corn borer populations capable of survival on commercial Bt corn plants were not available prior to the establishment of the Bt resistant *D. saccharalis* strain in our laboratory.

Previous studies on Bt resistance management have been focused on *O. nubilalis* and *D. grandiosella*. Limited research on Bt resistance has included *D. saccharalis* (Huang et al. 2006b). Information for use of the current “high dose/ refuge” IRM strategy for managing *D. saccharalis*

resistance to Bt corn is limited. It is necessary to validate if the currently adopted IRM strategy for *O. nubilalis*/*D. grandiosella* is also an effective approach for management of Bt resistance in *D. saccharalis*. Two objectives of the current study were designed to verify two of these four key assumptions of the “high dose/ refuge” strategy for managing *D. saccharalis* resistance to Bt corn.

High Dose Clarification of Bt Corn Against *D. saccharalis*

Larval survival of a Dipel[®]-resistant strain of *O. nubilalis* on Bt corn plants has been evaluated in the greenhouse (Huang et al. 2002). The results showed that the laboratory selected Dipel[®]-resistant strain of *O. nubilalis* could not survive on commercial Bt corn hybrids that expressed a high level of Cry1Ab toxin. To determine if Bt corn commonly planted in Louisiana qualifies as “high dose” against *D. saccharalis* as required for the “high/dose refuge” IRM strategy, larval survival of Cry1Ab-susceptible, -resistant, and heterozygous genotypes of *D. saccharalis* on several commercial Bt corn hybrids were evaluated in the greenhouse at different plant stages (Objective 1). Larval survival data of the heterozygous genotypes for Bt resistance were used to determine if a Bt hybrid qualifies as “high dose” based on the US EPA definition described above.

Cross-Resistance

Information on cross-resistance of *D. saccharalis* to various Bt toxins is useful in understanding resistance mechanisms and developing management strategies. Cross-resistance among Bt toxins has been evaluated in several insect species. Most published data indicated that selection for resistance to one Bt Cry toxin can lead to resistance to others (Tabashnik 1994b; Tabashnik et al. 1994, 1996, 2000; Gould et al. 1995; Sayyed and Wright 2001; Ferré and Van Rie 2002; Li et al. 2005). However, the patterns of cross-resistance can be varied in different

insect species and even among insect strains of a same species. Cross-resistance of *D. saccharalis* to Cry toxins has not been examined. In this study, susceptibility of the Cry1Ab-resistant *D. saccharalis* to four Bt Cry proteins was assayed in the laboratory (Objective 2). These Cry proteins included Cry1Aa, Cry1Ac, Cry1A.105, and Cry2Ab2. Cry1A.105 and Cry2Ab2 are the two Bt proteins expressed in a new Bt corn event, MON 89034 (Johnson 2007). This Bt event corn will offer broad control of Lepidopteran corn pests and may offer a valuable resistance management tool because of the presence of the two individually effective insecticidal Bt proteins against Lepidopteran pests. This new generation of Bt corn product has been approved by the U.S. regulatory authorities for commercial use beginning in 2009 under the trade name of YieldGard VT Pro™. Results generated from this study should provide valuable information about the ability of MON 89034 hybrids to control *D. saccharalis* populations in the mid-southern region and in development of future IRM strategies for Bt corn.

Fitness of Bt Resistance

Fitness costs are often associated with resistance and can be used for Bt resistance management. Genes which provide resistance to novel challenges such as pesticides, toxins and pathogens often impose fitness costs on resistant phenotypes (Raymond et al. 2005). Significant non-recessive fitness costs associated with resistance imply that the resistance allele frequency in field insect populations will decrease once selection pressure is removed. For non-recessive fitness costs, resistance development in field insect populations can be significantly delayed or even be reversed, if the absence of selection pressure is long enough (Tabashnik et al. 2005). Therefore, understanding the fitness of insecticide resistance is important in development of effective IRM strategies.

Studies on the fitness of Bt resistance have been conducted in several insect species targeted by transgenic Bt crops (e.g. Bt corn or Bt cotton) (Groeters et al. 1993, Oppert et al.

2000, Liu et al. 2001a, Akhurst et al. 2003, Snow et al. 2003, Bird and Akhurst 2004, Cerda and Wright 2004, Vacher et al. 2004, Carrière et al. 2005, Higginson et al. 2005, Raymond et al. 2005, Anilkumar et al. 2008).

In most cases, Bt resistance is associated with fitness costs and most are recessive (Anilkumar et al. 2008). In some circumstances, fitness costs associated with Bt resistance can interact with environmental factors such as host plants (Carrière et al. 2005, 2006; Janmaat and Myers 2005, 2006; Bird and Akhurst 2005, 2007). In this study, larval growth and development of the Cry1Ab-susceptible, -resistant, and their F₁ progeny of *D. saccharalis* were evaluated on a meridic diet with/without Bt toxin in the laboratory and on conventional non-Bt corn plants in the greenhouse (Objective 3). Data generated from this study should provide valuable information in understanding resistance mechanisms and developing appropriate strategies for managing *D. saccharalis* resistance to Bt corn.

Inheritance of Bt Resistance

Knowledge about the genetic modes of Bt resistance can improve resistance detection and monitoring, risk assessment, modeling, and development of IRM strategies (Bourguet 2004, Tabashnik and Carrière 2007). The genetic basis of Bt resistance has been assessed in many insect species (Heckel 1994; Ferré 1997; Liu and Tabashnik 1997; Tabashnik et al. 1997, 2002a, 2004; Huang et al. 1999b; Bourguet et al. 2000; Sayyed et al. 2000; Ferré and Van Rie 2002; Morin et al. 2003; Sayyed and Wright 2004; Alves et al. 2006; Tabashnik and Carrière 2007). In most cases, a high level of Bt resistance is controlled by one or a few autosomal, recessive or incompletely recessive gene(s) (Liu et al. 2001b, Tabashnik et al. 2002b, Augustin et al. 2004, Kain et al. 2004, Sayyed et al. 2004, Liang et al. 2008). In contrast, low levels of resistance could be more dominant (Gould et al. 1992, Huang et al. 1999b, Kranthi et al. 2005). There are also

some exceptions (Chaufaux et al. 1997, Tang et al. 1997, Sayyed et al. 2003, Janmaat et al. 2004, Alves et al. 2006, Jackson et al. 2006). Inheritance of Bt resistance in corn stalk boring species has been examined for only three laboratory selected strains of *O. nubilalis* (Huang et al. 1999b, Alves et al. 2006). None of these three Bt-resistant strains of *O. nubilalis* have demonstrated the ability to survive on commercial Bt corn plants (Huang et al. 2002, Bourguet 2004). The availability of a Cry1Ab-resistant strain of *D. saccharalis* provided an opportunity to assess the genetic basis of Bt resistance for this species. Various cross-mating studies were used to characterize the genetic basis of Bt resistance in *D. saccharalis* and determine if resistance is controlled by a recessive gene (Objective 4).

The specific objectives of this project include:

Objectives

- I. Evaluate the performance of transgenic Bt corn hybrids against Cry1Ab-susceptible and -resistant *D. saccharalis* to determine if Bt corn varieties commonly planted in Louisiana qualify as high dose as required for the current “high dose/refuge” IRM strategy for Bt corn;
- II. Determine susceptibilities of Cry1Ab-resistant and -susceptible *D. saccharalis* to four other Bt toxins to analyze the cross-resistance pattern and generate information needed for developing new Bt corn for managing *D. saccharalis*;
- III. Assess relative fitness of Cry1Ab-susceptible and -resistant *D. saccharalis* on diet and conventional corn plants to determine if fitness costs are associated with Cry1Ab resistance in *D. saccharalis*; and

IV. Characterize the inheritance of Cry1Ab resistance in *D. saccharalis* to verify if Bt resistance is controlled by a recessive gene as defined in the “high dose/refuge” IRM strategy for Bt corn.

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CHAPTER 2

EVALUATION OF TRANSGENIC *BACILLUS THURINGIENSIS* CORN HYBRIDS AGAINST CRY1AB-SUSCEPTIBLE AND -RESISTANT SUGARCANE BORER (LEPIDOPTERA: CRAMBIDAE) *

Introduction

Field corn, *Zea mays* L, represents substantial acreage and contributes significant crop value to agriculture in the mid-southern region of the United States (National Agricultural Statistics Service 2006). Transgenic field corn expressing Cry1Ab protein from *Bacillus thuringiensis* (Bt) was commercialized in 1999 in the region. Adoption of this technology has increased rapidly across the region because of increasing yield losses from a complex of field cornstalk borers. Bt corn currently accounts for >40% of the total corn acreage in this area. However, the widespread acceptance of transgenic Bt corn could accelerate development of resistance, raising concerns about the long-term sustainability of Bt corn as an effective pest management tool (Tabashnik 1994, Ostlie et al. 1997, Gould 1998, Baute 2004).

To ensure the long-term sustainability of Bt corn, an insect resistance management (IRM) plan has been implemented as a mandatory requirement for planting Bt corn in the United States (US EPA 2001). The current IRM strategy is termed the “high dose/refuge” strategy, and it was developed specifically for managing European corn borer, *Ostrinia nubilalis* (Hübner), and southwestern corn borer, *Diatraea grandiosella* Dyar, resistance (Ostlie et al. 1997). Although *O. nubilalis* and *D. grandiosella* are the two most important corn borer species across the North Central and Midwestern regions of the Corn Belt, in the mid-southern region IRM strategies for sugarcane borer, *Diatraea saccharalis* (F.) (Lepidoptera: Crambidae), should be considered equally important. In recent years, *D. saccharalis* has expanded its geographic range, and it has

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become a dominant cornstalk-boring species in some areas of mid-southern states, especially in Louisiana and Texas (Castro et al. 2004a, Porter et al. 2005, Huang et al. 2006a). *D. saccharalis* infestations also have been reported on field corn in Mississippi and Arkansas (Davis et al. 1999, Huang et al. 2006b). For these reasons, *D. saccharalis* was recently listed as a target pest of Bt corn by the United States Environmental Protection Agency (United States Environmental Protection Agency 2005a, b). During 2004-2005, a major Cry1Ab resistance allele was documented in a Louisiana *D. saccharalis* population (Huanget al. 2007a, c). The resistance detected in this population is the first major resistance to the Cry 1Ab protein in commercial Bt corn documented for any cornstalk borer species. In addition, laboratory bioassays have shown that *D. saccharalis* is less susceptible to Bt corn cultivars expressing the Cry1Ab protein than the European corn borer and southwestern corn borer (Huang et al. 2006b). Two recent field studies from Louisiana also indicated that field performance of Bt corn against *D. saccharalis* varied among Bt corn cultivars (Castro et al. 2004b, Mc-Allister et al. 2004).

The success of the “high-dose/refuge” IRM strategy requires that Bt corn plants must produce a high dose that can kill the heterozygous genotype for Bt resistance in the target species. The available information supporting the use of this strategy to manage Bt resistance in *D. saccharalis* is very limited. Therefore, it is necessary to validate this strategy for this species. The objectives of this study are to evaluate the performance of common Bt corn hybrids planted in Louisiana against a Cry1Ab-resistant *D. saccharalis* strain and thus to assess if these Bt corn hybrids qualify as high dose for managing this cornstalk-boring pest as required for the current IRM strategy for Bt corn.

Material and Methods

Insect Sources. Three genotypes of *D. saccharalis* were tested in this study. A Cry1Ab-susceptible strain (Bt-SS) was established using larvae collected from cornfields near Winnsboro

in northeastern Louisiana during 2004. The Cry1Ab-resistant strain (Bt-RR) was developed from a single iso-line family by using an F₂ screen (Huang et al. 2007a). These Cry1Ab-resistant insects can complete larval development on commercial Bt corn hybrids and show a significant resistance level to the trypsin-activated Cry1Ab toxin (Huang et al. 2007c). During confirmation of Bt resistance, individuals of the Cry1Ab-resistant strain were backcrossed with those of the Cry1Ab-susceptible strain and reselected for Bt resistance in the F₂ generation of the backcross (Huang et al. 2007a). The heterozygous genotype (Bt-RS: F₁) was developed by crossing the Cry1Ab-resistant and -susceptible moths.

Corn Hybrids. Five commercial non-Bt corn hybrids and seven YieldGard Bt corn hybrids adapted to Louisiana's environment were evaluated in a greenhouse experiment. The five non-Bt corn hybrids included DK697 (Monsanto, St. Louis, MO), DG5515 (Dyna-Gro Seed, UAP Mid-South, Cordova, TN), Golden Acres 2995RR (Golden Acres Genetics, Waco, TX), Pioneer 31G66 (Pioneer Hi-Bred International, Johnston, IA), and TV 2140 (Terral Seed, Inc., Lake Providence, LA). The seven Bt corn hybrids were DKC69-70 (YGCB) (Monsanto), DKC69-71 (RR2/ YGCB) (Monsanto), DG5528BT (Dyna-Gro Seed, UAP Mid-South), FFR900BT (FFR Seed, Southaven, MS), Golden Acres 2841 RRB (Golden Acres Genetics), Pioneer 31B13 (Pioneer Hi-Bred International), and TV2160Bt (YieldGard) (Terral Seed, Inc.). The Bt corn hybrids produced from a seed company are genetically similar to the corresponding non-Bt corn hybrid from the same company. Expression of Bt Cry1Ab toxin in plants was verified with Cry1Ab/1Ac Lateral Flow QuickStix Strip Kit (Envirologix, Portland, ME).

Two independent experiments validated the performance of the Bt corn cultivars against the Bt-SS, -RS, and -RR genotypes in 2005 and 2006. In each experiment, five corn seeds were planted in 18.9-liter pots containing ≈5 kg of a standard potting soil mixture (Perfect Mix, Expert

Gardener Products, St. Louis, MO), which were placed in a Louisiana State University Agricultural Center greenhouse located in Baton Rouge, LA. Seedlings were thinned to three plants per pot at 2 wk after seedling emergence. The plants were irrigated and fertilized for optimum growth during the tests.

Insect Inoculation. Neonates (<24 h old) of the Bt-SS, -RS, and -RR genotypes of *D. saccharalis* were infested into plant whorls during vegetative stages (V7–V10) or in the collar of the leaf directly above or below the uppermost ear during reproductive stages (R1–R3) (Ritchie et al. 1993). A single plant for each corn hybrid was inoculated with 20 insects of a genotype. In 2005 (experiment 1), *D. saccharalis* neonates were infested on V7–V8 (50 d after planting) and R1 (80 d after planting) stage corn plants to simulate damage from the first and second field generations, respectively. In 2006 (experiment 2), larvae were infested on V9–V10 (60 d after planting) and R1–R3 (90 d after planting) stage corn plants. Corn plants were dissected at 21 d after larval inoculation. The number of surviving larvae was recorded for each infested plant. In each experiment, three replicates (pots) were used for each combination of insect genotype and corn hybrid, and each pot contained two to three plants.

Data Analysis. Larval survivorship on non-Bt and Bt corn hybrids was analyzed using a two-way analysis of variance (ANOVA) (SAS Institute 1999) with the insect genotype and corn hybrid as the two main factors. The LSMEANS test at the $P = 0.05$ level was used to determine treatment differences. Percentages estimating larval survivorship were subjected to the arcsine ($x^{0.5}$) transformation before analysis with ANOVA (Zar 1984). Non-transformed data are presented in the figures. In addition, larval mortality of the Bt-RS genotype was used to determine whether a Bt corn hybrid would qualify as high dose for *D. saccharalis*. A Bt corn

hybrid is judged as high dose if it kills $\geq 95\%$ heterozygotes (RS) for Bt resistance (FIFRA Scientific Advisory Panel 1998, United States Environmental Protection Agency 2001).

Results

Larval Survivorship of *D. saccharalis* on Non-Bt Corn, 2005. There were no significant differences in larval survivorship 21 d after infestation among the three *D. saccharalis* genotypes on vegetative stage plants of the five non-Bt corn hybrids ($F = 0.05$; $df = 2, 30$; $P = 0.9505$ for insect genotype, $F = 1.70$; $df = 4, 30$; $P = 0.1760$ for corn hybrid, and $F = 1.83$; $df = 8, 30$; $P = 0.1107$ for interaction) (Fig. 2.1A). The overall larval survival rate across the three genotypes on non-Bt corn plants was $37.9 \pm 1.6\%$ (mean \pm SEM).

In contrast, larval survivorship of *D. saccharalis* on reproductive stage non-Bt corn plants was significantly different among insect genotypes ($F = 6.03$; $df = 2, 30$; $P = 0.0063$) (Fig. 2.1B). The survival rates of Bt-RS and -RR genotypes were significantly higher ($P < 0.05$) than that of the Bt-SS genotype. An average of 32.3 ± 1.9 , 47.3 ± 3.4 , or $41.6 \pm 3.5\%$ larvae survived on non-Bt plants infested with Bt-SS, -RS, or -RR insects, respectively. The effect of non-Bt corn hybrid on larval survivorship was not significant ($F = 1.93$; $df = 4, 30$; $P = 0.1312$) and the interactions between insect genotype and corn hybrid also were not significant ($F = 0.37$; $df = 8, 30$; $P = 0.9293$).

Larval Survivorship of *D. saccharalis* on Bt Corn, 2005. Survivorship of *D. saccharalis* after 21 d on vegetative stage Bt corn plants was significantly different among insect genotypes ($F = 31.83$; $df = 2, 42$; $P < 0.0001$) (Fig. 2.2A).

The overall survival rate of the Bt-RR genotype among the seven Bt corn hybrids was $9.6 \pm 1.8\%$, and was significantly greater ($P < 0.05$) than those of the Bt-SS ($0.5 \pm 0.3\%$) and -RS ($1.4 \pm 0.4\%$) genotypes. Difference in larval survivorship between Bt-SS and -RS genotypes was not

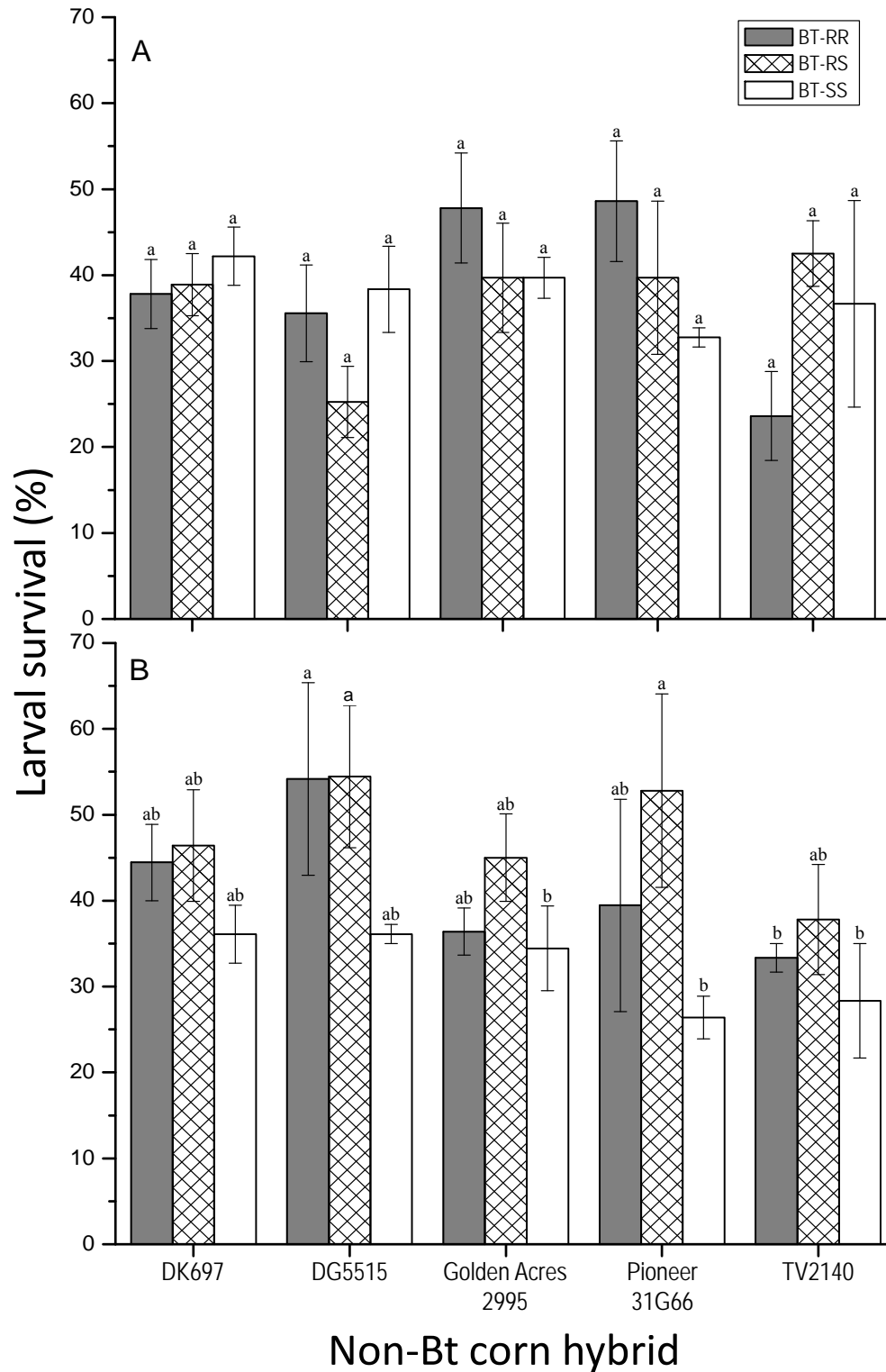


Figure 2.1. Larval survivorship (% mean \pm SEM) of Cry1Ab-susceptible (SS), -heterozygous (RS), and -resistant (RR) *D. saccharalis* on vegetative (A) and reproductive (B) stage plants of five non-Bt corn hybrids, 2005.

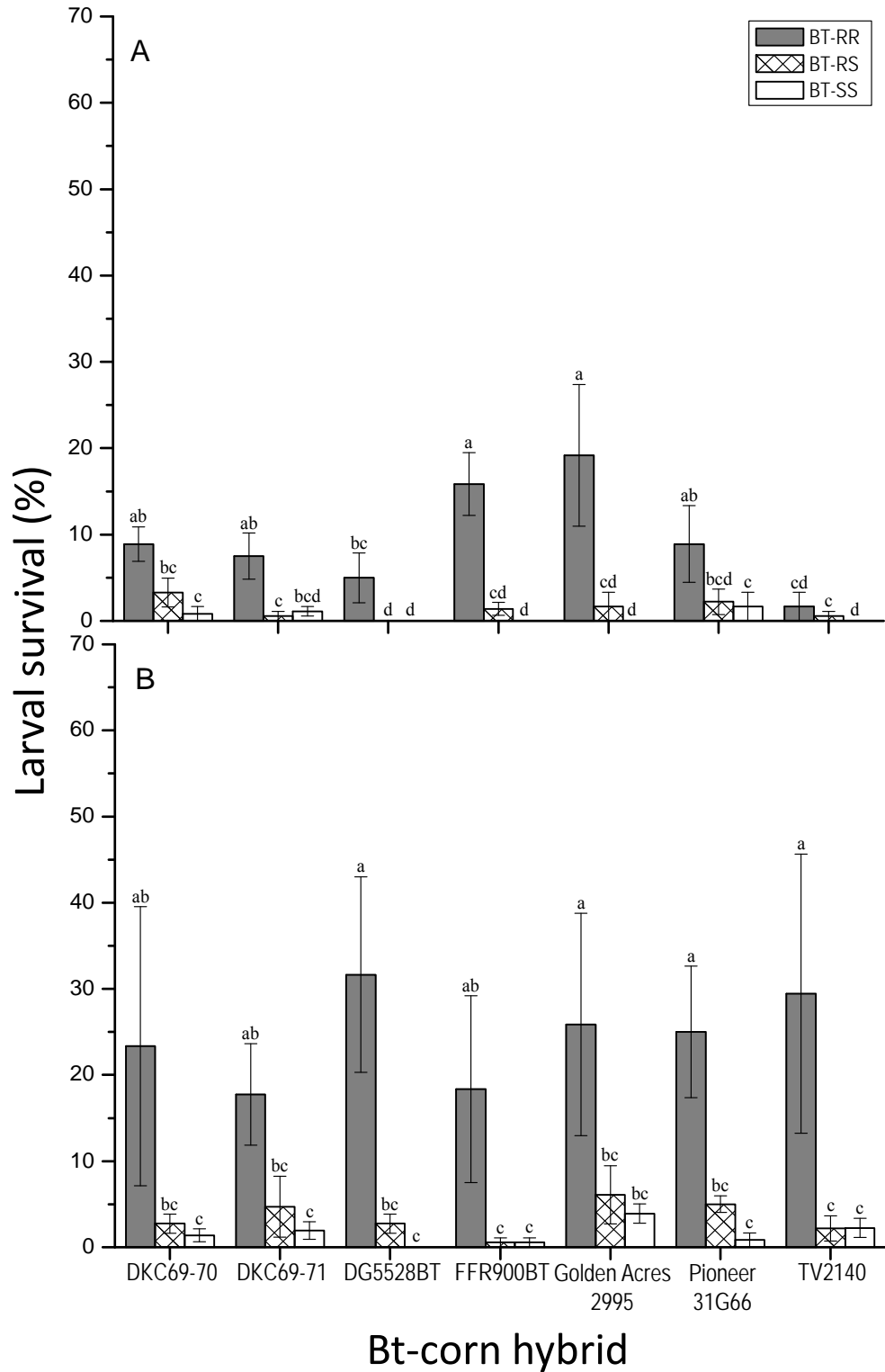


Figure 2.2. Larval survivorship (% mean \pm SEM) of Cry1Ab-susceptible (SS), -heterozygous (RS), and -resistant (RR) *D. saccharalis* on vegetative (A) and reproductive (B) stage plants of seven Bt corn hybrids, 2005.

significant ($P > 0.05$). Larval survivorship varied among Bt hybrids ($F = 2.52$; $df = 6, 42$; $P = 0.0359$). Survivorship of Bt-RR larvae was significantly lower ($P < 0.05$) on the TV2160Bt hybrid than that on the DKC69-70, DKC69-71, FFR 900BT, Golden Acres 2841RRB, and Pioneer 31B13 hybrids. Larval survivorship of Bt-RR insects on DG5528BT was also significantly less ($P < 0.05$) than on the FFR900BT and Golden Acres 2841RRB hybrids. The interaction between insect genotype and corn hybrid was not significant ($F = 1.48$; $df = 12, 42$; $P = 0.1693$). Larval survivorship of the Bt-RS genotype was $<5\%$ across the seven Bt corn hybrids.

Larval survivorship of *D. saccharalis* on reproductive stage Bt corn plants was significantly different among insect genotypes ($F = 34.99$; $df = 2, 42$; $P < 0.0001$) (Fig. 2.2B). An average of $1.5 \pm 0.4\%$ Bt-SS, $3.4 \pm 0.8\%$ Bt-RS, and $24.5 \pm 4.3\%$ Bt-RR larvae survived after 21 d. Larval survivorship between the Bt-SS and -RS genotypes was not significant ($P > 0.05$). The main effect of corn hybrid and the interaction between insect genotype and corn hybrid on larval survival were not statistically different ($F = 0.60$; $df = 6, 42$; $P = 0.7295$ for corn hybrid and $F = 0.37$; $df = 12, 42$; $P = 0.9667$ for interaction). Approximately 7% of Bt-RS larvae survived on the Bt corn hybrid, Golden Acres 2841RRB. Survivorship of the Bt-RS genotype also reached 5% on DKC69-71 and Pioneer 31B13, whereas it was $<5\%$ for other Bt corn hybrids.

Larval Survivorship of *D. saccharalis* on Non-Bt Corn, 2006. Differences in larval survivorship were significant on non-Bt plants during vegetative stages among the three insect genotypes ($F = 15.3$; $df = 2, 30$; $P < 0.0001$) but was not significant for corn hybrid ($F = 2.05$; $df = 4, 30$; $P < 0.1120$). The interaction of insect genotype and corn hybrid was also significant ($F = 2.57$; $df = 8, 30$; $P = 0.0289$). Survivorship of Bt-RS and -RR on DK697, DG5515, and Pioneer 31G66 was significantly higher ($P < 0.05$) than that of the Bt-SS strain (Fig. 2.3A). Larvae of Bt-

RR genotype also demonstrated a greater ($P < 0.05$) survivorship on Golden Acres 2995 ($57.5 \pm 5.2\%$) than Bt-SS larvae ($34.2 \pm 2.2\%$).

Larval survivorship of *D. saccharalis* on reproductive stage plants of non-Bt corn was significantly different among insect genotypes ($F = 5.23$; $df = 2, 30$; $P = 0.0113$). Larval survivorship of the Bt-SS genotype was significantly greater ($P < 0.05$) than that of the Bt-RS and -RR genotypes (Fig. 2.3B). Survivorship of the Bt-SS, Bt-RS, and Bt-RR genotypes was 43.2 ± 3.0 , 32.0 ± 3.4 , and $31.2 \pm 2.7\%$, respectively, across the five non-Bt corn hybrids. The effect of corn hybrid on larval survivorship was not significant ($F = 1.36$; $df = 4, 30$; $P = 0.2729$) and the interaction between insect genotype and corn hybrid also was not significant ($F = 0.76$, $df = 8, 30$, $P = 0.6369$).

Larval Survivorship of *D. saccharalis* on Bt Corn, 2006. Larval survivorship was significantly different among insect genotypes on vegetative stage plants ($F = 56.11$; $df = 2, 42$; $P < 0.0001$) (Fig. 2.4A). An average of $14.3 \pm 2.5\%$ larvae of the Bt-RR genotype survived on the seven Bt corn hybrid and was significantly higher ($P < 0.05$) than that observed with Bt-SS ($0.0 \pm 0.0\%$) and -RS ($3.5 \pm 0.6\%$) genotypes. Larval survivorship of the Bt-RS genotype was significantly greater than that of the Bt-SS genotype ($P < 0.05$). The main effect of corn hybrid and the interaction between insect genotype and corn hybrid was not significant ($F = 1.82$; $df = 6, 42$; $P = 0.1175$ for corn hybrid and $F = 0.74$; $df = 12, 42$; $P = 0.7075$ for interaction). Larval survivorship of the Bt-RS genotype was $\leq 5\%$ across the seven Bt corn hybrids. Larval survivorship was significantly different among insect genotypes on reproductive stage plants ($F = 25.98$; $df = 2, 42$; $P < 0.0001$). An average of $18.1 \pm 2.4\%$ Bt-RR larvae survived on Bt corn plants 21 d after infestation. Bt-RR larval survivorship was significantly greater ($P < 0.05$) than those of the Bt-SS ($2.7 \pm 1.40\%$) and -RS ($6.8 \pm 1.6\%$) insects (Fig. 2.4B).

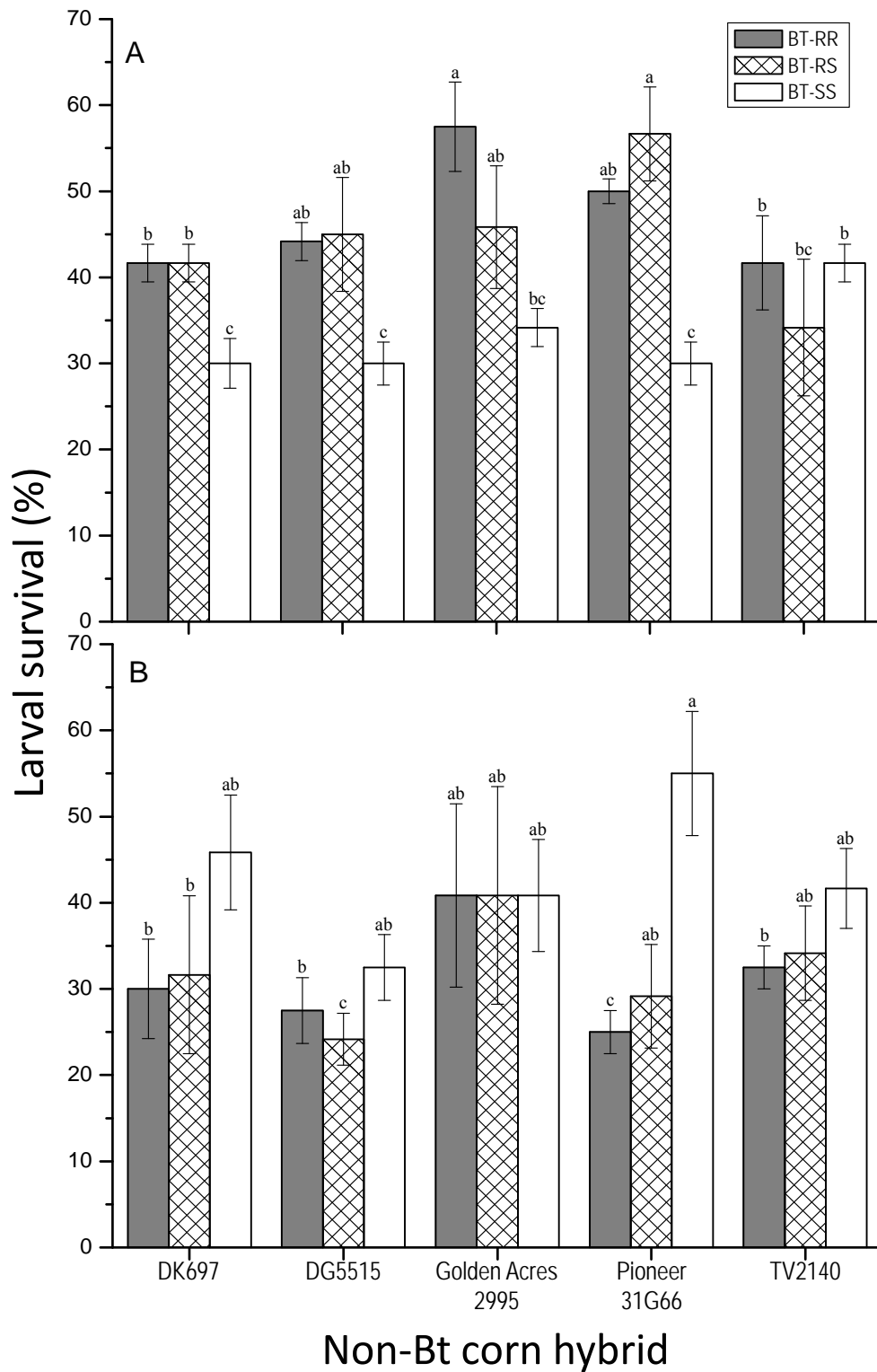


Figure 2.3. Larval survivorship (% mean \pm SEM) of Cry1Ab-susceptible (SS), -heterozygous (RS), and -resistant (RR) *D. saccharalis* on vegetative (A) and reproductive (B) stage plants of five non-Bt corn hybrids, 2006.

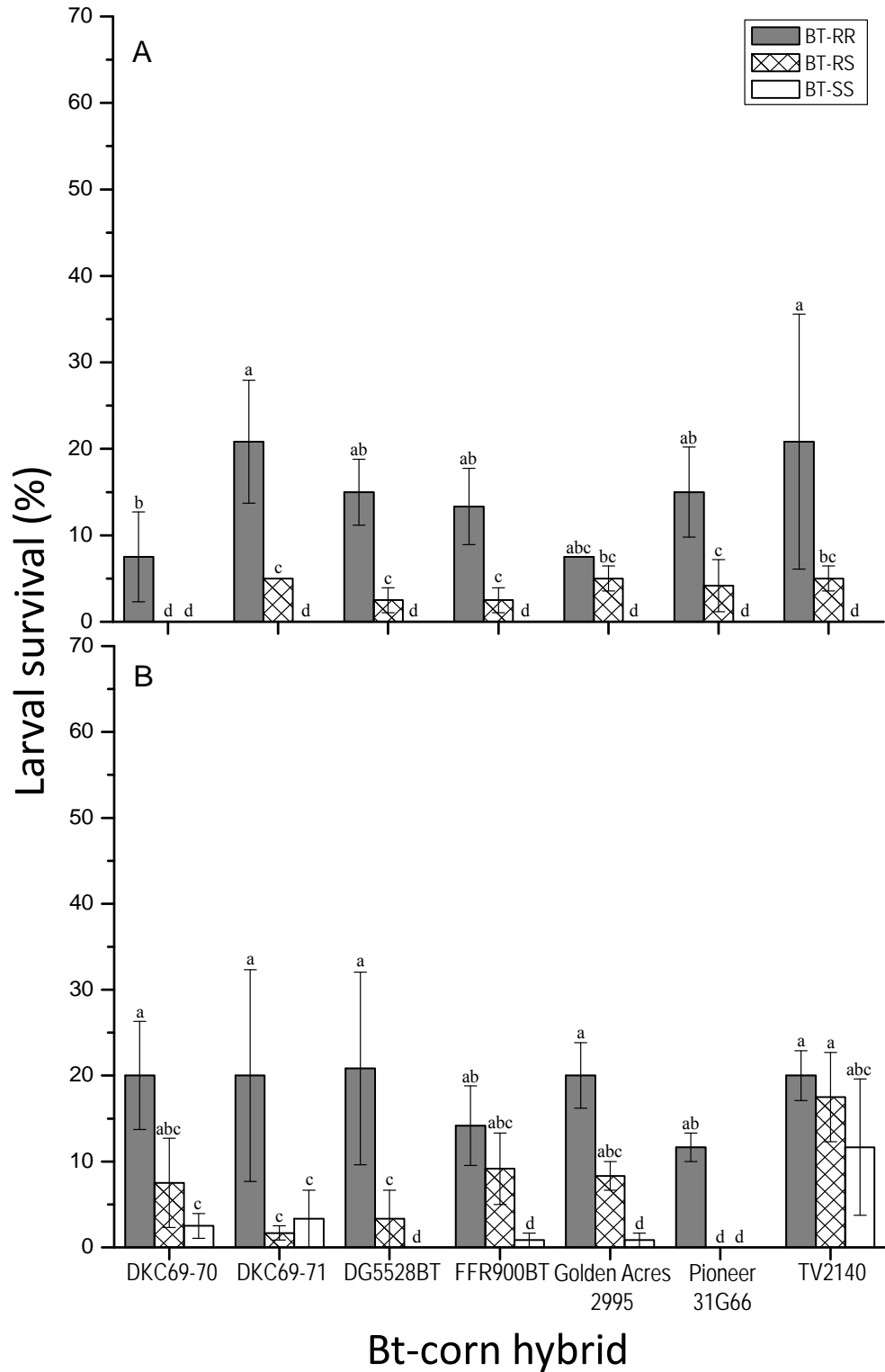


Figure 2.4. Larval survivorship (% mean \pm SEM) of Cry1Ab-susceptible (SS), -heterozygous (RS), and -resistant (RR) *D. saccharalis* on vegetative (A) and reproductive (B) stage plants of seven Bt corn hybrids, 2006.

Survivorship of the Bt-SS and -RS genotypes also was significantly different ($P < 0.05$). The main effect of corn hybrid on survivorship was significant ($F = 3.32$, $df = 6, 42$; $P = 0.0091$). More larvae ($P < 0.05$) (mainly from Bt-RS and Bt-SS genotypes) survived on the TV2160Bt hybrid than on DKC69-70, DKC69-71, DG5528BT, FFR900BT, and Pioneer31B13 hybrids. Larval survivorship on Golden Acres 2841RRB also was significantly higher than that on Pioneer31B13. The interaction between insect genotype and corn hybrid was not significant ($F = 0.78$; $df = 12, 42$; $P = 0.6634$). Survivorship of the Bt-RS genotype was $>5\%$ for TV2160Bt ($17.5 \pm 5.2\%$), FFR900BT ($9.2 \pm 4.2\%$), Golden Acres 2841RRB ($8.3 \pm 1.7\%$), and DKC69-70 ($7.5 \pm 5.2\%$). Larval survivorship of Bt-RS genotype on TV2160Bt, FFR900BT, Golden Acres 2841RRB, and DKC69-70 was significantly greater ($P < 0.05$) than that on Pioneer31B13. Survivorship on TV2160Bt was also significantly higher ($P < 0.05$) than that on DKC69-71 and DG5528BT.

Discussion

The Cry1Ab-resistant strain of *D. saccharalis* used in this study was previously confirmed to carry a major resistance gene(s) that allowed the insects to complete larval development (from neonate to pupal stage) on the Bt corn hybrid DKC69-70 (Huang et al. 2007a). Data from the current study showed that the larval survivorship of the Cry1Ab-resistant insects was considerably higher than that of the Bt-SS and -RS genotypes across the seven commercial Bt corn hybrids at both the vegetative and reproductive stages in both experiments. The relatively high survivorship of the Cry1Ab-resistant genotype further confirms that this Cry1Ab-resistant strain carries at least one major resistance gene to the Cry1Ab protein expressed in the Bt corn.

Development of Bt resistance in insect pests is a primary concern for long-term success of transgenic Bt corn technology. To delay resistance development in target insect pests, the high-

dose/refuge structured strategy has been implemented in the United States (FIFRA Scientific Advisory Panel 1998, United States Environmental Protection Agency 2001) and Canada (Baute 2004). The success of this strategy requires that Bt corn plants produce a sufficient concentration of Bt protein to ensure that heterozygotes (RS) for Bt resistance can be killed (Ostlie et al. 1997, Bourguet et al. 2003). The high-dose qualification has not been directly evaluated for European corn borer and southwestern corn borer because major resistance traits have not been found in these species (FIFRA Scientific Advisory Panel 1998, Bourguet et al. 2003), in spite of extensive sampling (Andow et al. 1998, 2000; Bourguet et al. 2003; Stodola et al. 2006; Huang et al. 2007b). Therefore, an indirect criterion of high dose was suggested by the EPA Scientific Advisory Panel on Bt Plant-Pesticides and Resistance Management (FIFRA Scientific Advisory Panel 1998, United States Environmental Protection Agency 2001). The panel used empirical data to suggest that a definition of high dose should include “a dose 25 times the toxin concentration needed to kill Bt-susceptible larvae.” This indirect measurement of high dose has been used to evaluate the high dose qualification of Bt corn hybrids against corn stalk borers and other target insect species (United States Environmental Protection Agency 2001). The panel also recognized that it is conceivable that a Bt-resistant heterozygote may develop with higher than 25-fold resistance (FIFRA Scientific Advisory Panel 1998, United States Environmental Protection Agency 2001). Field and laboratory data have shown that many of the commercial Bt corn hybrids are very effective against European corn borer and southwestern corn borer (Buschman et al. 1999, Huang et al. 1999). Based upon those results, all commercial Bt corn hybrids are likely to fulfill the high-dose requirement for European corn borer and southwestern corn borer (United States Environmental Protection Agency 2001).

The availability of a Cry1Ab-resistant *D. saccharalis* strain provided an opportunity to test directly the high-dose assumption by measuring larval survivorship of heterozygous genotype for Bt resistance on Bt corn plants. The low larval survivorship ($\leq 5\%$) of the Bt-RS genotype on seven Bt corn hybrids at vegetative plant stages suggests all seven corn hybrids expressed a high dose of Bt protein against *D. saccharalis* during early plant stages as defined by the FIFRA Scientific Advisory Panel (FIFRA Scientific Advisory Panel 1998). The high efficacies ($\leq 5\%$ larval survivorship) of the three Bt corn hybrids (DKC69-71, DG5528BT, and Pioneer 31B13) against the Bt-RS genotype during reproductive stages indicate that these hybrids might also express a high dose of Bt protein at the later plant stages. However, survivorship of the Bt-RS genotype on TV2160Bt, Golden Acres 2841RRB, FFR 900BT, and DKC69-70 Bt corn hybrids during later reproductive stages was $> 5\%$, suggesting that these hybrids did not produce a high dose of Bt protein during those reproductive stages.

To ensure no insects from escaping isolation within the greenhouse, all experiments were terminated before larvae developed to the pupal stage (21 d after larval infestation). Additional insect mortality of Bt-SS, -RS, and -RR genotypes on Bt corn plants might have occurred if they were exposed to plant tissue for a longer period. The laboratory bioassay with Bt corn plant tissue showed that additional mortality of Cry1Ab-resistant larvae was low 15 d after inoculation (Huang, data not shown), suggesting that 21 d-survivorship data are reasonable for estimating the efficacy of Bt corn plants against the three genotypes of *D. saccharalis*.

Larvae of the Bt-SS, -RS, and -RR genotypes adapted well ($> 23\%$ survivorship) to plants of the five non-Bt corn hybrids in the greenhouse. However, the performance of the three insect genotypes on the five non-Bt corn hybrids was not consistent across plant stages, or between the two experiments (2005 and 2006). Natural resistance in specific corn hybrids and developmental

stages within a hybrid could produce variable effects on *D. saccharalis* (Kumar and Mihm 1996, Davis et al. 1999, Maredia and Mihm 1999). The differences in plant stages and planting date between 2005 and 2006 may provide a partial explanation for the differences in larval survivorship between the two experiments.

With the exception of the TV2160Bt hybrid at reproductive stages, very low survivorship (0–3.5%) of the Bt-SS insects were observed across the treatment combinations of Bt corn hybrids and plant growth stages in the two experiments. The results showed the YieldGard trait in these Bt corn hybrids expressing the Cry1Ab protein was effective against Cry1Ab-susceptible *D. saccharalis*. Similar results were reported from two previous studies (Castro et al. 2004b, McAllister et al. 2004). Castro et al. (2004b) reported that several locally adapted hybrids from the MON810 Bt corn event were very effective against southwestern corn borer, whereas significant variation in efficacy among those Bt hybrids was observed against *D. saccharalis*. McAllister et al. (2004) also found plant stage, plant structure, and Bt corn variety could affect the efficacy of the Cry protein in Bt corn against *D. saccharalis*.

In addition, this study showed that larval survivorship of the Bt-RS genotype of *D. saccharalis* on Bt corn plants was considerably lower than that of the Bt-RR insects across all treatments in the 2-yr study. However, survivorship of the Bt-RS genotype on Bt corn plants was consistently greater than that of the Bt-SS genotype. The difference in larval survivorship on Bt corn plants between Bt-SS and -RS genotypes was significant ($P < 0.05$) in two of the four treatment combinations of plant growth stage and experimental time. These results indicate that Bt resistance in *D. saccharalis* may be inherited as a partially recessive trait. More detailed segregation analysis is needed to determine whether Bt resistance in *D. saccharalis* is controlled

by a single recessive or at least partially recessive as required for the high-dose/refuge IRM strategy for Bt corn (Ostlie et al. 1997, Bourguet et al. 2003).

In summary, this study demonstrated that the Bt resistant strain of *D. saccharalis* can survive on the common commercial Bt corn hybrids in Louisiana. The results from this study suggested that a high-dose Bt corn for European corn borer and southwestern corn borer may not qualify as high dose for *D. saccharalis*. Furthermore, a high dose expressed by a Bt corn hybrid during vegetative plant stages may not produce a high dose during reproductive stages of development. Variability in performance of different Bt corn hybrids and in different plant growth stages within the same Bt corn hybrid also suggests a requirement to validate high dose qualification against *D. saccharalis* for each Bt corn hybrid, at different plant stages, and even perhaps in different environments. In addition, the natural resistance levels of a corn hybrid and the expression of Cry1Ab protein in Bt corn plants might be different in open field conditions. Therefore, careful monitoring of *D. saccharalis* survivorship is necessary after a greenhouse verified high dose Bt corn cultivar is planted in open fields.

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CHAPTER 3

SUSCEPTIBILITY OF CRY1AB-SUSCEPTIBLE AND -RESISTANT SUGARCANE BORER (LEPIDOPTERA: CRAMBIDAE) TO FOUR *BACILLUS THURINGIENSIS* PROTEINS *

Introduction

In recent years, the sugarcane borer, *Diatraea saccharalis* (F.) (Lepidoptera: Crambidae), has expanded its geographic range and has become a dominant corn borer species across many areas of the mid-southern region of the United States, especially in Louisiana and Texas (Castro et al. 2004, Porter et al. 2005, Huang et al. 2007a). In Louisiana, field corn was seriously damaged by late-season populations of *D. saccharalis* during 2002 and 2003. Yield losses to non-Bt corn from this stalk boring pest exceeded 30% in many fields during these two years. A four-year corn stalk-boring pest survey (2004 – 2007) in Louisiana showed that *D. saccharalis* accounted for more than 80% of the total corn borer populations (Huang and Leonard 2008). In Texas, field corn was severely damaged by *D. saccharalis* in 2005. Economic infestations were reported from Corpus Christi to Victoria, and north to the Dallas area (Porter et al. 2005). Field samples collected during 2006 and 2007 showed that *D. saccharalis* accounted for >95% of the total corn borer populations in these areas (RP and FH, unpublished data).

Since 1999, transgenic corn hybrids, *Zea mays* L., expressing the *Bacillus thuringiensis* (Bt) insecticidal Cry1Ab protein (e.g. YieldGard® corn) have been used successfully for managing corn borer problems in the mid-southern region. Acreage of Cry1Ab-expressing Bt corn is currently near the maximum level (50%) allowed by the United States Environmental Protection Agency for Bt resistance management in this region. More recently, genes encoding for Bt Cry1A.105 and Cry2Ab2 proteins have been transferred into corn hybrids with a novel method called Vector-Stack transformation (or VecTran technology) (Monsanto 2007), resulting in an

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event, MON 89034, that contains both of these Bt proteins expressed at high levels. These hybrids will offer control of a broad spectrum of Lepidopteran corn pests and may serve as a valuable resistance management tool because of the presence of the two Lepidopteran active and highly effective insecticidal Bt proteins (Monsanto 2007). This new generation of Bt corn products has been approved by U.S. regulatory authorities for commercial use beginning 2009 under the trade name of YieldGard VT Pro™.

During 2004-2007, a major Cry1Ab resistance allele was documented in three populations of *D. saccharalis* collected from northeast and central Louisiana (Yue et al. 2008, Huang and Leonard 2008). These Cry1Ab-resistant insects can complete larval development on commercial Cry1Ab corn hybrids (YieldGard) (Huang et al. 2007a, Wu et al. 2007) and showed a significant level of resistance (> 100-fold) to a trypsin-activated Cry1Ab toxin (Huang et al. 2007b). The resistance allele detected in *D. saccharalis* represents the first major resistance allele to the proteins found in commercial Bt corn for any corn stalk borer species. Recently, field resistance to Bt corn was reported for the stem borer, *Busseola fusca* (Fuller), in South Africa (van Rensburg 2007).

Information on cross-resistance of insect pests to insecticides is essential in understanding resistance mechanisms and development of management strategies. Cross-resistance among Bt toxins has been evaluated in several insect species. Most published data suggest that selection for resistance to one Bt Cry toxin can lead to resistance to others (Tabashnik 1994; Tabashnik et al. 1994, 1996, 2000; Gould et al. 1995; Ferré and Van Rie 2002; Li et al. 2005), but the patterns of cross-resistance vary among insect species and even among insect strains within the same species. The objective of this study was to determine the susceptibility of the Cry1Ab-resistant

D. saccharalis to four Bt Cry proteins, including the proteins that are present in MON 89034: Cry1Aa, Cry1Ac, Cry1A.105, and Cry2Ab2.

Materials and Methods

Cry1Ab-Susceptible and -Resistant Strains of *D. saccharalis*. A Cry1Ab-susceptible strain (Cry1Ab-SS) of *D. saccharalis* was established from larvae collected from corn fields near Winnsboro in Northeast Louisiana during 2004 (Huang et al. 2006). A Cry1Ab-resistant strain (Cry1Ab-RR) of *D. saccharalis* was developed from a single two-parent family-line collected from the same location as the Cry1Ab-susceptible strain and was identified as carrying major resistance alleles by using an F₂ screen (Huang et al. 2007a). These Cry1Ab-resistant insects completed larval development on commercial Bt corn hybrids expressing the Cry1Ab protein and demonstrated a significant resistance level (> 100-fold) to purified trypsin-activated Cry1Ab toxin, while the Cry1Ab-SS strain was susceptible to the Bt corn (Huang et al. 2007a, Wu et al. 2007) and purified Cry1Ab protein (Huang et al. 2007b). During confirmation of Bt resistance, individuals of the Cry1Ab-resistant strain were backcrossed with those of the Cry1Ab-susceptible strain and re-selected for Bt resistance with Cry1Ab corn leaf tissue in the F₂ generation of the backcross. The backcrossed and reselected resistant strain was used in the current study.

Sources of Cry Proteins. Susceptibility of the Cry1Ab-SS and -RR strains of *D. saccharalis* was evaluated for four individual Cry proteins: Cry1Aa, Cry1Ac, Cry1A.105, and Cry2Ab2. Purified (99.9%) Cry1Aa and Cry1Ac proteins were obtained from Dr. Marianne Puztai-Carey, Case Western Reserve University, Ohio. The Cry proteins were produced using recombinant *Escherichia coli* culture and were subsequently activated with trypsin. The activated Cry proteins were lyophilized before they were used in the bioassays. The purity of these two

proteins was determined using high-performance liquid chromatography and sodium dodecyl sulfate polyacrylamide gel electrophoresis (Pusztai-Carey et al.1995, Masson et al. 1998). Cry1A.105 and Cry2Ab2 were provided by Monsanto Company (St. Louis, MO). The Cry1A.105 was produced in a culture of *E. coli* containing the pMON96851 expression plasmid and had a molecular weight of 58.1 – 131.5 kDa. This protein was 80% pure and was provided at a concentration of 1.2 mg/mL in a buffer (named as buffer A) consisting of 25 mM CAPS, pH 10.3, 1 mM benzamidine-HCl, 0.1 mM EDTA, and 0.2 mM DTT. The Cry2Ab2 protein was produced in a culture of *E. coli* containing the pMON70520 expression plasmid and had a molecular weight 61.1 kDa. The protein was provided in a buffer named as buffer B consisting of 50 mM CAPS, pH 11, and 2 mM DTT with a purity of 87% and a concentration of 0.5 mg/mL.

Insect Bioassay. Susceptibility of Cry1Ab-SS and –RR strains of *D. saccharalis* to the four Cry proteins was determined using a method similar to that described by Huang et al. (2007b). Each individual Cry protein was incorporated into a meridic diet prepared for rearing *D. saccharalis* (Bio-Serv, Frenchtown, NJ). Each bioassay included seven Bt concentrations and one (for assaying with Cry1Aa and Cry1Ac) or two (for assaying with Cry1A.105 and Cry2Ab2) non-Bt controls. The Cry protein concentrations used in each bioassay ranged from 0.016 to 64 µg /g for Cry1Aa and Cry1Ac and 0.031 to 128 µg /g for Cry1A.105 and Cry2Ab2. Diet mixed with distilled water only was included as a blank control in all bioassays. In testing with Cry1A.105 and Cry2Ab2, diet mixed with buffer only was used as an additional negative control. For diet incorporation assays, individual Cry proteins were suspended and diluted in distilled water or in buffer solution. The desired Cry protein concentrations were achieved by mixing appropriate volumes of Cry protein solution into the diet just prior to dispensing the diet into individual cells of 128-cell trays (Bio-Ba-128, C-D International, Pitman, NJ). In the bioassay,

approximately 0.7 ml of treated diet was poured into each cell using 10 or 20-ml syringes (Becton, Dickinson and Company, Franklin Lakes, NJ). One neonate (< 24-h old) was placed on the diet surface of each cell. Each combination of insect strain by Cry protein concentration was replicated four times with 16 to 32 larvae in each replication. The bioassay trays were held in an environmental chamber maintained at 28 °C, 50% RH, and a photoperiod of 16:8 (L:D) h. Larval mortality, larval weight, and the number of surviving larvae that did not gain significant weight (< 0.1 mg per larva and still in first instar) were recorded on the 7th d after inoculation.

Data Analysis. As described in Huang et al. (2007b), larval mortality was analyzed with two criteria: actual larval mortality and ‘practical’ mortality. Actual larval mortality was calculated using the number of dead larvae divided by the total number of larvae assayed. The practical mortality was obtained using the equation: Practical mortality (%) = 100 x (number of dead larvae + number of surviving larvae that had a body weight of < 0.1 mg per larva) / total number of insects assayed. The ‘practical’ mortality criterion is a more feasible and accurate measurement than the actual mortality in determining susceptibility of *D. saccharalis* to Bt toxins because it takes into account both actual mortality and larval growth inhibition (Huang et al. 2007b). A similar mortality criterion has been used in previous studies to measure Bt susceptibility in several lepidopteran species (Sims et al. 1996, Marçon et al. 1999).

The actual larval mortality of the Cry1Ab-RR strain was low (< 62%) for three of the four Cry proteins at all concentrations tested. These data could not be analyzed with probit analysis for determining lethal concentrations (e.g. LC₅₀). Therefore, the actual mortality data observed from both insect strains were transformed using an arcsine transformation ($x^{0.5}$) and were subjected to a two-way analysis of variance (ANOVA) (SAS institute 2007) with insect strain

and Cry protein concentration as the two main factors. Treatment differences were determined using the least squares difference test at the $\alpha = 0.05$ level.

Data on the ‘practical mortality’ of *D. saccharalis* observed on Cry protein-treated diet were corrected for the mortality on non-treated control diet or the buffer-treated diet (Abbott 1925) and then were subjected to probit analysis using the SAS Proc probit program (SAS institute 2007) to calculate the median lethal concentration (LC_{50}) and the corresponding 95% confidence intervals (CI). Resistance ratios for each Cry protein were calculated using the LC_{50} value of Cry1Ab-resistant strain divided by the LC_{50} of the Cry1Ab-susceptible strain. The lethal concentration ratio test described in Robertson and Preisler (1992) was used to determine if the differences in LC_{50} s between the two insect strains was significant at the $\alpha = 0.05$ level for each Cry protein.

Percentage of *D. saccharalis* growth inhibition on a Bt-treated diet was calculated using the formula described in Huang et al. 2007b: larval growth inhibition (%) = 100 x (body weight of larvae feeding on non-treated control diet – body weight of larvae feeding on Bt diet or on buffer-treated diet) / (body weight of larvae feeding on non-treated control diet). The growth inhibition data were transformed using arcsine ($x^{0.5}$) before being subjected to statistical analysis. A two-way ANOVA was used to analyze the larval growth inhibition data with insect strain and Bt concentration as the two main factors, followed by the least squares difference test at $\alpha = 0.05$ level to compare treatments (SAS institute 2007).

Results

Actual Larval Mortality. Actual mortality of the Cry1Ab-SS and Cry1Ab-RR strains on the non-Bt control diet at 7 d was low (0 - 8.6 %). The effects of Cry protein concentration and insect strain on larval mortality were significant for all Cry proteins ($F \geq 26.98$; $df = 6-8, 39-54$;

$P < 0.0001$) and insect strains ($F \geq 7.28$; $df = 1, 39-54$; $P \leq 0.0103$). The interaction between Cry concentration and insect strain was also significant for the four Cry proteins ($F \geq 8.26$; $df = 5 - 8, 39 - 54$; $P < 0.0001$). Larval mortality at Cry1Aa concentrations of $\leq 4 \mu\text{g/g}$ was low ($< 25\%$) for both insect strains (Fig.3.1).

Larvae of the Cry1Ab-RR strain feeding on Cry1Aa-treated diet at $16 \mu\text{g/g}$ had significantly lower ($P < 0.05$) mortality than the Cry1Ab-SS strain. Mortality of both insect strains at $64 \mu\text{g/g}$ was high ($> 90\%$) and did not differ significantly ($P > 0.05$) between the two strains. In the Cry1Ac bioassays, larval mortality of the two insect strains increased as the Cry1Ac concentration increased. At concentrations of $\geq 0.25 \mu\text{g/g}$, mortality of Cry1Ab-RR larvae was significantly lower ($P < 0.05$) than that of the Cry1Ab-SS strain. For the Cry1A.105, at concentrations of $\leq 0.5 \mu\text{g/g}$, differences in mortality between the two insect strains were not significant ($P > 0.05$). At concentrations of $\geq 2 \mu\text{g/g}$, mortality of the Cry1Ab-SS strain was significantly greater ($P < 0.05$) than that of the Cry1Ab-RR strain. Compared to larvae feeding on control diet, significantly higher mortality was observed in Cry1Ab-SS strain at concentrations $\geq 0.125 \mu\text{g/g}$ and mortality reached 90% at $128 \mu\text{g/g}$. In contrast to the Cry1Ab-SS strain, significant mortality of the Cry1Ab-RR strain occurred only at the Cry1A.105 concentration of $8 \mu\text{g/g}$, but mortality reached only 33% at $128 \mu\text{g/g}$. Treated with the Cry2Ab2 diet, Cry1Ab-SS and Cry1Ab-RR strains expressed relatively low mortality levels compared to the other three Cry proteins. No significant larval mortality was observed for both insect strains until the concentration of Cry2Ab2 reached $128 \mu\text{g/g}$. At this concentration, approximately 72% of the Cry1Ab-SS strain were killed, which was significantly higher ($P < 0.05$) than that (27%) for the Cry1Ab-RR strain.

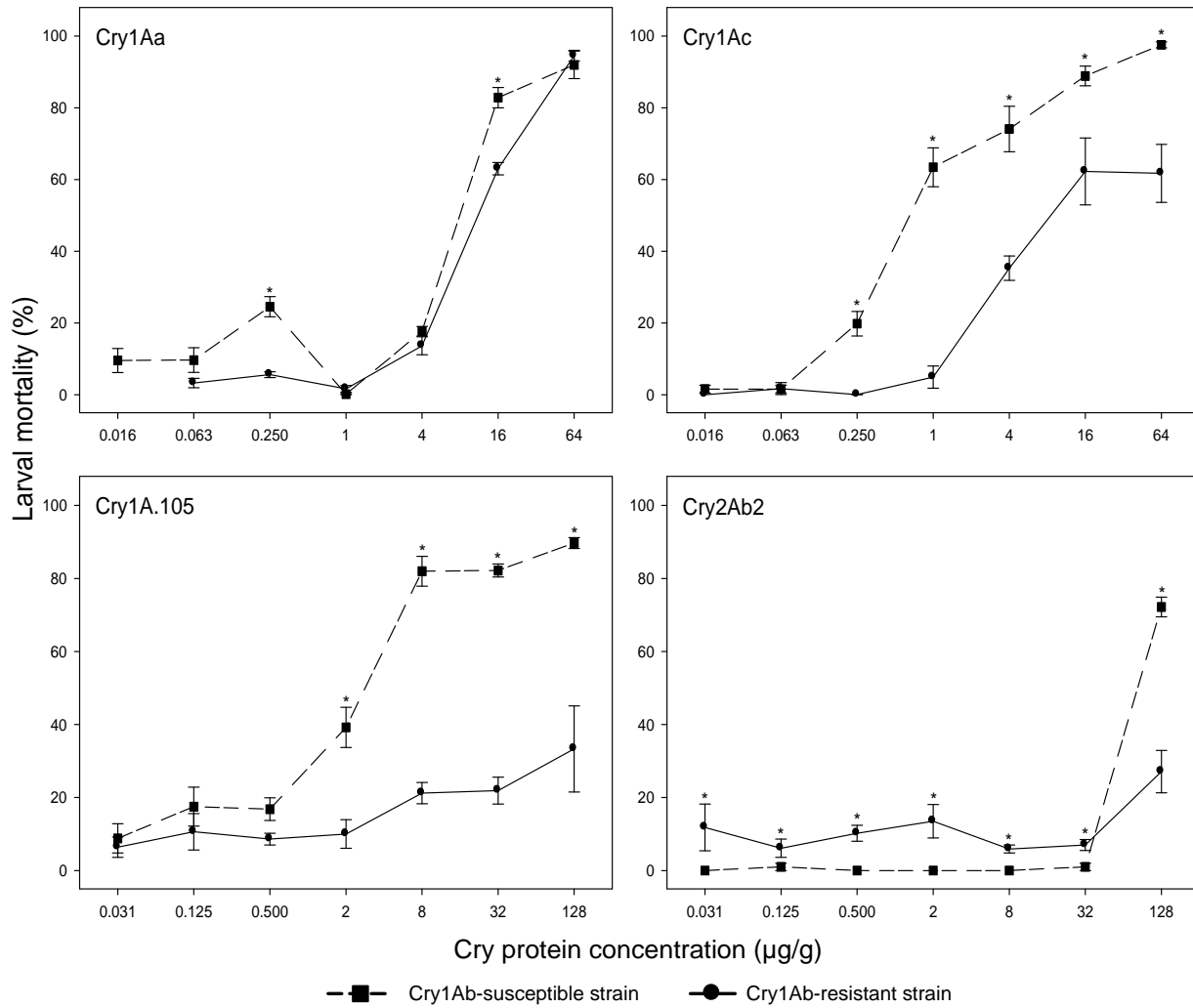


Figure 3.1. Actual larval mortality (% mean \pm SEM) of the Cry1Ab-susceptible and -resistant strains of *D. saccharalis* exposed to a diet treated with *Bacillus thuringiensis* Cry1Aa, Cry1Ac, Cry1A.105, or Cry2Ab2 proteins at 7 day after inoculation. For each Cry protein concentration, mean values followed by asterisk * are significantly different between the two insect strains ($P < 0.05$; LSMEANS test).

Practical Mortality. Practical mortality of the Cry1Ab-SS and Cry1Ab-RR strains of *D. saccharalis* on non-Bt control diet ranged from 0 – 15% across all the bioassays. The practical mortality of Cry1Ab-SS strain on Cry1Aa-treated diet was high (> 96%) even at the lowest concentration (0.016 µg/g) tested, and it reached 100% at 0.063 µg/g. LC₅₀ values of Cry1Aa against the Cry1Ab-SS strain could not be calculated, but based on the current bioassays, it should be less than 0.016 µg/g. Larval practical mortality of the Cry1Ab-RR strain ranged from 3.3 ± 1.3 % (mean ± SEM) at the Cry1Aa concentration of 0.063 µg/g to 100% at 16 µg/g. The calculated LC₅₀ value of Cry1Aa based on practical mortality was 1.25 µg/g with a 95% CI of 0.99 to 1.58 µg/g (Table 3.1). With the larval mortality of the Cry1Ab-SS strain as a standard, the resistance ratio of the Cry1Ab-RR strain was > 80-fold for the Cry1Aa protein. The Cry1Ab-RR strain also demonstrated a significant level of cross-resistance to Cry1Ac. Practical mortality of the Cry1Ab-SS strain was 2.7 ± 1.2 % at the Cry1Ac concentration of 0.016µg/g and reached 100% at 1 µg/g, while the Cry1Ab-RR strain required higher concentrations to generate the similar mortality levels. The calculated LC₅₀ value of Cry1Ac for Cry1Ab-RR strain was 3.61 µg/g, which was 45-fold greater than that for the Cry1Ab-SS strain (Fig 3.2, Table 3.1).

The difference in the LC₅₀ values of the two strains was significant ($P < 0.05$) based on the lethal dose ratio tests. Practical mortality levels of the Cry1Ab-SS larvae on Cry1A.105 treated-diet ranged from 12.8 ± 5.5% at 0.031 µg/g to 100% at 8 µg/g . Practical mortality levels of the Cry1Ab-RR strain ranged from 6.4 ± 2.8% at 0.031 µg/g to 100% at 32 µg/g. The calculated LC₅₀ values of Cry1A.105 for the Cry1Ab-SS and Cry1Ab-RR strains were 0.27 and 1.11µg/g, respectively (Table 3.1). The 4-fold difference in the LC₅₀s between the two strains was statistically significant ($P < 0.05$) based on the lethal dose ratio test. Practical mortality levels of the Cry1Ab-SS strain on Cry2Ab2 treated-diets ranged from 15.0 ± 15.0% at 0.031µg/g to 100%

at 128 $\mu\text{g/g}$. Practical mortality levels of the Cry1Ab-RR strain ranged from $10.3 \pm 0.8\%$ at 0.125 $\mu\text{g/g}$ to 94.7 % at 128 $\mu\text{g/g}$. The calculated LC_{50} values for Cry2Ab2 against the Cry1Ab-SS and Cry1Ab-RR strains were 11.87 and 6.01 $\mu\text{g/g}$, respectively, and did not differ significantly between the two insect strains (Fig. 3.2, Table 3.1).

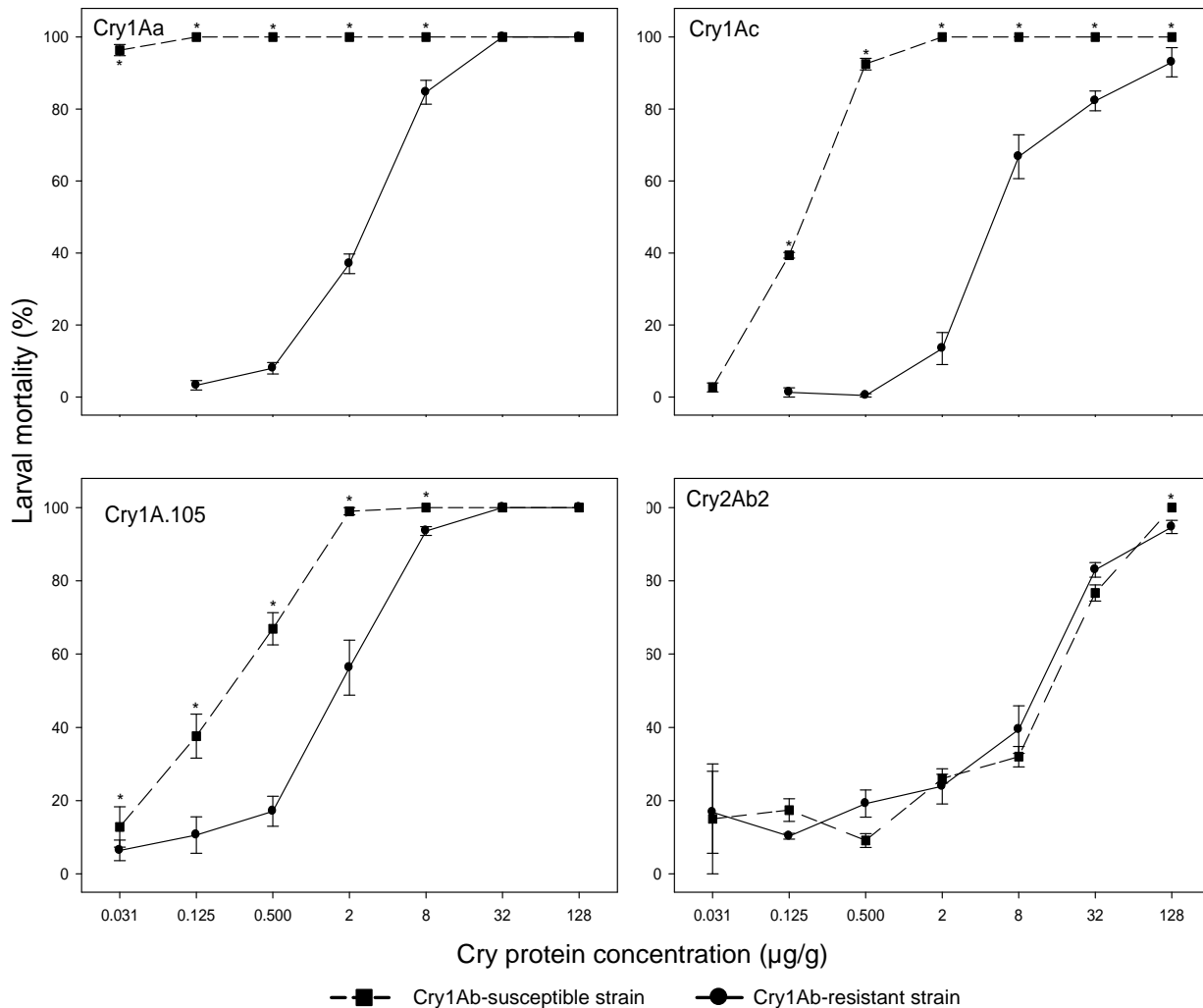


Figure 3.2. Practical mortality (% mean \pm SEM) of the Cry1Ab-susceptible (SS) and Cry1Ab-resistant (RR) strains of *D. saccharalis* exposed to a diet treated with *Bacillus thuringiensis* Cry1Aa (a), Cry1Ac (b), Cry1A.105 (c), or Cry2Ab.280 (d) proteins at 7 day after inoculation. For each Cry protein concentration, mean values followed by asterisk * are significantly different between the two insect strains ($P < 0.05$; LSMEANS test).

Table 3.1. Median lethal concentrations (LC₅₀) and 95% confidence intervals (CI) based on practical mortality of *B. thuringiensis* Cry1Ab-susceptible and -resistant strains of *D. saccharalis* to four other Cry proteins^a.

Toxin	Insect Strain	n ^b	Slope ± SE	<i>P</i> -value χ^2 test	LC ₅₀ (95%CI) (µg/g)	Resistance Ratio ^d
Cry1Aa	Susceptible	862	---	---	<< 0.0156 ^c	---
	Resistant	624	1.87±0.15	0.0680	1.25 (0.99-1.58)	> 80
Cry1Ac	Susceptible	476	2.78±0.23	0.9977	0.08 (0.07-0.09)	---
	Resistant	529	1.45±0.02	<0.0001	3.61 (2.25-5.46)	45 ^s
Cry1A.105	Susceptible	379	1.89 ± 0.22	0.0816	0.27 (0.20-0.36)	---
	Resistant	491	1.42 ± 0.16	0.0007	1.11 (0.75-1.67)	4.1 ^s
Cry2Ab2	Susceptible	604	0.76 ± 0.11	<0.0001	11.87 (5.91-30.83)	---
	Resistant	556	0.72 ± 0.13	<0.0001	6.01 (2.49-18.97)	- 0.51 ^{ns}

^a ‘Practical mortality’ is defined as the total of dead larvae and surviving larvae that did not demonstrate significant weight gain (<0.1 mg/larva and still in first instar) in a 7-day bioassay divided by the total number of larvae in the test.

^b n = total number of neonates assayed.

^c Mortality of the Cry1Ab-SS strain of *D. saccharalis* on Cry1Aa treated diet was high across all the concentrations tested. It was 96% at 0.016 µg/g, the lowest concentration assayed.

^d Resistance ratio to a Cry protein was calculated using the LC₅₀ value of the Cry1Ab resistant-strain divided by the LC₅₀ of the Cry1Ab-susceptible strain.

^s Resistance ratios were significant ($P < 0.05$) based on the lethal dose ratio test (Robertson & Preisler 1992).

^{ns} Resistance ratios were not significant ($P > 0.05$) based on the lethal dose ratio test.

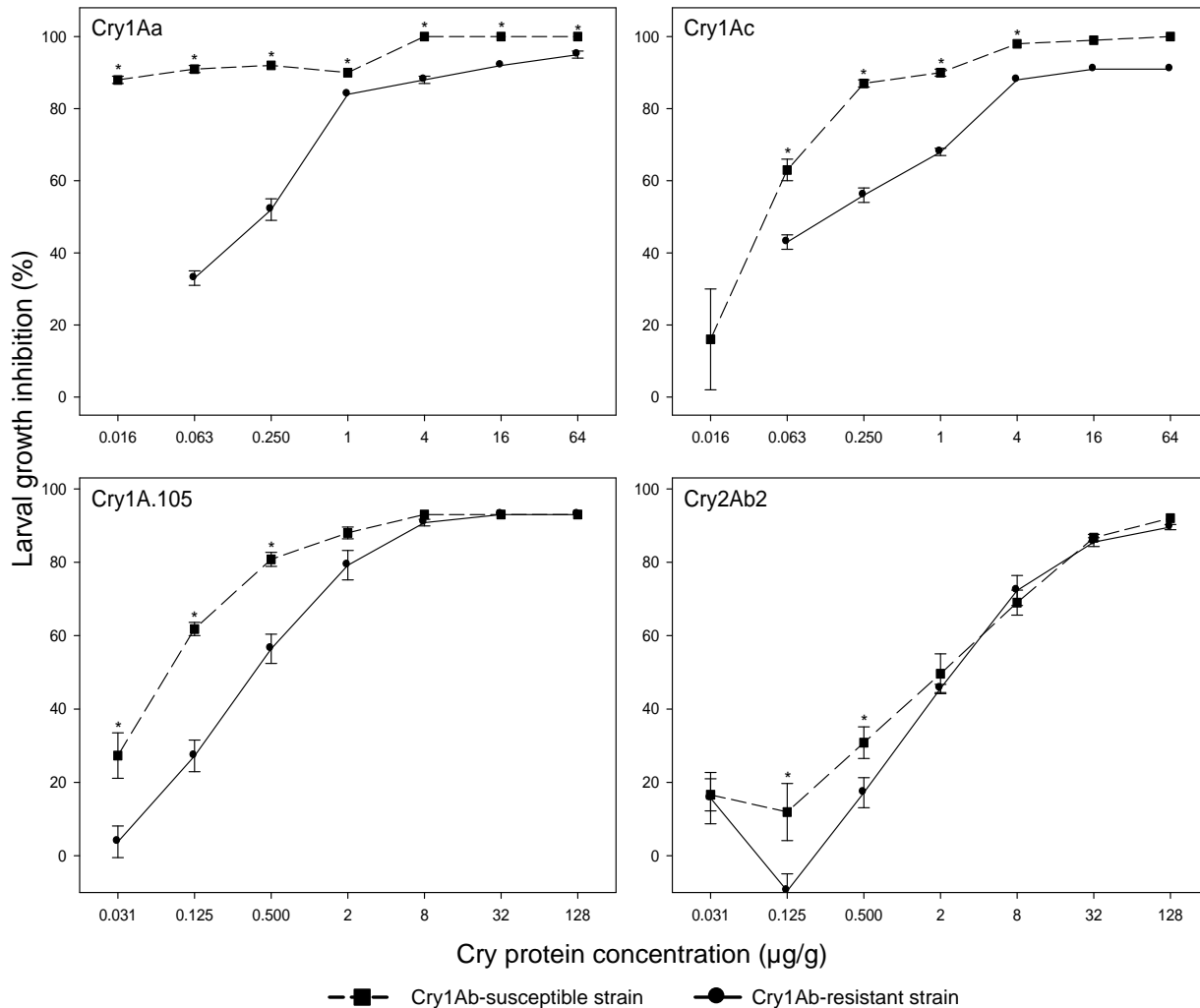


Figure 3.3. Larval growth inhibition (% mean \pm SEM) of the Cry1Ab-susceptible and -resistant strains of *D. saccharalis* exposed to a diet treated with *B. thuringiensis* Cry1Aa, Cry1Ac, Cry1A.105, or Cry2Ab2 proteins at 7 day after inoculation. These values were calculated using the formula: growth inhibition (%) = $100 \times (\text{body weight of larvae feeding on non-treated control diet} - \text{body weight of larvae feeding on Cry protein-treated diet or buffer-treated diet}) / (\text{body weight of larvae feeding on non-treated control diet})$. For each Cry protein concentration, mean values followed by asterisk * are significantly different between the two insect strains ($P < 0.05$; LSMEANS test).

Larval Growth Inhibition. Both Cry1Ab-SS and Cry1Ab-RR strains developed normally on diet treated with buffer compared to the insects reared on the blank control diet. Growth inhibition of larvae feeding on buffer-treated diet was low (-0.7 to 11%) and not significant across insect strains and Cry proteins (Fig. 3.3). The effects of Cry protein concentration and

insect strain on larval growth inhibition were significant for each of the four Cry proteins ($F \geq 97.39$; $df = 6-7, 42-48$; $P < 0.0001$ for protein concentration and $F \geq 4.74$; $df = 1, 42-48$; $P \leq 0.0352$ for insect strain). The interaction between Cry protein concentration and insect strain was significant for Cry1Aa, Cry1Ac, and Cry1A.105 ($F \geq 4.41$; $df = 5-7, 45-48$; $P \leq 0.0041$), but not significant for Cry2Ab2 ($F = 1.94$; $df = 6, 42$; $P = 0.2626$).

In general, growth inhibition of both Cry1Ab-SS and Cry1Ab-RR strains increased as Cry protein concentrations increased (Fig.3.3). At most of the concentrations tested, growth of the Cry1Ab-RR strain was less affected than the Cry1Ab-SS insects, regardless of Cry protein. Cry1Aa and Cry1Ac at 0.063 $\mu\text{g/g}$ inhibited larval growth by 91% and 63%, respectively, in the susceptible strain, whereas only 33% and 43% inhibition was observed in the resistance strain, respectively, at the same concentrations. In the bioassays with Cry1A.105, significant growth inhibition of the Cry1Ab-SS strain was observed at 0.031 $\mu\text{g/g}$, the lowest Bt concentration tested, while more protein (0.125 $\mu\text{g/g}$) was needed to achieve a similar level of inhibition in the Cry1Ab-RR strain. For Cry1A.105, approximately 8 $\mu\text{g/g}$ toxin completely inhibited larval growth of the Cry1Ab-SS strain, while 32 $\mu\text{g/g}$ toxin was necessary to completely inhibit growth in the Cry1Ab-RR strain. For Cry2Ab2, growth of both Cry1Ab-SS and Cry1Ab-RR strains was significantly inhibited at concentrations $\geq 0.5 \mu\text{g/g}$ and growth inhibition significantly increased in direct proportion to Bt concentrations. Larval growth of both insect strains was virtually completely inhibited at 128 $\mu\text{g/g}$ of Cry2Ab2.

Discussion

In general, meridic diet treated with the buffer only did not cause significant larval mortality or growth inhibition of *D. saccharalis* in the bioassays with Cry1A.105 and Cry2Ab2 for either Cry1Ab-SS or Cry1Ab-RR strains. No buffer was used in the testing with Cry1Aa and Cry1Ac.

These results indicate that the observed larval mortality and growth inhibition can be attributed to the toxicity of the respective Cry proteins.

Among the four Cry proteins tested in the present study, Cry1Aa was most toxic against both the Cry1Ab-SS and Cry1Ab-RR strains, followed by the Cry1A.105 protein. Larvae of the Cry1Ab-SS strain were also very sensitive to Cry1Ac, but the Cry1Ab-RR larvae were more resistant to the Cry1Ac than to Cry1Aa and Cry1A.105. The Cry2Ab2 protein demonstrated the least efficacy against both insect strains. Based upon the results of a previous assay with purified trypsin-activated Cry1Ab protein (Huang et al. 2007b), Cry1Ac and Cry1A.105 were equally effective against the Cry1Ab-SS strain, but Cry1A.105 was more effective against the Cry1Ab-RR insects than the Cry1Ab protein.

Cross-resistance of different Bt toxins have been investigated in several lepidopteran species targeted by Bt cotton and Bt corn (Tabashnik et al. 2000, Gould et al. 1995, Ferré and Van Rie 2002, Li et al. 2005). Evidence is mounting that selection for resistance to one Bt toxin can lead to resistance in other related toxins. Cross resistance patterns and their underlying physiological mechanisms are complex and somewhat unpredictable (Bauer 1995). In general, the spectrum of cross resistance can vary for different Bt toxins, insect species, and even among insect strains of a same species. One of the most likely factors underlying the cross-resistance patterns may be the specific Bt binding sites in the midgut brush border membrane of an insect species.

Unfortunately, the specific binding sites of Bt toxins remains unknown for most insect species.

In Bt-SS diamondback moth, *Plutella xylostella* (L.), it is believed that there may be four different Bt binding sites in the midgut brush border membrane: site 1 for Cry1Aa; site 2 for Cry1Aa, Cry1Ab, Cry1Ac, Cry1F, and Cry1B; site 3 for Cry1B; and site 4 for Cry1C (Ferré and Van Rie 2002). Results of the present study showed that the Cry1Ab-RR strain of *D. saccharalis* was also resistant to Cry1Aa and Cry1Ac, indicating these three Bt toxins could share a similar

binding sites, as suggested in *P. xylostella*. In contrast with Cry1Aa and Cry1Ac, the Cry1Ab-RR *D. saccharalis* demonstrated only a low level (4-fold) of cross-resistance to Cry1A.105 and had no cross-resistance to the Cry2Ab2, suggesting these two toxins may have independent mechanisms or target sites.

The first generation of transgenic Bt corn hybrids (e.g. YieldGard® corn) expressed only a single cry protein and thus had a relatively narrow target spectrum. This single Bt protein expression in crop plants has been of considerable concern in many pest management systems because the target insects may be able to more rapidly adapt to single toxins than to multiple toxins. To broaden the insecticidal spectrum and/or delay resistance development in target insect pests, a gene-stacking strategy has been used to develop transgenic plants that express two or more Bt proteins. It is believed that stacking two or more Bt genes with different insecticidal mechanisms (e.g. Bt binding sites) into one plant for controlling target species should delay Bt resistance development (Roush 1998). Recently, genes encoding for Cry1A.105 and Cry2Ab2 proteins were transferred into corn plants (Monsanto 2007). The resulting Bt event, MON 89034, with stacked-genes encoding both the Cry1A.105 and Cry2Ab2 proteins is expected to become commercially available in the United States in 2009 under the trade name of YieldGard VT Pro™ (Monsanto 2007). Bioassay data from the present study indicate that Cry1Ab-RR *D. saccharalis* demonstrates relatively low cross-resistance (4-fold) to the Cry1A.105 protein and no cross-resistance to Cry2Ab2. Albeit limited, these results do provide some evidence that the new Bt corn with stacked genes of Cry1A.105 and Cry2Ab2 may provide a means to delay resistance development in *D. saccharalis*. In addition, Cry1Aa was the highly efficacious against both the Cry1Ab-SS and Cry1Ab-RR strains indicates that the Cry1Aa protein may be a good candidate for use in Bt corn to manage *D. saccharalis*.

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CHAPTER 4

RELATIVE FITNESS OF CRY1AB-SUSCEPTIBLE AND -RESISTANT SUGARCANE BORER (LEPIDOPTERA: CRAMBIDAE) ON MERIDIC DIET AND NON-BT CORN PLANTS

Introduction

Genes which provide resistance to novel challenges such as pesticides, toxins or pathogens often impose fitness costs on individuals that display a resistant phenotype (Raymond et al. 2005). Significant non-recessive fitness costs associated with resistance imply that a frequency of resistance alleles in field insect populations will decrease once the selection agent is removed. For a non-recessive fitness cost, resistance development in field populations can be significantly delayed or even be reversed if the absence of selection pressure is sufficient long (Tabashnik et al. 2005). Therefore, understanding the fitness of resistant insects is important in development of effective resistance management strategies. Studies on the relative fitness of *Bacillus thuringiensis* (Bt) resistant populations have been conducted on several insect species targeted by transgenic Bt crops (e.g. Bt corn or Bt cotton) (Akhurst 2003; Snow et al. 2003; Bird and Akhurst 2004; Cerda and Wright 2004; Vacher et al. 2004; Carrière et al. 2005, 2006; Higginson et al. 2005; Raymond et al. 2005; Anilkumar et al. 2008). In most cases, Bt resistance is associated with fitness costs and most of the fitness costs were associated with recessive inheritance (Anilkumar et al. 2008). In some cases, fitness costs associated with Bt resistance can interact with environmental factors such as host plants (Carrière et al. 2004, 2005; Janmaat and Myers 2005, 2006; Raymond et al. 2006; Bird and Akhurst 2005, 2007).

Since 1999, transgenic corn, *Zea mays* L., expressing Bt toxins (e.g., YieldGard corn), has been widely and successfully planted for managing corn stalk borer pests in the mid-southern region of United States. Bt corn acreage in this region is currently close to the maximum level

(50%) allowed by the United States Environmental Protection Agency for Bt resistance management. The sugarcane borer, *Diatraea saccharalis* (F.), is the most economically important corn stalk boring pest and the primary target of transgenic Bt corn in Louisiana and some other areas of the mid-southern region (Castro et al. 2004, Huang and Leonard 2008). A Cry1Ab-resistant *D. saccharalis* strain capable of completing larval development on commercial Cry1Ab corn has been selected using a novel F₂ screening technique (Huang et al. 2007a). The resistance allele identified in this *D. saccharalis* population represents the first major resistance allele documented in any corn stalk boring specie worldwide.

The availability of this Cry1Ab-resistant strain provides opportunities to examine the relative fitness of Bt resistant *D. saccharalis*. In this study, larval growth and development of Cry1Ab-susceptible, -resistant, and their F₁ progeny of *D. saccharalis* were evaluated on a meridic diet with/without a Bt toxin (Cry1Ab) in the laboratory and on conventional non-Bt corn plants in the greenhouse. Data generated from this study should provide valuable information in developing appropriate strategies for managing resistance of *D. saccharalis* to Bt corn.

Materials and Methods

Insect Sources. A Cry1Ab-susceptible strain (Bt-SS) of *D. saccharalis* was established from larvae collected in corn fields near Winnsboro in Northeast Louisiana during 2004 (Huang et al. 2007a). A Cry1Ab-resistant strain of *D. saccharalis* was originated from a single two-parent family-line using an F₂ screen (Huang et al. 2007a). The two-parent family-line carrying the Cry1Ab resistance allele was developed from a field collection in 2004 near the same location as the Bt-SS strain. During confirmation of Bt resistance, individuals of the original Bt resistant strain were backcrossed with those of the Bt-SS strain and re-selected for Bt resistance with Cry1Ab corn leaf tissue in the F₂ generation of the backcross. The re-selected Cry1Ab-

resistant strain (Bt-RR) completed larval development on commercial Bt corn hybrids expressing the Cry1Ab protein and demonstrated significant resistance levels (≈ 100 -fold) to purified trypsin-activated Cry1Ab toxin (Huang et al. 2007a, 2007b; Wu et al. 2007). The Bt-RR strain had been maintained in the laboratory with continuous selection for approximately two years when this study was started. Another Bt resistant strain (Bt-R'R') was developed by backcrossing the Bt-RR with the Bt-SS and reselection for Bt resistance on Cry1Ab corn leaf tissue in the F₂ generation. Progeny of the subsequent generation of the reselected Bt-R'R' were used in the current study. In addition, two F₁ genotypes (Bt-R'_mS_f and Bt-R'_fS_m) were developed by reciprocal crosses of Bt-SS with Bt-R'R'. In this study, all five genotypes of *D. saccharalis* were assayed on meridic diet with/without Cry1Ab toxin and on intact conventional non-Bt corn plants to determine the relative fitness across a range of Bt exposure.

Source of Cry1Ab Toxin. Purified (99.9%) Cry1Ab protein was obtained from Dr. Marianne Puztai-Carey, Case Western Reserve University, Ohio. The Cry1Ab was produced using recombinant *Escherichia coli* cultures and was subsequently activated with trypsin. The activated Cry protein was lyophilized before it was used in the bioassays.

Corn Plants. A commercial non-Bt corn hybrid, DK697 (Monsanto, St. Louis, MO) was planted in 18.9-liter pots containing approximately 5 kg of a standard potting soil mixture (Perfect Mix, Expert Gardener Products, St. Louis, MO) in a greenhouse at the Louisiana State University Agricultural Center, Baton Rouge, LA. Five corn seeds were planted in each pot. Seedlings were thinned to two plants per pot at 2 wk after seedling emergence. The plants were irrigated and fertilized for optimum growth during the tests.

Fitness of Five *D. saccharalis* Genotypes on Non-Treated Diet or Cry1Ab-Treated Diet. Larvae of the five *D. saccharalis* genotypes were inoculated on a non-treated regular diet and

three diets containing low concentrations (0.01, 0.05, 0.1 µg/g) of Cry1Ab toxin. The three low concentrations used in the current study were determined from previous bioassays (Huang et al. 2007b). At these Cry1Ab concentrations, larval mortality of *D. saccharalis* at 7 days after inoculation (DAI) was expected to be very low (Huang et al. 2007b). The Cry1Ab treated diets were prepared by mixing appropriate volumes of Cry1Ab solutions into the diet. An equivalent amount of distilled water was added to the non-treated diet so that the total amount of water in the Cry1Ab-treated diet was equal to that in the non-treated diet. Immediately after mixing, 1.0 ml of Cry1Ab treated or non-treated diet was placed into each cell of the 128-cell trays (Bio-Ba-128, C-D International Inc. Pitman, NJ) using 20-ml syringes (Becton, Dickinson and Company, Franklin Lakes, 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 28 °C, 50% RH, and a photoperiod of 16:8 (L:D) h. There were four replications for each combination of insect strain by Cry1Ab concentration with 30 larvae in each replication. After 10 days, survivors were transferred into 30-ml-cups (1 larva/cup) (SOLO, Chicago, IL) containing approximately 5 ml of diet at the same Cry1Ab concentrations and were allowed to develop to pupation. After the first pupa was observed, all cups were observed daily until all insects pupated or died. Pupation time, pupation success, sex ratio, and pupal weight were recorded for each treatment.

Fitness of Five *D. saccharalis* Genotypes on Non-Bt Corn Plants/Non-Treated Diet. At 50 days after planting, 10 neonates (>24 h old) from each of the five genotypes were manually released into the whorl of a corn plant in the greenhouse. There were three replications (3 pots or 6 plants of non-Bt corn hybrid, DK697) for each insect genotype. Corn plants were dissected at 19 DAI. The number of surviving larvae and insect body weight were recorded. At this time, most (>90%) survivors were in larval stages. All larvae recovered from plants were transferred into 30-ml plastic cups (1 larva/cup) containing approximately 5 ml of non-treated diet and

allowed to develop to pupation (thereafter refer to as combined rearing) in growth chambers at the same environmental conditions described previously. Insects were evaluated daily until all insects pupated or died. Pupation time, pupation success, sex ratio, and pupal weight were recorded for each treatment.

Data Analysis. Larval development time and larval/pupal body weight for each insect genotype were transformed to $\log(x + 1)$ scale, while survival/pupation rate were transformed to angular values to normalize treatment variances before statistical analysis (Zar 1984). Transformed data were subjected to a two-way analysis of variance (ANOVA) with the insect genotype and Cry1Ab concentration as the two main factors (SAS Institute 2008). Treatment means among insect genotypes within same Cry1Ab concentrations were separated using the least square difference test at the $\alpha = 0.05$ level (SAS Institute 2008). Data associated with the tests on corn plants were analyzed using one-way ANOVA and differences among insect genotypes were separated using the Fisher's Protected LSD test at the $\alpha = 0.05$ level (SAS Institute, 2008). Untransformed means and standard errors are presented in the figures and tables.

Results

Neonate-to-Pupa Development for *D. saccharalis* on Non-Treated Diet or Cry1Ab-Treated Diet. Larval-to-pupa development times of both male and female *D. saccharalis* were significantly different among Cry1Ab concentrations ($F > 17.56$; $df = 3, 60$; $P < 0.0001$) and insect genotypes ($F \geq 4.12$; $df = 4, 60$; $P \leq 0.0051$). The interaction of Cry1Ab concentration and insect genotype was also significant ($F \geq 3.71$; $df = 12, 60$; $P \leq 0.0003$). Compared to Cry1Ab-susceptible insects (Bt-SS), Bt- R'R' did not show fitness costs in larval development on non-treated diet. The neonate-to-pupa development time of male *D. saccharalis* was not significantly different ($P > 0.05$) among the five genotypes on non-treated diet and at the Cry1Ab concentration of $0.01 \mu\text{g/g}$ (Fig. 4.1). Male neonates took an average of 21.8 days to become

pupae on non-Bt diet. Male development time to pupation was significantly ($P < 0.05$) delayed for Bt-SS genotypes at the two higher Cry1Ab concentrations (e.g. 0.05 and 0.1 $\mu\text{g/g}$). The larval development time of the Bt-R'_mS_f genotype was also significantly longer ($P < 0.05$) than that of the other three genotypes at these two Cry1Ab concentrations. A similar pattern was observed for the female *D. saccharalis* among the five genotypes (Fig. 4.1). The average time needed for female neonate to pupa was 1.5 days longer than that of the males. Except the Bt-RR on non-treated diet, there were no significant differences ($P > 0.05$) in larval development time among all genotypes on non-treated diet and diet treated with 0.01 $\mu\text{g/g}$ Cry1Ab. As observed for the males, larval development of Bt-SS females was significantly ($P < 0.05$) delayed at Cry1Ab concentrations of 0.05 and 0.1 $\mu\text{g/g}$.

Pupal Weight of *D. saccharalis* on Non-Treated Diet or Cry1Ab-Treated Diet. No significant difference in pupal weight was observed among the five genotypes on non-treated diet. Cry1Ab at all concentrations did not significantly reduce pupal weight (Fig. 4.2). The main effect of Cry1Ab concentration and insect genotype on male pupal weight was not significant ($F = 1.49$; $df = 3, 60$; $P = 0.2259$ for Cry1Ab concentration and $F = 1.68$; $df = 4, 60$; $P = 0.1657$ for insect genotype). The interaction of the two factors also was not significant ($F = 1.15$; $df = 12, 60$; $P = 0.3393$). On non-treated diet, a male pupa had an average weight of 94.2 mg. Male pupal weight did not differ significantly ($P > 0.05$) among the five genotypes at all Cry1Ab concentrations (Fig. 4.2). Female pupae of *D. saccharalis* weighed more than males with an average weight of 158.6 mg/pupa. The main effects of Cry1Ab concentration and insect genotype on female pupal weight was significant ($F = 4.96$; $df = 3, 60$; $P = 0.0016$ for Cry1Ab concentration and $F = 6.75$; $df = 4, 60$; $P = 0.0005$ for insect genotype). However, there were no significant differences among the five genotypes on non-treated diet or at each of the three Cry1Ab concentrations (Fig. 4.2).

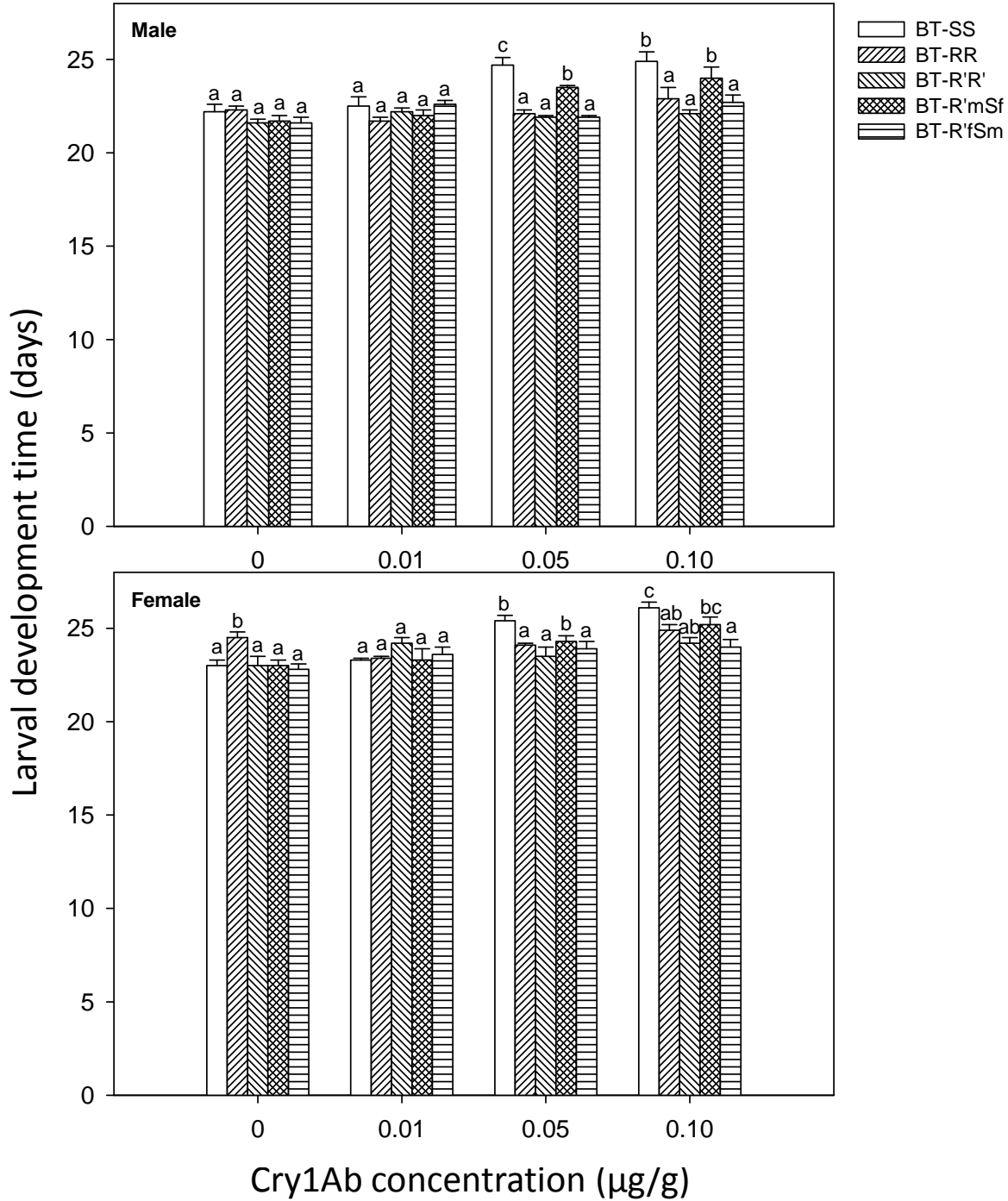


Figure 4.1. Neonate-to-pupa development time (day: mean \pm SEM) of five genotypes of *D. saccharalis* on non-treated diet and diet treated with low concentrations of *B. thuringiensis* Cry1Ab toxin. Insect genotypes: Bt-SS = Cry1Ab-susceptible; Bt-RR = original Cry1Ab-resistant; Bt-R'R' = Bt-RR back-crossed with Bt-SS and re-selected resistant; Bt-R'_mS_f = F₁ of Bt-R'R' male \times Bt-SS female; and Bt-R'_fS_m = F₁ of Bt-R'R' female \times Bt-SS male. Mean values within a Cry1Ab concentration across five insect genotypes followed by a same letter are not significantly different ($P > 0.05$; LSMEANS test).

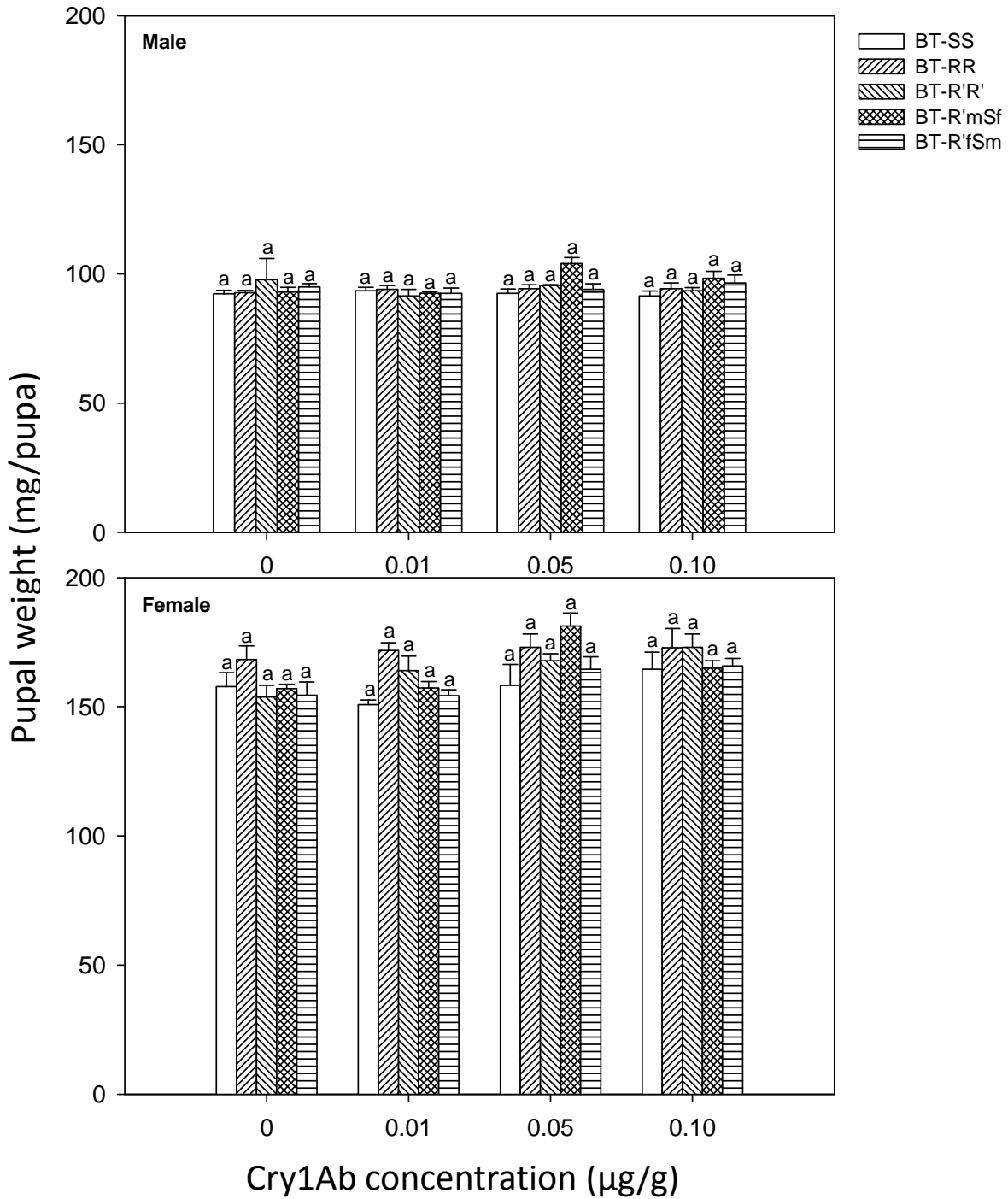


Figure 4.2. Pupal weight (mg/pupa, mean \pm SEM) of five genotypes of *D. saccharalis* on non-treated diet and diet treated with low concentrations of *B. thuringiensis* Cry1Ab toxin. Insect genotypes: Bt-SS = Cry1Ab-susceptible; Bt-RR = original Cry1Ab-resistant; Bt-R'R' = Bt-RR back-crossed with Bt-SS and re-selected resistant; Bt-R'mSf = F₁ of Bt-R'R' male \times Bt-SS female; and Bt-R'fSm = F₁ of Bt-R'R' female \times Bt-SS male. Mean values within a Cry1Ab concentration across five insect genotypes followed by a same letter are not significantly different ($P > 0.05$; LSMEANS test).

Pupation Success of *D. saccharalis* on Non-Treated Diet or Cry1Ab-Treated Diet.

There were no obvious fitness differences in pupation success rates among the five genotypes on non-treated diet. However, pupation success rates on Cry1Ab- treated diet were considerably different among the insect genotypes across Cry1Ab concentrations (Fig. 4.3). The main effect of Cry1Ab concentration and insect genotype on pupation rate was significant ($F = 20.19$; $df = 3, 60$; $P < 0.0001$ for Cry1Ab concentration and $F = 5.98$; $df = 4, 60$; $P = 0.0004$ for insect genotype). The interaction of the two factors was also significant ($P = 6.77$; $df = 12, 60$; $P < 0.0001$). Pupation success rates on non-treated diet ranged from 76.6 to 93.8% among the five genotypes and but was not significantly different ($P > 0.05$) between the Cry1Ab-susceptible and -resistant strains.

The pupation success rate was lower on diet treated with 0.05 or 0.1 $\mu\text{g/g}$ Cry1Ab for all genotypes, but the reduction was significantly greater ($P < 0.05$) for Bt-SS than that for the other four genotypes. The Bt-R'R' at 0.1 $\mu\text{g/g}$ appeared to have a higher pupation success rate than the two heterozygous and Bt-RR genotypes (Fig. 4.3).

Sex Ratio of *D. Saccharalis* on Non-Treated Diet or Cry1Ab-Treated Diet. There were no significant fitness differences in sex ratio among the five genotypes on non-treated diet (Fig. 4.4). Cry1Ab in diet at the concentrations tested also did not have a significant effect on sex ratio (Fig. 4.4). The main effect of Cry1Ab concentration and insect genotype, and their interaction on sex ratio were not significant ($F \leq 2.65$; $df = 3, 4; 60$; $P \geq 0.0566$ for the two main factors and $F = 0.86$; $df = 12, 60$; $P = 0.5881$ for the interaction).

The overall sex ratio of male: female across insect genotypes and Cry1Ab concentrations was 1.33: 1.

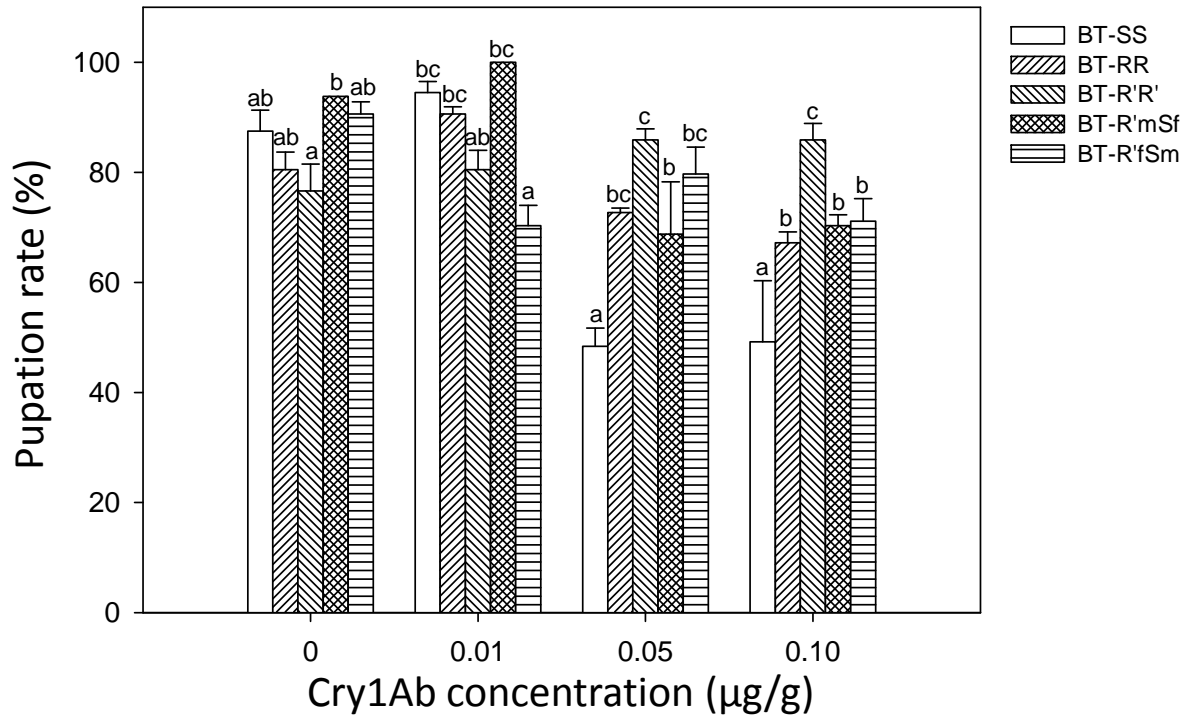


Figure 4.3. Pupation rate (%: mean \pm SEM) of five genotypes of *D. saccharalis* on non-treated diet and diet treated with low concentrations of *B. thuringiensis* Cry1Ab toxin. Insect genotypes: Bt-SS = Cry1Ab-susceptible; Bt-RR = original Cry1Ab-resistant; Bt-R'R' = Bt-RR back-crossed with Bt-SS and re-selected resistant; Bt-R'mSf = F₁ of Bt-R'R' male \times Bt-SS female; and Bt-R'fSm = F₁ of Bt-R'R' female \times Bt-SS male. Mean values within a Cry1Ab concentration across five insect genotypes followed by a same letter are not significantly different ($P > 0.05$; LSMEANS test).

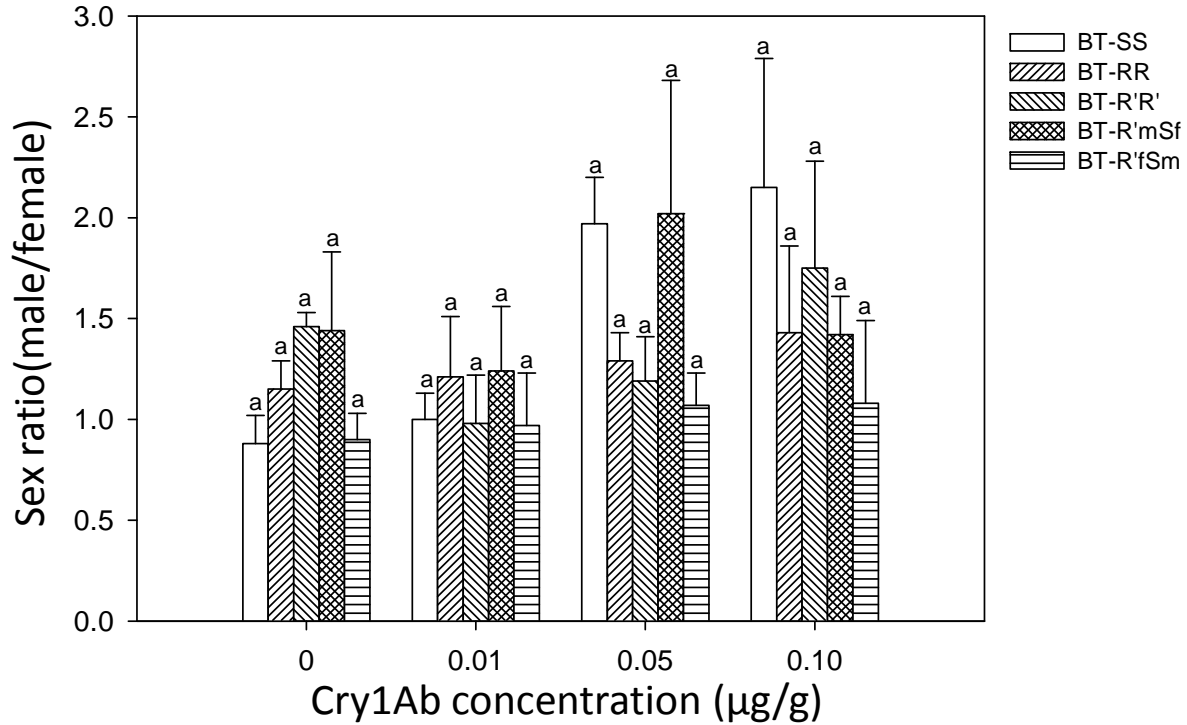


Figure 4.4. Sex ratio (male/female: mean \pm SEM) of five genotypes of *D. saccharalis* on non-treated diet and diet treated with low concentrations of *B. thuringiensis* Cry1Ab toxin. Insect genotypes: Bt-SS = Cry1Ab-susceptible; Bt-RR = original Cry1Ab-resistant; Bt-R'R' = Bt-RR back-crossed with Bt-SS and re-selected resistant; Bt-R'mSf = F₁ of Bt-R'R' male \times Bt-SS female; and Bt-R'fSm = F₁ of Bt-R'R' female \times Bt-SS male. Mean values within a Cry1Ab concentration across five insect genotypes followed by a same letter are not significantly different ($P > 0.05$; LSMEANS test).

Larval Survival and Growth of *D. Saccharalis* on Non-Bt Corn Plants. There were no significant fitness differences in larval survival and development on non-Bt corn plants associated with Bt resistance in *D. saccharalis*. Larval survival rates of the five *D. saccharalis* genotypes on non-Bt corn plants in the greenhouse were not significantly different ($F = 1.98$; $df = 4, 10$; $P = 0.1733$) with an overall average survival rate of 51% after 19 days (Table 4.1). Larval body weights were also not significantly different among the genotypes ($F = 0.84$; $df = 4, 10$; $P = 0.5324$) with an average of 94 mg/larva after feeding on non-Bt corn plants for 19 days (Table 4.1).

Table 4.1. Larval survival (% mean \pm SEM) and growth (% mean \pm SEM) of five genotypes of *D. saccharalis* on a conventional non-Bt corn hybrid (DK697).

Genotype ¹	Survival rate ²	Larval body weight ^{2,3}
	(%)	(mg/larva)
Bt-SS	42.8 \pm 3.4 a	87.8 \pm 6.0 a
Bt-RR	53.9 \pm 6.4 a	103.0 \pm 10.5 a
Bt-R'R'	55.0 \pm 4.9 a	99.2 \pm 3.4 a
Bt-R'mSf	56.6 \pm 1.1 a	93.5 \pm 8.1 a
Bt-R'fSm	48.6 \pm 1.7 a	87.4 \pm 7.5 a

¹ *D. saccharalis* genotypes: Bt-SS = Cry1Ab-susceptible; Bt-RR = original Cry1Ab-resistant; Bt-R'R' = Bt-RR back-crossed with Bt-SS and re-selected resistant; Bt-R'mSf = F₁ of Bt-R'R' male \times Bt-SS females; and Bt-R'fSm = F₁ of Bt-R'R' female \times Bt-SS male.

² Means within a column followed by a same letter are not significantly different ($P < 0.05$; by Fisher's Protected LSD test).

³ Larval body weight was measured at 19 DAI.

Table 4.2. Larval development (% mean \pm SEM), pupation rate (% mean \pm SEM), and sex ratio (mean \pm SEM) of five *D. saccharalis* genotypes on a non-Bt conventional corn hybrid (DK697) and meridic diet.

Insect genotype ¹	Neonate-pupa development time ²		Pupation rate ²	Pupal weight ²		Sex ratio ²
	(day)			(mg/pupa)		
	Male	Female	(%)	Male	Female	
Bt-SS	23.2 \pm 0.7 a	24.6 \pm 0.9 a	42.8 \pm 3.4 a	84.7 \pm 1.6 a	142.5 \pm 5.3 a	1.66 \pm 0.23 a
Bt-RR	22.3 \pm 1.1 a	24.2 \pm 0.2 a	53.9 \pm 6.4 a	86.2 \pm 1.5 a	161.3 \pm 5.4 a	1.08 \pm 0.33 a
Bt-R'R'	25.4 \pm 1.3 a	26.2 \pm 1.4 a	52.5 \pm 6.4 a	88.5 \pm 4.3 a	156.4 \pm 2.2 a	1.14 \pm 0.09 a
Bt-R'mSf	22.8 \pm 1.1 a	25.2 \pm 1.0 a	56.7 \pm 1.1 a	89.0 \pm 1.2 a	155.0 \pm 1.4 a	0.94 \pm 0.13 a
Bt-R'fSm	24.0 \pm 0.6 a	25.0 \pm 0.9 a	44.6 \pm 4.0 a	88.0 \pm 2.1 a	155.6 \pm 6.5 a	1.71 \pm 0.29 a

¹ *D. saccharalis* genotypes: Bt-SS = Cry1Ab-susceptible; Bt-RR = original Cry1Ab-resistant; Bt-R'R' = Bt-RR back-crossed with Bt-SS and re-selected resistant; Bt-R'mSf = F₁ of Bt-R'R' male \times Bt-SS female; and Bt-R'fSm = F₁ of Bt-R'R' female \times Bt-SS male.

² Means within a column followed by a same letter is not significantly different ($P < 0.05$; by Fisher's Protected LSD test).

Relative Fitness of *D. Saccharalis* on Non-Bt Corn Plants with Transfer to Non-Treated

Diet. No notable fitness differences for all biological parameters were observed among the five genotypes for the combined rearing on corn plant and diet (Table 4.2). Neonate-to-pupa development time was not significantly different among the five insect genotypes on the combined rearing ($F = 1.37$; $df = 4, 0$; $P = 0.3102$ for male and $F = 0.58$; $df = 4, 10$; $P = 0.6850$ for female), with an overall average development time of 23.5 days (male) and 25.0 days (female). Approximately 50% neonates of the five genotypes successfully pupated and there were no differences among the genotypes ($F = 1.7$; $df = 4, 10$; $P = 0.2252$) (Table 4.2). There were also no significant differences in pupal weight among the insect genotypes ($F = 0.52$; $df = 4, 10$; $P = 0.7267$ for male and $F = 2.42$; $df = 4, 10$; $P = 0.1162$ for female) (Table 4.2). An average male pupa weighed 87.3 mg and a female weighed 154.2 mg. Sex ratios among the five genotypes was also not significantly different ($F = 3.32$; $df = 4, 10$; $P = 0.1284$) with an ratio of 1.31:1(male: female) (Table 4.2).

Discussion

Results of this study showed that there was no major fitness costs associated with Cry1Ab resistance in *D. saccharalis*. All biological parameters (e.g. larval growth, development, and survival, pupation rate, larval and pupal weight, sex ratio) of *D. saccharalis* on non-treated meridic diet and on conventional non-Bt corn plants were similar among the five genotypes with few exceptions.

Bt resistance in other insect species targeted by Bt crops, however, was often associated with fitness costs. For example, significant fitness costs in insect growth and development, larval survival including overwintering survival, and/or mating success and reproductive capacity have been documented in several Bt-resistant populations of the pink bollworm, *Pectinophora*

gossypiella (Saunders), cotton bollworm, *Helicoverpa armigera* (Hübner) and *H. zea* (Boddie) (Carrière et al. 2001, 2005; Tabashnik et al. 2003; Bird and Akhurst 2004, 2005; Anilkumar et al. 2008; Liang et al. 2008; Zhao et al. 2008). Significant fitness costs also were associated with Bt resistance in *Chrysomela tremulae*, a target pest of transgenic-Bt poplar trees (Wenes et al. 2006), and *Trichoplusia ni* (Janmaat and Myers 2005, 2006), an insect species that has developed high levels of resistance to Bt insecticide sprays.

However, there are some exceptions. A Bt-resistant strain of the tobacco budworm, *Heliothis virescens* (F.), a primary target of Bt cotton in the United States, did not differ in larval development and survival on non-Bt diet compared to Bt-susceptible larvae (Gould and Anderson 1991). Both field and laboratory-selected Bt resistant populations of diamondback moth, *Plutella xylostella* (L.), another major pest that has developed high level of resistance to Bt insecticide spray in the field, did not show fitness costs compared to Bt-susceptible strains (Tang et al. 1997, Sayyed and Weight 2001). Larval development and survival of a Dipel-resistant strain of European corn borer, *Ostrinia nubilalis* (Hübner), on non-Bt diet was similar compared to those of Bt-susceptible strain (Huang et al. 2005). Larval development time and survivorship indicated that a fitness cost was associated with resistance to Bt in some Bt-resistant colonies, but not in others such as Indianmeal moth, *Plodia interpunctella* (Hübner) (Oppert et al. 2000).

The lack-of-fitness costs in Bt resistant *D. saccharalis* could make it an even greater challenge in managing Bt resistance for this important corn stalk boring species. Previous studies have shown that *D. saccharalis* is much less susceptible to Cry1Ab compared to the other two major corn borer pests, *O. nubilalis* and southwestern corn borer, *D. grandiosella* Dyar (Huang et al. 2006). Bt resistance allele frequency in field populations of *D. saccharalis* was estimated to be greater than that of *O. nubilalis* and *D. grandiosella* (Huang and Leonard 2008). In addition, a

greenhouse study suggested that most current commercial Bt corn (e.g. YieldGard corn) may express a marginal “high dose” to *D. saccharalis* (Wu et al. 2007). All these results suggest that there may be a relatively higher potential for *D. saccharalis* populations to develop resistance to Bt corn. Information generated from the current and previous studies support the need for a scientifically sound resistance management plan to ensure the long-term success of Bt corn technology in the mid-southern region of the United States.

Response of insects to low concentrations of Bt toxins has usually been reported to reduce larval growth, development, and reproduction (Gould et al. 1991, Nyouki et al. 1996, Liu et al. 2001, Huang et al. 2005). The results observed in the current study with *D. saccharalis* appear to be in agreement with those previous published results. Several past studies also have compared the effects of low concentrations of Bt toxins on development and reproduction of Bt-susceptible and -resistant strains of several important agricultural lepidopteran species (Brewer 1991, Gould and Anderson 1991, Liu et al. 2001). Compared to Bt-susceptible insects, resistant larvae reared on diet treated with low concentrations of Bt toxins usually had lower larval mortality, greater larval and pupal weights, and higher fecundity. In the current study, larval development and pupation of Cry1Ab-susceptible larvae of *D. saccharalis* was significantly affected by the low concentrations of Cry1Ab in diet. However, the effect was not significant on larval development and less significant on pupation success rates on Bt-RR, Bt-R'R' and Bt-R'S genotypes. The overall performance of the two F₁ genotypes on the Cry1Ab treated diet, especially at 0.01 and 0.05 µg/g, was similar to the two Cry1Ab-resistant strains, suggesting a dominant inheritance of the Bt resistance at the low concentrations. Cry1Ab-resistance in this *D. saccharalis* strain was inherited as an incompletely recessive gene at higher Cry1Ab concentrations and commercial Cry1Ab corn plants or completely recessive at a very high Cry1Ab concentration (e.g. 128 µg/g)

(Wu et al. unpublished data). It is possible to develop Bt corn plants that express a sufficient dose of Bt proteins to “make” Bt resistance in *D. saccharalis* functionally (completely) recessive.

In the current study, fitness of the original Cry1Ab-resistant strain (Bt-RR) of *D. saccharalis* on diet and plants was evaluated along with the backcross-and-reselected genotype (Bt-R'R') and Bt-SS. Except for the neonate-to-pupa development time on non-Bt diet, all biological parameters measured on both non-Bt diet and non-Bt corn plants were not significantly different among these three genotypes. In addition, all biological parameters measured on diet treated at the three concentrations of Cry1Ab were similar between the Bt-RR and Bt-R'R' strains except for the pupation rate at 0.1 µg/g.

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CHAPTER 5

INHERITANCE OF RESISTANCE TO *BACILLUS THURINGIENSIS* CRY1AB PROTEIN IN THE SUGARCANE BORER (LEPIDOPTERA: CRAMBIDAE)

Introduction

Information about the genetic background of *Bacillus thuringiensis* (Bt) resistance in insect can improve resistance detection and monitoring, risk assessment, modeling, and development of insecticide resistance management (IRM) strategies (Bourguet 2004, Tabashnik and Carrière 2007). The current IRM strategy for Bt corn is termed the “high dose/refuge” strategy (Ostlie et al. 1997, US EPA 2001). This strategy relays on a high dose of Bt toxins expressed in Bt corn plants to kill all or nearly all resistant heterozygotes. Sufficient number of susceptible moths developed on non-Bt refuge plants are then available to mate with the rare resistant homozygotes that survived on Bt corn plants. Therefore, most individuals carrying Bt resistance alleles of the target insect species will be heterozygous in the field. These heterozygous individuals should be killed with high dose Bt corn such that resistance allele frequency will be maintained at a very low level in the field insect populations for a long period of time. One of the key assumptions for the success of the “high/dose refuge” IRM strategy is that the resistance of the target pest to Bt toxins should be recessive or at least incompletely recessive (Ostlie et al. 1997, US EPA 2001).

The genetic basis of Bt resistance has been assessed in many insect species (Heckel 1994, Tabashnik et al. 1997, Huang et al. 1999, Bourguet 2004, Ferré and Van Rie 2002, Alves et al. 2006, Tabashnik and Carrière 2007). In most of these reports, a high level of resistance was controlled by one or a few autosomal, recessive or incompletely recessive gene(s) (Liu et al. 2001; Tabashnik et al. 2002; Sayyed et al. 2003, 2004; Kain et al. 2004; Augustin et al. 2004; Liang et al. 2008). In contrast, low levels of resistance could exhibit a more dominant trait compared to those instances of intense levels of resistance (Gould et al. 1992, Huang et al. 1999,

Kranthi et al. 2005). There are also some exceptions (Chaufaux et al. 1997, Tang et al. 1997, Sayyed et al. 2004, Janmaat et al. 2004, Alves et al. 2006, Jackson et al. 2006).

The sugarcane borer, *Diatraea saccharalis* (F.), is the primary corn borer pest species in Louisiana and some areas of Texas (Porter et al. 2005, Huang and Leonard 2008). Transgenic Bt corn (e.g. YieldGard corn) currently is the most important tool for managing corn stalk boring pests in the United States including Louisiana (Huang and Leonard 2008, Yue et al. 2008). During 2004, a major Bt resistance allele was detected in a *D. saccharalis* population collected from non-Bt corn fields in northeast Louisiana (Huang et al. 2007a). The Cry1Ab-resistant strain survived and completed larval development (neonate to pupa) on intact commercial Bt corn plants and showed a significant resistance level to purified trypsin-activated Cry1Ab toxin (Huang et al. 2007a, 2007b; Wu et al. 2007). Inheritance of Bt resistance in corn stalk boring species has been examined for three laboratory- selected strains of the European corn borer, *Ostrinia nubilalis* (Hübner), (Huang et al. 1999, Alves et al. 2006). None of these three Bt-resistant *O. nubilalis* strains has demonstrated the ability to survive on commercial Bt corn plants (Bourguet et al. 2000, Huang et al. 2002). The availability of the Cry1Ab-resistant strain of *D. saccharalis* provided an opportunity to assess the genetic basis of Bt resistance for this corn borer species. In this study, several genetic cross experiments were conducted to determine the inheritance of Bt resistance in *D. saccharalis*. The results of this study should provide useful information to improve IRM strategies for Bt corn in *D. saccharalis* and other related corn borer species.

Materials and Methods

Sources of Cry1Ab-Susceptible and -Resistant *D. saccharalis*. A Cry1Ab-susceptible strain (Cry1Ab-SS) of *D. saccharalis* was established from larvae collected from corn fields near

Winnsboro in Northeast Louisiana during 2004 (Huang et al. 2006). A Cry1Ab-resistant strain (Cry1Ab-RR) of *D. saccharalis* was developed from a single two-parent family-line by screening 230 F₂ family-lines collected from non-Bt corn fields at the same location as the Cry1Ab-SS strain (Huang et al. 2007a). The Cry1Ab-resistant strain of *D. saccharalis* was able to survive and complete entire larval development (neonate to pupal stage) on commercial transgenic corn plants expressing the Cry1Ab protein (Huang et al. 2007a, Wu et al. 2007).

Genetic Crosses. To develop the genotypes with appropriate genetic backgrounds to analyze Bt resistance inheritance in *D. saccharalis*, pupae were classified by sex. Females from one population were mass-crossed with males from the other population in 3.8-liter cardboard cartons (Neptune Paper Products, Newark, NJ). The design of the cardboard cartons for the genetic crosses was similar to the containers used for adult mating and oviposition described in Huang et al. (2007b). For egg collection, a piece of wax paper was lined against the inside wall of the cartons to serve as the oviposition site. The cardboard cartons were placed in an environmental chamber maintained at 28°C, 65% RH, and a 14:10 (L: D) h photoperiod. Wax paper containing eggs was removed from the carton and immediately washed with 75% alcohol solution and dried in room condition. Eggs on the wax paper were placed in the wells of the eight-well trays (Bio-Ba-8, C-D International, Pitman, NJ) containing wetted-filter paper for maintaining appropriate moisture levels for egg hatching. Trays containing eggs were placed in a growth chamber maintained at 28°C, 50% RH, and a 16:8 (L: D) h photoperiod.

Three types with a total of eight different crosses were conducted (Fig. 5.1). These genetic crosses included (a) two reciprocal parental crosses between Cry1Ab-SS and Cry1Ab-RR strains; b) two F₁ by F₁ crosses; and c) four backcrosses of F₁ with Cry1Ab-RR strain. Bioassays with two F₁ populations showed Cry1Ab resistance in this *D. saccharalis* strain was incompletely

recessive, therefore, backcrosses were conducted by crossing F_1 insects with the Cry1Ab-RR parents. The F_1 populations produced from two reciprocal crosses used in this study were F_{1a} : progeny of Cry1Ab-RR males crossed with Cry1Ab-SS females, and F_{1b} , progeny of Cry1Ab-RR females crossed with Cry1Ab-SS males. The two F_2 populations were F_{2a} : progeny of the sib-mating within F_{1a} , and F_{2b} : progeny of the sib-mating within F_{1b} . The four backcross populations were Backcross A: progeny of F_{1a} males and Cry1Ab-RR females, Backcross B: progeny of F_{1a} females and Cry1Ab-RR males, Backcross C: progeny of F_{1a} males and Cry1Ab-RR females, and Backcross D: progeny of F_{2a} females and Cry1Ab-RR males (Fig. 5.1). Neonates from the eight cross-populations and the two parental populations (Cry1Ab-SS and Cry1Ab-RR) were used in this study to estimate the inheritance of Cry1Ab resistance in this population of *D. saccharalis*.

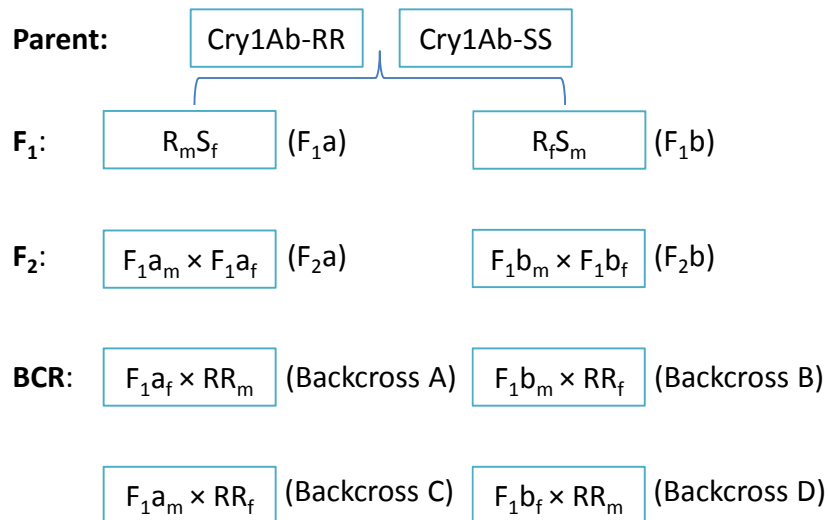


Figure 5.1. Illustration of various genetic crosses for determining inheritance of Cry1Ab resistance in *D. saccharalis*. Cry1Ab-SS = parental Cry1Ab-susceptible strain, Cry1Ab-RR = parental Cry1Ab-resistant strain, F_{1a} = progeny of Cry1Ab-RR male crossed with Cry1Ab-SS female, F_{1b} = progeny of Cry1Ab-RR female crossed with Cry1Ab-SS male, F_{2a} = progeny of sib-mating within F_{1a} , F_{2b} = progeny of sib-mating within F_{1b} , Backcross A = progeny of F_{1a} male and Cry1Ab-RR female, Backcross B = progeny of progeny of F_{1a} female and Cry1Ab-RR male, Backcross C = progeny of F_{1a} male and Cry1Ab-RR female, and Backcross D = progeny of F_{2a} female and Cry1Ab-RR male.

Source of Cry1Ab Toxin. Purified (99.9%) Cry1Ab protein was obtained from Dr. Marianne Puztai-Carey, Case Western Reserve University, Ohio. The Cry1Ab was produced using recombinant *Escherichia coli* cultures and was subsequently activated with trypsin. The activated Cry1Ab protein was lyophilized before it was used in the bioassays.

Dose-Response Bioassays. A diet incorporation method described in Huang et al. (2007b) was used to determine the response of the Cry1Ab-SS, Cry1Ab-RR, and the eight cross-populations to purified Cry1Ab toxin. Seven Cry1Ab concentrations, 0.016, 0.063, 0.25, 1, 4, 16, and 64 µg/g, plus a control were used in each bioassay. Bioassays were conducted in the 128-cell trays (Bio-Ba-128, C-D International, Pitman, NJ). To achieve the desired concentrations for bioassay, lyophilized Cry1Ab toxin was dissolved in distilled water. The appropriate Cry1Ab concentration was added into diet just prior to dispensing into the cells. Diet treated with distilled water only was used for the control treatment. In the bioassay, approximately 0.7 ml of treated diet was poured into each cell using 10 or 20-ml syringes (Becton, Dickinson and Company, Franklin Lakes, NJ). One neonate (< 24-h old) was manually infested onto the surface of each cell. There were four replications for each combination of insect population and Cry1Ab concentration with 32 neonates for each replication. After insect infestation, the bioassay trays were held in an environmental chamber maintained at 28 °C, 50% RH, and a photoperiod of 16:8 (L:D) h. The number of dead larvae or survivors that did not gain significant weight (< 0.1 mg per larva and still in first instar) was recorded on the 7th d after inoculation.

Larval Mortality on Bt Corn Leaf Tissue. A conventional non-Bt corn hybrid, DK 697 (Monsanto, St. Louis, MO), and a Bt corn hybrid, DKC69-70 (Monsanto, St. Louis, MO), were planted in a greenhouse at the Louisiana State University in Baton Rouge, LA, with the similar methods as described in Wu et al. (2007). DKC69-70 contains the Cry1Ab gene and is genetically similar to DK 697 hybrid. At the V6-V8 plant stage, fully-expanded leaves were

removed from corn plants and dissected into 7-cm in length small pieces. Larval survival of the *D. saccharalis* genotype populations was assayed on corn leaf tissue in the 8-well C-D international tray (Bio-Smart-8, C-D International, Pitman, NJ) using a similar method described in Huang et al. (2006). In the bioassays, six to eight pieces of corn leaf tissues were placed in each well of the 8-well trays containing approximately 10 ml of 2% solidified agar solution for maintaining a relatively high moisture level. Twenty-five neonates from each of the 10 genotype populations were placed in each well of the 8-well trays. There were four replications for each combination of insect population and corn hybrid. After insect infestation, bioassay trays were placed in an environmental chamber maintained at 28°C, 50% RH, and a photoperiod of 16:8 (L:D) h. Larval mortality was recorded on the 7th d after inoculation.

Larval Mortality on Intact Bt Corn Plants. Larval mortalities of the Cry1Ab-SS, Cry1Ab-RR, and a heterozygous (Cry1Ab-RS) population of *D. saccharalis* were evaluated in the greenhouse on seven commercial transgenic corn hybrids expressing the Cry1Ab protein and five conventional non-Bt corn hybrids. Detailed information on the corn hybrids, planting, insect infestation, and experimental design were described in Wu et al. (2007). For each test, 20 neonates of the three insect populations were manually infested on each plant at the vegetative (V7-V10) and reproductive (R1-R3) plant stages, respectively. Larval mortality was recorded at 21 d after insect infestation. For each plant stage, there were three replications for each combination of insect population and corn hybrid with three pots (or 6 to 9 plants) per replication. The experiment was repeated two times during 2005-2006. *D. saccharalis* survival among corn hybrids and plant stages was previously reported in Wu et al. (2007). In this paper, larval mortality data were pooled across all Bt corn hybrids and all non-Bt corn hybrids, respectively. The pooled mortality data were used to determine the dominance levels of the Cry1Ab resistance in *D. saccharalis* on intact Bt corn plants.

Data Analysis. A measurement of ‘practical mortality’ described in Huang et al. (2006) was used for analyzing the data obtained from the dose-response bioassays. Larval mortality at a specified Cry1Ab concentration was calculated based on the number of dead larvae plus the number of survivors that did not gain significant weight after 7 d for a total number of Bt affected larvae. This total of affected larvae was then divided by the total larvae assayed. Larval mortality was then corrected for control mortality on non-treated diet using the methods of Abbott (1925), followed by Probit Analysis (SAS Institute 2008) to calculate the lethal concentration that caused 50% mortality and the corresponding 95% confidence intervals (CI).

The maternal effect of Bt resistance in *D. saccharalis* was assessed by comparing the dose response curves (e.g. LC₅₀s, slopes) of the two F₁ populations from the reciprocal crosses. Significant differences in LC₅₀s between the two F₁ populations were determined using a lethal dose ratio test (Robertson and Preisler 1992). A similar dose response curve between the two F₁ populations would suggest non-sex linkage and an autosomal inheritance, while a significant difference indicates the resistance is sex-related.

Dominance levels of Bt resistance in *D. saccharalis* were estimated using two methods. The first method is the Stone’s dominance “D” value, which was calculated using the following formula described in Stone (1968).

$$D = \frac{2\log LC_{RS} - \log LC_{RR} - \log LC_{SS}}{\log LC_{RR} - \log LC_{SS}} \quad (1)$$

Where, LC_{RS}, LC_{SS} and LC_{RR} are the LC₅₀ values of the F₁ (heterozygous), Cry1Ab-SS and Cry1Ab-RR populations, respectively. LC₅₀ values used to compute the D value were obtained from the dose-response bioassays. The D value ranges from -1 to 1, a value of -1 indicating resistance is completely recessive; a value of 0 suggesting resistance is additive; and a value of 1 implying resistance is completely dominant. The LC₅₀ values of the two F₁ populations from

the reciprocal crosses between Cry1Ab-SS and Cry1Ab-RR populations did not differ significantly. Therefore, the LC₅₀ calculated based on the pooled bioassay data of the two F₁ populations was used to determine the dominance D value.

The second method is a single concentration method and is termed “effective dominance”, D_{ML} (Roush and McKenzie 1987, Bourguet et al. 2000), which can be calculated using the following formula:

$$D_{ML} = \frac{ML_{RS} - ML_{SS}}{ML_{RR} - ML_{SS}} \quad (2)$$

Where, ML_{RS} , ML_{SS} and ML_{RR} refer to the mortality levels of heterozygous, susceptible and resistant insects, respectively. D_{ML} ranges between 0 and 1. $D_{ML} = 0$ refers to a completely recessive resistance and $D_{ML} = 1$ means the resistance is completely dominant. In this study, D_{ML} was estimated using mortality measured for three procedures, (1) mortality at each of the seven Cry1Ab concentrations in the dose-response bioassays, (2) mortality observed on Bt corn leaf tissue, and (3) mortality on intact Bt plants.

Chi-square (χ^2) tests were used to determine if the observed mortality in the F₂ and backcross populations on Cry1Ab treated diet and on Bt corn leaf tissue fitted the single gene Mendelian model, respectively (Lande 1981, Tabashnik 1991).

$$\chi^2 = \frac{(O - E)^2}{np(1 - p)} \quad (3)$$

Where O is the observed number of dead larvae of the F₂ or backcross populations at a certain Cry1Ab concentration or Bt corn leaf tissue, E is the expected number of dead larvae, n is the number of total larvae tested and p is the expected mortality. The test statistic, χ^2 , was compared with a χ^2 -distribution with 1 degree of freedom. The null hypothesis will be rejected if the test

shows that $P < 0.05$ (Tabashnik 1991). Larval mortality of the pooled F_2 and pooled backcross populations observed at three Cry1Ab concentrations, 1, 4, and 16 $\mu\text{g/g}$, were used to fit the Mendelian monogenic model. At these concentrations, the three genotypes (Cry1Ab-SS, Cry1Ab-RR, Cry1Ab-RS) were best discriminated based on the dose response bioassays. In addition, the observed larval mortality of these pooled populations on Bt corn leaf tissue also were used to fit the monogenic model.

Results

Overall Response of Different Genotype Populations of *D. saccharalis* to Cry1Ab in Diet. The dose-mortality data of the 10 genotype populations of *D. saccharalis*, generally, fitted the probit models well ($P > 0.05$) (Table 5.1, Fig. 5.2). The LC_{50} values of the Cry1Ab-SS and Cry1Ab-RR populations were 0.18 $\mu\text{g/g}$ with a 95% CI of 0.15-0.21 $\mu\text{g/g}$ and 7.81 $\mu\text{g/g}$ with a 95% of 6.53-9.33 $\mu\text{g/g}$, respectively. The difference in the LC_{50} s of two parental populations was 43.4 - fold, which was significant ($P < 0.05$) based on the lethal dose ratio test (Robertson and Preisler 1992). The LC_{50} values of the two F_1 populations ranged from 0.39 to 0.51 $\mu\text{g/g}$, which was 1.7 to 2.8-fold greater ($P < 0.05$) than that of the Cry1Ab-SS population, but it was considerably smaller (≈ 17 -fold, $P < 0.05$) than that of the Cry1Ab-RR population. The LC_{50} s of the two F_2 populations were not significantly different based on the lethal dose ratio test (Robertson and Preisler 1992), with a pooled LC_{50} of 1.09 $\mu\text{g/g}$ and a 95% CI of 0.92-1.31 $\mu\text{g/g}$. The LC_{50} s of the four backcross populations ranged from 1.26 to 4.19 $\mu\text{g/g}$. The LC_{50} calculated based on the pooled data of the four backcross populations was 2.12 $\mu\text{g/g}$ with a 95% CI of 1.81-2.50 $\mu\text{g/g}$. Based on the non-overlapping within one standard error, the slopes of the dose-mortality relationships of the two parental populations were significantly greater than these of the other eight populations. Slopes of the two F_1 populations appeared to be somewhat greater than those of the F_2 and backcross populations.

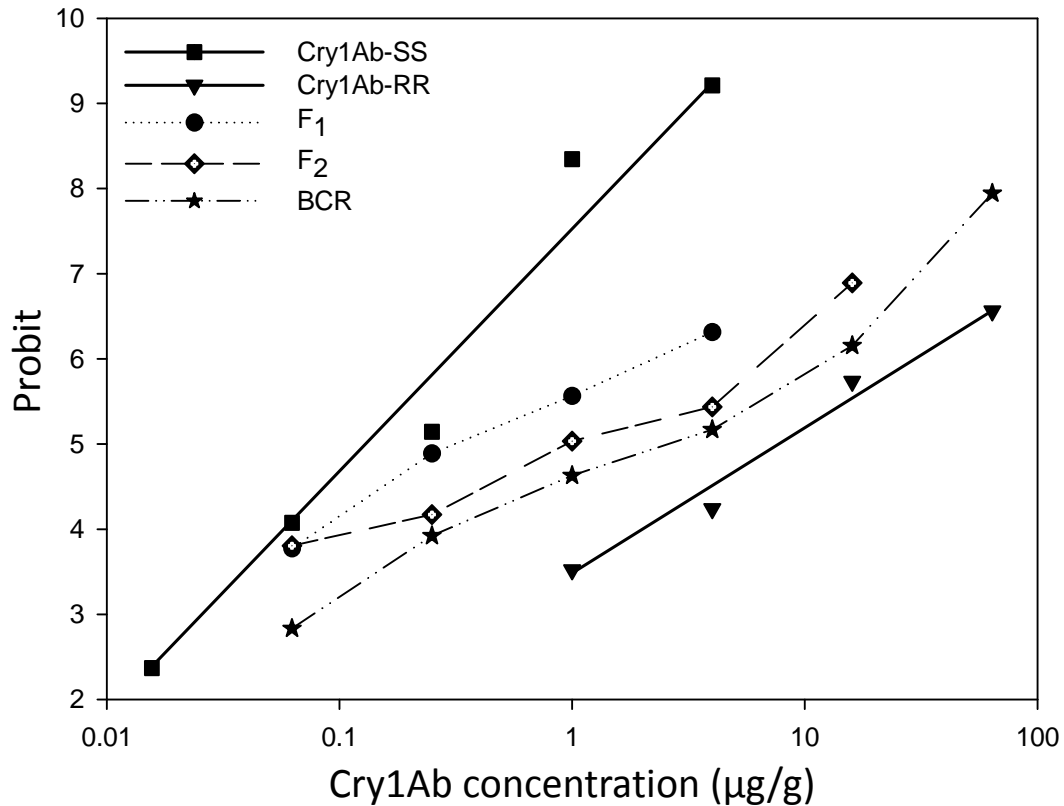


Figure 5.2. Dose-response of various genotype populations of *D. saccharalis* to Cry1Ab in diet. Cry1Ab-SS = parental Cry1Ab-susceptible strain, Cry1Ab-RR = parental Cry1Ab-resistant strain, F₁ = pooled response of the two parental reciprocal crosses between Cry1Ab-SS and Cry1Ab-RR strains, F₂ = pooled response of the sib-mating within each of the two F₁ genotypes, BCR = pooled responses of the four backcrosses between F₁ and Cry1Ab-RR populations.

Sex Linkage and Maternal Effects of Cry1Ab Resistance in *D. saccharalis*. The dose-mortality relationship curves were similar between the two F₁ populations (Table 5.1). The LC₅₀ values of the two F₁ populations were not different significantly ($P > 0.05$) based on the lethal dose ratio test (Robertson and Preisler 1992). The slopes of dose-response curves for the two F₁ populations also did not differ significantly based on the overlapping of the two slopes within their standard errors. Therefore, maternal effects and sex linkage of Cry1Ab resistance were not evident in this study. The results suggest that Cry1Ab resistance in *D. saccharalis* was autosomally inherited.

Table 5.1. Response of different genotype populations of *D. saccharalis* larvae to Cry1Ab toxin in diet.

Insect population*	n	Slope \pm SE	LC ₅₀ (95%CI) (μ g/g)	P-Value χ^2 test	Resistance factor [#]
Cry1Ab-SS	592	1.99 \pm 0.14	0.18 (0.15 – 0.21)	0.1162	1.0
Cry1Ab-RR	506	1.80 \pm 0.13	7.81 (6.53 – 9.33)	0.3292	43.4
F _{1a}	583	1.21 \pm 0.09	0.51 (0.40 - 0.64)	0.6735	2.8
F _{1b}	532	1.40 \pm 0.14	0.30 (0.22 - 0.40)	0.0962	1.7
Pooled F ₁	1115	1.33 \pm 0.07	0.38 (0.33 - 0.45)	0.1232	2.1
F _{2a}	760	1.03 \pm 0.06	1.09 (0.86 – 1.40)	0.2234	6.1
F _{2b}	675	1.05 \pm 0.07	1.07 (0.83 - 1.39)	0.6284	5.9
Pooled F ₂	1435	1.07 \pm 0.05	1.09 (0.92 - 1.31)	0.5403	6.1
Backcross A	562	1.16 \pm 0.08	1.26 (0.98 - 1.61)	0.1046	7.0
Backcross B	539	1.02 \pm 0.09	1.82 (1.40 - 2.39)	0.4258	10.1
Backcross C	765	1.24 \pm 0.08	2.27 (1.84 – 2.86)	0.3080	12.6
Backcross D	634	1.50 \pm 0.11	4.29 (3.50 - 5.35)	0.5829	23.8
Pooled backcross	2500	1.13 \pm 0.05	2.12 (1.81 - 2.50)	0.0001	11.8

* F_{1a} = F₁ progeny of Cry1Ab-RR male and Cry1Ab-SS female; F_{1b} = F₁ progeny of Cry1Ab-RR female and Cry1Ab-SS male; F_{2a} = progeny of sib-mating within F_{1a}; F_{2b} = progeny of sib-mating within F_{1b}; Backcross A = progeny of F_{1a} male \times Cry1Ab-RR female; Backcross B = progeny of F_{1a} female \times Cry1Ab-RR male; Backcross C = progeny of F_{1a} male \times Cry1Ab-RR female; and Backcross D = progeny of F_{1a} female \times Cry1Ab-RR male.

Resistance ratio was calculated using the LC₅₀ value of the tested population divided by the LC₅₀ of the Cry1Ab-SS strain.

Dominance Levels of Cry1Ab Resistance in *D. saccharalis* Estimated Using LC₅₀

Values of Dose-Response Bioassays. The LC₅₀ of the pooled F₁ population was significantly ($P < 0.05$) lower than that of the Cry1Ab-RR strain but it was significantly ($P < 0.05$) greater than that of the Cry1Ab-SS strain based on the lethal dose ratio test (Robertson and Preisler 1992). The results suggest that the resistance was neither completely dominant nor completely recessive. The calculated dominance D value based on Stone's method (equation 1) (Stone 1968) was -0.60, suggesting that the Cry1Ab resistance in *D. saccharalis* was incompletely recessive.

Effective Dominance (D_{ML}) of Cry1Ab Resistance in *D. saccharalis* at Single Cry1Ab Concentrations. The effective dominance, D_{ML} , calculated based on the mortality observed from single Cry1Ab concentrations of the dose-response bioassays using the equation 2 was concentration-dependent (Table 5.2). D_{ML} values decreased as Cry1Ab concentration increased. For example, D_{ML} was 0.84 at 0.016 µg/g, indicating a nearly completely dominant inheritance at this low concentration, while it was 0.03 at 16 µg/g and became zero when Cry1Ab concentration increased to 64 µg/g, suggesting the resistance was completely or nearly completely recessive at these two higher concentrations. D_{ML} ranged from 0.38 to 0.10 when Cry1Ab concentrations increased from 0.062 to 4 µg/g, indicating that resistance was incompletely recessive between the two concentrations.

Effective Dominance (D_{ML}) of Cry1Ab Resistance in *D. saccharalis* Estimated on Bt Corn Leaf Tissue and Intact Bt Corn Plants. Effective dominance, D_{ML} , calculated based on larval mortality of Cry1Ab-SS, Cry1Ab-RR, and the pooled F₁ populations on Bt corn leaf tissue was 0.26, suggesting that Cry1Ab resistance was incompletely recessive (Table 5.2). D_{ML} measured based on larval mortality on intact Bt plants was 0.24 at the vegetative plant stages and 0.26 at the reproductive plant stages for the 2006 trial, also suggesting an incompletely recessive

resistance. The resistance appeared to be more recessive according to the data observed from the 2005 trial with a D_{ML} value of 0.10 at the vegetative plant stage and 0.08 at the reproductive plant stage.

Testing for Fitting the Mendelian Monogenic Model. χ^2 tests showed that the observed mortality at the three Cry1Ab concentrations (1, 4, and 16 $\mu\text{g/g}$) fitted well for both the backcross and F_2 populations (Table 5.3). The observed mortality of the two populations at each of the three Cry1Ab concentrations was not significantly different ($P > 0.05$), compared to the expected values calculated based on the Mendelian single gene model. These results suggest that the Cry1Ab resistance in the *D. saccharalis* population was controlled by a single gene. In addition, the observed mortality on Cry1Ab corn leaf tissue also fitted the Mendelian monogenic model well for both the F_2 and backcrossed populations ($P > 0.05$) (Table 5.3). The results observed from Bt corn leaf tissue confirmed that one gene (or a few linked genes) influenced Cry1Ab resistance in this *D. saccharalis* strain.

Discussion

Larval mortality of the Cry1Ab-SS, Cry1Ab-RR, and populations from various crosses on Cry1Ab diet, Bt corn leaf tissue, and on Bt corn plants showed that Cry1Ab resistance in *D. saccharalis* was controlled by a single autosomal gene. The dominance levels of the resistance were dose-dependent. On Cry1Ab-treated diet, resistance, in most cases, was incompletely recessive. However, at low Cry1Ab concentrations (e.g. 0.062 $\mu\text{g/g}$), resistance was nearly completely dominant, while it was completely recessive at high concentrations (e.g. 64 $\mu\text{g/g}$). In testing using Bt corn leaf tissue or intact Bt corn plants, the Cry1Ab resistance was found to be incompletely or nearly completely recessive.

Table 5.2. Effective dominance (D_{ML}), of Cry1Ab resistance in *D. saccharalis* in three test procedures.

Testing material	Cry1Ab concentration ($\mu\text{g/g}$) or corn stage	Effective dominance (D_{ML})	Result
Purified Cry1Ab toxin in diet	0.016	0.84	Near a completely dominant
	0.062	0.38	Incompletely recessive
	0.25	0.17	Incompletely recessive
	1.0	0.29	Incompletely recessive
	4.0	0.10	Nearly completely recessive
	16.0	0.03	Nearly completely recessive
	64.0	0	completely recessive
Bt corn leaf tissue	Vegetative plant stages	0.26	Incompletely recessive
Intact Bt corn plants *	Vegetative, 2005	0.10	Nearly completely recessive
	Reproductive, 2005	0.08	Nearly completely recessive
	Vegetative, 2006	0.24	Incompletely recessive
	Reproductive, 2006	0.26	Incompletely recessive

* For the trial in 2005, neonates of *D. saccharalis* were infested at the V7-V8 (vegetative) and R1 (reproductive) plant stages, respectively, while for the trial in 2006, insects were infested at the V9-V10 (vegetative) and R1-R3 (reproductive) plant stages, respectively.

Table 5.3. Testing for fitting the Mendelian monogenic model for Cry1Ab resistance in *D. saccharalis* *

Test	Cry1Ab concentration ($\mu\text{g/g}$)	Pooled F ₂					Pooled backcross				
		n	No. dead larvae		χ^2 test		n	No. dead larvae		χ^2 test	
			Expected	Observed	χ^2	P-value		Expected	Observed	χ^2	P-value
Purified Cry1Ab in diet	1	230	104.5	115.6	2.18	0.1398	503	194.7	186.3	0.59	0.4424
	4	244	145.0	158.0	2.89	0.0891	508	289.3	275.9	1.44	0.2301
	16	243	210.8	218.7	2.22	0.1362	505	431.3	435.3	0.26	0.6101
Bt corn leaf tissue	n/a	200	153.1	164.7	3.75	0.0528	400	250.4	261.5	1.30	0.2542

* Observed larval mortality was corrected for control mortality on non-Bt diet or non-Bt corn leaf tissue.

Inheritance of Bt resistance in insects has been assessed in many agriculturally important insect pests including several target pest species of transgenic Bt cotton and Bt corn. In most cases, a high level of resistance was controlled by a single (or a few) autosomal gene (Heckel 1994, Tabashnik et al. 1997, Bourguet et al. 2000, Ferré and Van Rie 2002, Alves et al. 2006, Tabashnik and Carrière 2007). However, the predicted dominance levels of resistance are often dependent on assay methods, testing materials, and toxin concentrations (Liu and Tabashnik 1997, Bourguet et al. 2000, Liu et al. 2001, Tabashnik and Carrière 2007). For example, resistance of the pink bollworm, *Pectinophora gossypiella* (Saunders), a key target pest of Bt cotton in the southwestern United States and some areas of China and India, to Cry1Ac was controlled by a single or a few autosomal genes (Liu et al. 2001, Tabashnik et al. 2002). The dominance level of Cry1Ac resistance in *P. gossypiella* was toxin concentration dependent. Similar results were also found in the tobacco budworm, *Heliothis virescens* (F.), the primary target of Bt cotton in the United States (Gould et al. 1992, 1995) and in the cotton bollworm, *Helicoverpa armigera* (Hübner), the primary target pest of Bt cotton in Australia, China, and India (Kranthi et al. 2005, Liang et al. 2008). The results of the current study with *D. saccharalis* resistance to Cry1Ab toxin appear to agree with the results of these previous studies. However, Bt resistance in *O. nubilalis*, the primary target of Bt corn in the United States, appears to have a different genetic basis. Two laboratory selected strains of *O. nubilalis* with >1000-fold resistance to Cry1Ab were inherited polygenically (Alves et al. 2006). A Dipel[®]-resistant strain of *O. nubilalis* exhibited inheritance as an incompletely dominant gene when it was measured using Stone's method (Huang et al. 1999). None of the *O. nubilalis* Bt resistant strains have survived on commercial Cry1Ab corn plants (Huang et al. 2002, Bourguet 2004).

Results of this study provide additional information that should be considered when developing new Bt corn technologies and improving IRM plans for managing *D. saccharalis* in the mid-southern region. *D. saccharalis* is naturally more tolerant to Cry1Ab proteins compared to other corn stalk borer pests (Huang et al. 2006). The relatively lower sensitivity coupled with the incompletely recessive resistance trait to Bt proteins in *D. saccharalis* might enhance the ability of resistant heterozygotes to survive on some plant tissues with lower levels of Bt expression, especially for those plants expressing only a marginal dose to kill this corn borer species (Castro et al. 2004, McAllister et al. 2004, Wu et al. 2007). The varied dominance levels of Cry1Ab resistance in *D. saccharalis* on Bt plants suggest that Bt corn hybrids must express a high enough dose of Bt proteins to make the resistance functionally recessive. Resistant heterozygotes should be killed by the high-dose expressed in plants for the success of the “high/dose refuge” IRM strategy for Bt corn.

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CHAPTER 6

SUMMARY AND CONCLUSIONS

Transgenic *Bacillus thuringiensis* (Bt) corn is the primary tool for managing corn stalk borers across the United States including Louisiana. The rapid adoption of Bt corn necessitates implementation of an effective insecticide resistance management (IRM) plan to ensure the long-term success of Bt corn for suppressing corn borer populations. The currently adopted “high dose/refuge” IRM strategy for Bt corn was developed primarily based on the information generated from the European corn borer, *Ostrinia nubilalis* (Hübner), and the southwestern corn borer, *Diatraea grandiosella* Dyar. These two species are the most economically important corn borer pests in the North Central and Midwestern US Corn Belts. Recent studies have shown that management of resistance of the sugarcane borer, *Diatraea saccharalis* (F.) (Lepidoptera: Crambidae), to Bt corn in Louisiana and other areas of the mid-southern region of the United States is more important than that proposed for *O. nubilalis* and *D. grandiosella*. A recent survey of Louisiana corn stalk borer species found that *D. saccharalis* has been the dominant corn borer species in the state and the central gulf costal areas of Texas. Major resistance alleles to Bt corn have been found in three *D. saccharalis* populations collected from northeast and central Louisiana. This resistant *D. saccharalis* strain can survive and complete larval development on intact Bt corn plants. Previous studies on Bt resistance management generally have been focused on *O. nubilalis* and *D. grandiosella*. Research on Bt resistance in *D. saccharalis* has been limited. Information is needed for validating or improving the current Bt corn resistance management plan for *D. saccharalis*. In this study, four objectives were accomplished, which will provide essential information for addressing the concern of Bt resistance management in *D. saccharalis*.

Performance of transgenic Bt corn hybrids against Cry1Ab-susceptible and -resistant *D. saccharalis* was evaluated in the greenhouse to determine if Bt corn varieties commonly planted in Louisiana qualify as high dose as required for the current “high dose/refuge” IRM strategy for Bt corn. Larval survival of Cry1Ab-resistant, -susceptible, and -heterozygous genotypes of *D. saccharalis* was examined on vegetative and reproductive stages of five non-Bt and seven Bt field corn hybrids. Larval survival was recorded 21 days after infestation of neonates on plants. During the vegetative stages, all seven Bt corn hybrids were highly effective against Cry1Ab-susceptible and -heterozygous genotypes of *D. saccharalis*, while 8 -18% of the heterozygous genotype survived on reproductive stage plants for four Bt corn hybrids. This study demonstrated that the Cry1Ab-resistant strain of *D. saccharalis* can survive on commercial Bt corn hybrids recommended in Louisiana. The results suggest that a “high dose” Bt corn for *O. nubilalis* and *D. grandiosella* may not qualify as “high dose” for *D. saccharalis*. Furthermore, a “high dose” expressed by a Bt corn hybrid during vegetative plant stages may not produce a “high dose” during reproductive stages. Variability in performance of different Bt corn hybrids and in different plant growth stages within the same Bt corn hybrid also suggests a requirement to validate “high dose” qualification against *D. saccharalis* for each Bt corn hybrid, at different plant stages, and even perhaps in different environments.

Susceptibility of Cry1Ab-resistant and -susceptible *D. saccharalis* to four individual Bt toxins was assayed in the laboratory to assess the cross-resistance pattern and generate information needed for developing new Bt corn for managing *D. saccharalis*. Larval growth and mortality of Cry1Ab-susceptible and -resistant strains were evaluated on meridic diet containing one of the four selected Bt proteins: Cry1Aa, Cry1Ac, Cry1A.105, and Cry2Ab2. Cry1Aa was the most toxic protein against both insect strains, followed in decreasing potency by Cry1A.105,

Cry1Ac, and Cry2Ab2. Using practical mortality (larvae either died or no significant weight gain 7 days after infestation), the median lethal concentration (LC₅₀) of the Cry1Ab-resistant strain was estimated to be >80-, 45-, 4.1-, and -0.5-fold greater than that of the susceptible strain to Cry1Aa, Cry1Ac, Cry1A.105 and Cry2Ab2 proteins, respectively. The results showed that a Cry1Ab-resistant strain of *D. saccharalis* was also resistant to Cry1Aa and Cry1Ac, whereas it demonstrated relatively low cross-resistance to the Cry1A.105 protein and no cross-resistance to Cry2Ab2. Genes encoding for Bt Cry1A.105 and Cry2Ab2 proteins have been transferred into corn hybrids with a novel method called Vector-Stack transformation, resulting in the MON 89034 event. This event includes both proteins expressed at high levels. The results from this study provide some evidence that Bt corn with the stacked genes of Cry1A.105 and Cry2Ab2 may provide a means to delay resistance development in *D. saccharalis*. In addition, the high effectiveness of Cry1Aa against both the Cry1Ab-SS and Cry1Ab-RR strains indicates that the Cry1Aa protein may be a good candidate for use in Bt corn to manage *D. saccharalis*.

Relative fitness of Cry1Ab-susceptible and -resistant *D. saccharalis* on a meridic diet and conventional corn plants was examined in the laboratory and greenhouse to determine if fitness costs are associated with Cry1Ab resistance in *D. saccharalis*. Relative fitness on non-toxic diet, diet treated with three low concentrations (0.01, 0.05, and 0.1 µg/g) of Cry1Ab toxin, and on conventional corn plants was compared for five genotypes of *D. saccharalis* including a Cry1Ab-susceptible strain, a Cry1Ab-resistant strain, a backcross-reselected resistant strain, and two F₁ progeny of the susceptible and backcross-reselected strains. Biological parameters measured included neonate-to-pupa development time and pupation rate, larval survival, larval and pupal weight, and sex ratio. Larvae of the Cry1Ab-susceptible and backcross-reselected strains on non-toxic diet and non-Bt corn plants grew normally and not significantly different

between the two strains in all measured parameters, suggesting a lack-of-fitness cost of Cry1Ab resistance in *D. saccharalis*. Larval growth and larval survival of Cry1Ab-susceptible strain were significantly affected on diet treated with Cry1Ab toxin at 0.05 and 0.1 $\mu\text{g/g}$, while the effect of Cry1Ab resistant larvae was less significant. The lack-of-fitness costs of Bt resistance in *D. saccharalis* imply a greater challenge in managing Bt resistance for this corn borer species.

Inheritance of Cry1Ab resistance in *D. saccharalis* was characterized using various genetic crosses to verify if Bt resistance is controlled by a recessive gene as defined in the “high dose/refuge” IRM strategy for Bt corn. These genetic crosses included reciprocal parental crosses between Cry1Ab-susceptible and -resistant strains, F_1 by F_1 crosses, and backcrosses of F_1 with the Cry1Ab resistant population. Larval mortality of the parental and cross-populations was determined on Cry1Ab diet, Bt corn leaf tissue, and intact Bt corn plants. Maternal effects and sex linkage were examined by comparing the larval mortality between the two F_1 populations. Dominance levels of resistance were measured by comparing the larval mortality of the Cry1Ab-resistant, -susceptible, and -heterozygous populations. Number of genes associated with the resistance was determined by fitting the observed mortality of backcross and F_2 populations with Mendelian monogenic inheritance model. Cry1Ab resistance in *D. saccharalis* was inherited as a single autosomal gene. The resistance was incompletely or nearly completely recessive on Bt corn leaf tissue and intact Bt corn plants, while the dominance increased as Cry1Ab concentrations decreased using on diet treated with Cry1Ab toxin. The varied dominance levels of Cry1Ab resistance in *D. saccharalis* suggest that Bt corn hybrids must express a sufficient dose of Bt proteins to make the resistance functionally recessive. Thus, resistant heterozygous individuals will be killed as required in the “high dose/ refuge” resistance management strategy for Bt corn.

Results from this study will provide valuable information in understanding Bt resistance mechanisms and development of effective strategies for managing *D. saccharalis* resistance to Bt corn.

APPENDIX

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Fax: 301-731-4538
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A557 Life Sci Bldg

Dept of Entomology, LSU

Baton Rouge, LA 70803

Phone: 225-578-3265

xwu3@lsu.edu

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PhD Candidate
Dept of Entomology, LSU
Baton Rouge, LA 70803 USA
Cellphone: 225-454-9152
xwu3@lsu.edu
<http://home.paws.lsu.edu/xwu3:paws.lsu.edu/>

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VITA

Xiaoyi Wu is the eldest son of Mingxing Wu and Maobi Liu. He was born in October, 1970, in Kaijiang, Sichuan Province, China. Mr. Wu was married to Huifang Wang and they have a lovely daughter, Wenqi Wu, eight years old. Mr. Wu earned his Bachelor of Science (plant pathology) and Master of Science (entomology) from Nanjing Agricultural University, Nanjing, Jiangsu, China, during 1994 and 1997, respectively. After graduation, Mr. Wu served in the Jiangsu Agriculture and Forestry Department as a plant protection specialist for more than seven years (Aug 1997 to Dec 2004). Mr. Wu enrolled into graduate studies under the supervision of Dr. Fangneng Huang in the Department of Entomology at Louisiana State University and Agricultural and Mechanical College in 2005. He currently is a doctoral candidate in the Department of Entomology.