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Resistance risk assessment of *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) to Cry1F protein from *Bacillus thuringiensis* Berliner in Brazil

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Thesis submitted in order to obtain the degree of Doctor in Science. Area: Entomology

Piracicaba
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ABSTRACT

Resistance risk assessment of *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) to Cry1F protein from *Bacillus thuringiensis* Berliner in Brazil

The event TC1507 maize with *cry1F* gene from the bacterium *Bacillus thuringiensis* Berliner (Bt) was approved for commercial release in Brazil in 2008. The evolution of pest resistance to Bt plants has been a great concern to preserve the lifetime of this technology. Therefore, in this study we assess the risk of evolution of resistance to Cry1F protein in *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) populations from major maize-growing regions in Brazil. The baseline susceptibility to Cry1F was detemined with diet overlay bioassay for susceptible reference population and four field populations of *S. frugiperda*. Then, we monitored 43 populations of *S. frugiperda* sampled in nine different States of Brazil during 2010/2011, 2011/2012 and 2012/2013 crop seasons. Only 4-fold variation in susceptibility to Cry1F was detected among *S. frugiperda* from field populations in the baseline susceptibility study. Diagnostic concentration of 2,000 ng cm^{-2} was defined for monitoring the susceptibility to Cry1F in *S. frugiperda* populations. Survival at 2,000 ng cm^{-2} of Cry1F protein increased significantly throughout crop seasons in populations from São Paulo, Santa Catarina, Rio Grande do Sul, Bahia, Mato Grosso, Goiás, Mato Grosso do Sul, and Paraná, but not in Minas Gerais. We also sampled a population of *S. frugiperda* in TC1507 field failures in Bahia in October, 2011. This population was selected in laboratory with Cry1F protein up to 20,000 ng cm^{-2} and the resistance ratio of the selected resistant population (BA25R) was > 5,000-fold. This resistant population was able to survive in Cry1F maize from neonate till pupa and produce normal adult. The inheritance of *S. frugiperda* resistance to Cry1F protein was autosomal. To test the functional dominance, neonate larvae obtained from the cross of resistant and susceptible populations were tested in leaf bioassay, and around 8% of heterozygotes were able to survive and complete the larval development and produce normal adults on TC1507 leaves while susceptible larvae could not survive for up to five days after infestation. Dominance was estimated to be 0.15 ± 0.09, suggesting that resistance to Cry1F in TC1507 maize was incompletely recessive. We also conducted resistance selection studies in other seven *S. frugiperda* populations from six different Brazilian states to test whether the resistance alleles were at same locus or not. The F_1 larvae obtained from the cross between resistant population (BA25R) and each of the seven selected resistant populations were able to survive at 2,000 ng cm^{-2} of Cry1F protein in diet bioassay, and therefore they shared the same locus of resistance to Cry1F protein. We estimated the frequency of resistance allele to Cry1F protein in populations of *S. frugiperda* of main crop season 2011/2012 from five states. We stablished 517 isofemale lines using F_2 screen method. The total frequency of Cry1F resistance allele in Brazil was 0.088 with 95% confidence interval between 0.077 and 0.100. Based on results obtained in this study, the risk of resistance evolution to Cry1F protein by *S. frugiperda* is high in Brazil.

Keywords: Fall armyworm; *Bacillus thuringiensis*; Resistance management; TC1507; High-dose; Genetic basis

RESUMO

Avaliação do risco de resistência de *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) à proteína Cry1F de *Bacillus thuringiensis* Berliner no Brasil

O evento de milho TC1507 com gene *cry1F* da bactéria *Bacillus thuringiensis* Berliner foi aprovado comercialmente no Brasil em 2008. A evolução da resistência de pragas a plantas Bt tem sido uma grande preocupação na preservação desta tecnologia. Portanto, neste estudo foi avaliado o risco de evolução da resistência à proteína Cry1F em populações de *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) das principais regiões de cultivo de milho no Brasil. A linha-básica de suscetibilidade à proteina Cry1F foi determinada em bioensaio de aplicação superfícial na dieta para a população suscetível de referência e quatro populações de campo de *S. frugiperda*. Posteriormente, a suscetibilidade a Cry1F foi monitorada em 43 populações de *S. frugiperda* coletadas em nove Estados do Brasil nas safras agrícolas de 2010/2011, 2011/2012 e 2012/2013. A variação na suscetibilidade foi de apenas quatro vezes para Cry1F entre as populações de campo na linha-básica de suscetibilidade. A concentração diagnóstica de 2.000 ng cm^{-2} de proteína Cry1F foi definida para o monitoramento da suscetibilidade. A sobrevivência em 2.000 ng cm^{-2} de proteína Cry1F aumentou significativamente no decorrer das safras em populações de São Paulo, Santa Catarina, Rio Grande do Sul, Bahia, Mato Grosso, Goiás, Mato Grosso do Sul e Paraná, mas não em Minas Gerais. Além disso, uma população de *S. frugiperda* foi coletada em milho TC1507 com falha de controle na Bahia em outubro de 2011. Esta população foi selecionada no laboratório com a proteína Cry1F até 20.000 ng cm^{-2}, obtendo-se uma população resistente (BA25R) com razão de resistência >5000 vezes. Esta população resistente foi capaz de sobreviver no milho TC1507 desde larva neonata até a fase de pupa e com emergência de adultos normais. O padrão de herança da resistência de *S. frugiperda* a Cry1F foi autossômica. Para testar a dominância funcional, as larvas neonatas do cruzamento entre a população resistente e suscetível foram testadas em folhas do evento TC1507 e cerca de 8% dos heterozigotos foram capazes de sobreviver, completar o desenvolvimento e produzir adultos normais, enquanto as larvas da linhagem suscetível não sobreviveram por mais de cinco dias após a infestação. A dominância foi estimada em 0,15 ± 0,09; portanto, a resistência à proteína Cry1F no milho TC1507 foi incompletamente recessiva. A resistência foi selecionada para outras sete populações de seis Estados brasileiros para testar se os alelos de resistência estavam no mesmo locus. As larvas F_1 obtidas do cruzamento entre a população resistente (BA25R) e cada uma das sete populações selecionadas sobreviveram na concentração de 2,000 ng cm^{-2} de proteína Cry1F e, portanto, essas populações compartilharam o mesmo locus de resistência à proteína Cry1F. A freqüência do alelo resistente à proteína Cry1F foi estimada em populações de *S. frugiperda* coletadas em cinco Estados na safra 2011/2012. Foram estabelecidas 517 isolinhas utilizando o método de "F_2 screen". A freqüência total do alelo de resistência à proteína Cry1F no Brasil foi de 0,088, com intervalo de confiança de 95% entre 0,077 e 0,100. Com base nos resultados, o risco de evolução da resistência à proteína Cry1F por *S. frugiperda* é elevada no Brasil.

Palavras-chave: Lagarta-do-cartucho; *Bacillus thuringiensis*; Manejo da resistência; TC1507; Alta-dose; Base genética

1 INTRODUCTION

Genes c*ry* from bacterium *Bacillus thuringiensis* Berliner have been introduced in some plants to control insect pests in agriculture (SOBERÓN; GILL; BRAVO, 2009; JAMES, 2011). Cry proteins are produced continuously in tissues of Bt plants and protecting from degradation by environmental factors and increasing the insect control. Bt plants revolutionized the agriculture by replacing insecticides and reducing environmental impact (SOBERÓN; GILL; BRAVO, 2009). The event TC1507 maize with *cry1F* gene from *B. thuringiensis* that codes for Cry1F protein has been approved for commercial release in Brazil in 2008.

One of the target pests of the event TC1507 is the fall armyworm *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), a serious insect pest of maize in tropical region of America (ETCHEVERRY, 1957; ESTRADA, 1960; HOROVITZ, 1960; SIFUENTES, 1967; CRUZ, 1995). Many economically important crops such as cotton, rice, sugarcane, beans, maize, wheat, sorghum and soybeans are attacked by *S. frugiperda* (SILVA et al., 1968). There is a high genetic variability in this species (NAGOSHI; MEAGHER, 2008) and two host races have already been characterized (PASHLEY, 1986; BUSATO et al., 2004). Before releasing of Bt maize, *S. frugiperda* was controlled mainly with the use of insecticides in Brazil, but with low efficacy of this strategy because of high and multiple infestations of this species in different crops (SILVA, 1999) and evolution of resistance to major insecticides (DIEZ-RODRIGUEZ; OMOTO, 2001; CARVALHO et al., 2013). With the approval of Bt maize in Brazil, this technology has been an excellent option for *S. frugiperda* control.

The continuous expression of *cry* genes in Bt plants entails in strong selection for resistance in insect pest (McGAUGHEY; WHALON, 1992). Field evolution of resistance to Bt plants has been reported in many countries (VAN RENSBURG, 2007; STORER et al., 2010; DHURUA; GUJAR, 2011; GASSMANN et al., 2011), including *S. frugiperda* to Cry1F in Puerto Rico (STORER et al., 2010). To confirm the resistance, resistant insects to Bt crop should have the ability to survive on Bt plant, from egg to adult stage and produce viable offspring (ANDOW, 2008). But to prove that field failures are caused by resistance, it is necessary to show more details. The first step is show economic control of the insect by Bt crop. For example, Cry1F maize hybrids showed excellent control of *S. frugiperda* in the released commercial period (SIEBERT et al., 2008). The second step is necessary to show

economic damage in the Bt crop. There were reports in Puerto Rico about the unusual damage caused by *S. frugiperda* to Cry1F maize hybrids (STORER et al., 2010). The third step, the resistant insect needs to have the ability to survive in Bt plant. Resistant *S. frugiperda* was not showed to survive in Cry 1F maize yet, but resistant *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) larvae were able to survive in Cry1Ab maize (VAN RENSBURG, 2007). The last step, the resistance needs to be genetically inherited. *S. frugiperda* resistant from Puerto Rico showed inheritance autosomal, and incomplete recessive to Cry1F protein (STORER et al., 2010).

Evolution of resistance is one of major limitations to continued use of Bt crops under field conditions. The main goal of resistance management strategy is to delay or prevent the occurrence of field failures (ANDOW, 2008). High-dose/refuge is the main strategy to prevent Bt field failures. This strategy is a combination of high-dose plants and refuge areas (SHELTON et al., 2000). High-dose is the protein concentration sufficiently high to make the resistance functionally recessive (TAYLOR; GEORGHIOU, 1979; GOULD, 1998). Refuge is non-Bt field that provide susceptible individuals for mating with resistant (McGAUGHEY; WHALON, 1992; SHELTON et al., 2000). Besides, the initial frequency of allele resistance should be less 1×10^{-3} (ROUSH, 1994). If at least one these assumptions fail, resistance evolution will be faster. Besides, others parameters can have influence on evolution of resistance.

High-dose is a measure of dominance that is estimated by similarity of the heterozygote in relation of two homozygotes resistant (WRIGHT, 1929). Therefore, high-dose is the capacity of Bt event to kill all or nearly all heterozygous insect (TABASHNIK; CROFT, 1982; GOULD, 1998; TABASHNIK et al., 2004). Mathematical models have shown slower resistance evolution in high-dose events than low-dose (TABASHNIK; CROFT, 1982; TABASHNIK et al., 2004). Typically dominance has been measure at a fixed mortality, normally 50% or at a given Bt protein concentration (STONE, 1968; CURTIS; COOK; WOOD, 1978). The two ways do not have a huge importance for evolution of resistance. The best way is measure the fitness of three genotypes at plant concentration that is most relevant for resistance evolution (BOURGUET; GENISSEL; RAYMOND, 2000). Many methods have been proposed to estimate the high-dose concept in absence of a resistant population. The most accepted is based on dilution of plant tissue at 25-fold in artificial diet. In this dilution, mortality should be higher than 99% of susceptible

individuals at seven days after infestation of neonate larvae (US-EPA, 1998). Caprio; Sumerford and Sims (2000) suggested being 50-fold. Few works has showed the dominance with plant dilutions. Cry1Ac soybean events TIC107 and MON 87701 × MON 89788 showed to be high-dose for *Anticarsia gemmatalis* Hübner (Lepidoptera: Noctuidae), but were not able to kill all susceptible larvae of *Pseudoplusia includens* (Walker) (Lepidoptera: Noctuidae) in 25-fold tissues dilution, and therefore these events cannot be considered high-dose for this species (MacRAE et al., 2005; BERNARDI et al., 2012).

Resistance management strategies can be more accurately designed if the genetic basis of resistance to Bt crops are better understood (GOULD, 1998; FERRÉ; VAN RIE, 2002). Characterization of resistance to Cry proteins has shown dominance from recessive to incompletely recessive, and genes in autosomal chromosome (MAHON et al., 2007; PEREIRA; STORER; SIEGFRIED, 2008; PETZOLD-MAXWELL et al., 2012; ZHANG et al., 2012). *S. frugiperda* resistant to Cry1F from Puerto Rico was incomplete recessive and autosomal (STORER et al., 2010). In general, resistance to Cry protein has been determined as monogenic (TABASHNIK et al., 1997a; MAHON et al., 2007; PEREIRA; STORER; SIEGFRIED, 2008). Additionally, resistant populations could share the same resitant locus for the same Bt protein in many insect species (TABASHNIK et al., 1997b; TABASHNIK et al., 2004; MAHON; OLSEN; DOWNES, 2008; MAHON et al., 2010; FABRICK; TABASHNIK, 2012; MAHON; DOWNES; JAMES, 2012; ZHANG et al., 2012).

The key element to predict the rate of evolution of resistance is the frequency of resistance allele (FERRÉ; VAN RIE, 2002). The method called F_2 screen can detect the resistance allele even in low frequency (ANDOW; ALSTAD, 1998). This method is based on maintenance of genetic variation in isofemale line, and the resistance alleles are concentrating in homozygote genotype, where they are detected. The limitation of this method is labor intensive and expensive rearing requirements. Besides, this method is useful to determine the frequency of the resistance allele in order to evaluate of resistance management strategies (SIEGFRIED et al., 2007). Frequency of resistance allele to Bt proteins has been estimated by using F_2 screen in many insect species. In *Diatraea saccharalis* (Fabricius) (Lepidoptera: Crambidae) in U.S.A., the frequency of major resistance allele to Cry1Ab protein was estimated from 0.0023 to less than 0.0027 (HUANG; LEONARD; ANDOW, 2007; HUANG et al., 2008; HUANG et al., 2009).The estimated

frequency in *H. armigera* was 0.0146 to 0.052 to Cry1Ac in China (XU et al., 2009; ZHANG et al., 2012), and 0.0006 in Australia (DOWNES; MAHON; OLSEN, 2007).

The main field-evolved resistance was reported in Puerto Rico for *S. frugiperda* to Cry1F maize. After four years from commercial release, the the commercial sale of Cry1F maize in Puerto Rico was suspended. Evolution of resistance to Cry1F in Puerto Rico may have been due to the isolation of the island, tropical climate, high pest population and drought conditions in 2006/2007 crop season, which reduced the availability of alternative hosts for *S. frugiperda* (STORER et al., 2010). Most of Brazilian agriculture regions have similar climate conditions from Puerto Rico that allowed to cultivate crops all year round (STORER et al., 2012), and this scenario increases the risk of field evolution of resistance. In the Central-west and Western Bahia regions in Brazil, the winter season is dry and hot, but crops, including maiz, can be cultivated under center pivot irrigation systems. In this agricultural scenario, *S. frugiperda* is able to have continuous generations throughout the year which increases the pest problem (MARTINELLI et al., 2006; 2007). In Southern Brazil, despite cold winters, rainfall allows crop production during the winter in some regions, which again enables *S. frugiperda* to build up high population densities at the beginning of the summer season. These conditions that favor year-round use of Bt maize in Brazil, the risk of resistance evolution of *S. frugiperda* to Bt maize is high. Therefore, to assess the resistance risk of *S. frugiperda* to Cry1F and implement an Insect Resistance Management (IRM) program in Brazil, the major goals of this research were:

- To establish the baseline susceptibility and to monitor Cry1F protein susceptibility in Brazilian populations of *S. frugiperda* collected from different locations and crop seasons;

- To demonstrate that field failures in TC1507 event in Brazil were associated with field-evolved resistance to Cry1F protein in *S. frugiperda*;

- To determine the dominance of resistance of *S. frugiperda* to Cry1F directly (leaf bioassays) and indirectly (leaf tissue dilution in artificial diet);

- To test if Cry1F resistant *S. frugiperda* populations from different states of Brazil share the same locus of resistance;

- To conduct a F_2 screen to estimate the frequency of resistance allele to Cry1F protein in *S. frugiperda* in Brazil.

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2 GEOGRAPHICAL AND TEMPORAL VARIABILITY IN SUSCEPTIBILITY TO CRY1F PROTEIN FROM *Bacillus thuringiensis* IN *Spodoptera frugiperda* (LEPIDOPTERA: NOCTUIDAE) POPULATIONS IN BRAZIL

Abstract

The genetically modified maize TC1507 event with *cry1F* gene has been used to control *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) in Brazil since 2009/2010 cropping season. As part of the Insect Resistance Management program, we conducted studies to determine the baseline susceptibility to Cry1F prior to widespread plantings of TC1507 maize. Subsequently we evaluated the geographical and temporal variability of susceptibility to this protein in populations of *S. frugiperda* collected from major maize-growing regions in Brazil. The baseline susceptibility to Cry1F was detemined with diet-overlay bioassay for a susceptible reference population and four field populations of *S. frugiperda*. Then, we monitored the susceptibility to Cry1F in 43 populations of *S. frugiperda* sampled in nine States of Brazil between 2011 and 2013. In the baseline study, the MIC_{50} (concentration that inhibits molting to second instar in 50% of individuals) ranged from 3.59 to 72.47 ng of Cry1F protein cm^{-2}. Based on the upper limit of the MIC_{99} value of the joint analysis from the baseline susceptibility data, the concentration of 2,000 ng of Cry1F protein cm^{-2} was defined as a diagnostic concentration for potentially resistant individuals and used in monitoring the susceptibility of *S. frugiperda* to Cry1F. Survival at 2,000 ng of Cry1F protein cm^{-2} increased significantly throughout cropping seasons in *S. frugiperda* populations from São Paulo, Santa Catarina, Rio Grande do Sul, Bahia, Mato Grosso, Goiás, Mato Grosso do Sul, and Paraná. Highest survival (> 50%) was reached in populations collected from Bahia, Mato Grosso, Goiás, Mato Grosso do Sul, and Paraná during 2012/2013 cropping season. Therefore, significant decrease in susceptibility to Cry1F was detected in *S. frugiperda* throughout cropping seasons, especially in regions with intensive maize production in Brazil.

Keywords: Fall armyworm; *Bacillus thuringiensis*; Diagnostic concentration; Insect resistance management

2.1 Introduction

Spodoptera frugiperda (J.E. Smith) (Lepidoptera: Noctuidae) is one the most important pests of maize in Brazil (CRUZ, 1995). Before the Bt maize technology, the use of insecticides was very intense to control *S. frugiperda* and high frequencies of resistant individuals have been reported to most of the registered insecticides for this pest in Brazil (DIEZ-RODRIGUEZ; OMOTO, 2001; CARVALHO et al., 2013). Recently, with the commercial approval of Bt maize in Brazil in 2007, the adoption of Bt maize has increased significantly by reaching up to 80% of maize-cultivated area with hybrids during the 2012/2013 cropping season (CÉLERES, 2013), mainly with

the event TC1507. This event of Bt maize expresses cry1F gene from bacterium *Bacillus thuringiensis* Berliner and provides high levels of protection from *S. frugiperda* (WAQUIL et al., 2002; WAQUIL et al., 2004; SIEBERT et al., 2008). TC1507 maize has been available commercially since the 2009/2010 crop season in Brazil (STORER et al., 2012).

Brazilian agriculture is very intense and diverse with different cropping systems. Because of the tropical climate conditions, it is possible to have at least two cropping seasons in most of the agricultural regions with the main season planted in October/November and second season planted in February/March. In areas with irrigation systems, crops can also be cultivated during dry winter periods. Therefore, the risk of resistance evolution will be high if Bt maize is planted all year round. The potential of rapid field evolution to Cry1F in *S. frugiperda* has already been reported in Puerto Rico (STORER et al., 2010). To implement an Insect Resistance Management (IRM) program, we proposed to establish the baseline susceptibility to Cry1F protein in Brazilian populations of *S. frugiperda* and to monitor Cry1F susceptibility in populations of *S. frugiperda* collected from major maize-growing regions from 2011 to 2013.

2.2 Materials and methods

2.2.1 Insect population

S. frugiperda larvae were sampled in maize fields from nine States in Brazil during 2010/2011, 2011/2012 and 2012/2013 cropping seasons (Table 2.1 and Figure 2.1). Two populations were sampled in TC1507 maize, and the remaining 45 populations in non-Bt maize. The populations from 2010/2011 main season, prior to wide adoption of TC1507 maize, were used to establish the baseline susceptibility to Cry1F, subsequent listed in Table 2.1 were used to monitor the changes in the susceptibility to this protein throughout time. A susceptible laboratory population (SUS) was obtained from EMBRAPA Milho e Sorgo, Sete Lagoas, Minas Gerais, Brazil, and maintained on artificial diet (KASTEN JR; PRECETI; PARRA, 1978) in the absence of selection pressure with insecticides since 1995.

2.2.2 Insect rearing

S. frugiperda larvae collected in the field were individually placed in 50 ml plastic cups with 10 ml of artificial diet and sealed with an acrylic sheet, and remained in the cups until pupation. Pupae were placed in cylindrical PVC cages (10 cm diameter × 21 cm), lined with newsprint and closed at the top and bottom with Petri dishes (12 cm diameter x 1.5 cm), and the bottom Petri dish was lined with filter paper. Adult food was a solution of 10% honey in a plastic cup (50 ml) plugged with water absorbent cotton. Eggs were collected every three days and stored in plastic containers (500 ml) with filter paper moistened in distilled water. The neonate larvae (< 24 h) were inoculated in plastic cups (100 ml) with 20 ml of artificial diet, and at second instar larvae were placed individually in plastic cups (50 ml) with 10 ml of artificial diet and sealed with an acrylic sheet, and remained in the cups until pupation. Procedures were repeated with pupae, and adults of F_1 and eggs of F_2 generations. Larvae from F_2 generation were used to conduct the bioassays. Insects were reared at 25 ± 1°C, 60 ± 10% relative humidity, and 14:10 h (L:D) photoperiod.

Table 2.1 - Population of *S. frugiperda* tested in baseline susceptibility and monitoring studies

State	Location	Code	Maize[a]	Date	n[b]
Main season 2010/2011					
Paraná	Sabáudia	PR23	Non-Bt	Nov. 2010	838
Goiás	Rio Verde	GO15	Non-Bt	Nov. 2010	630
Bahia	São Desidério	BA22	Non-Bt	Dec. 2010	530
Minas Gerais	Uberlândia	MG12	Non-Bt	Dec. 2010	710
Second season 2010/2011					
Mato Grosso	Campo Verde	MT16	Non-Bt	Apr. 2011	144
Paraná	Londrina	PR27	Non-Bt	May 2011	690
Bahia	Luís Eduardo Magalhães	BA23	Non-Bt	May 2011	442
Main season 2011/2012					
Bahia	Barreiras	BA25	TC1507	Oct. 2011	94
São Paulo	Casa Branca	SP11	Non-Bt	Dec. 2011	923
Rio Grande do Sul	Santo Ângelo	RS9	Non-Bt	Dec. 2011	485
Rio Grande do Sul	Passo Fundo	RS10	Non-Bt	Dec. 2011	363
Santa Catarina	Xanxerê	SC1	Non-Bt	Dec. 2011	371
Paraná	Cascavel	PR30	Non-Bt	Dec. 2011	795
Goiás	Rio Verde	GO18	Non-Bt	Dec. 2011	893
Goiás	Planaltina	GO20	Non-Bt	Dec. 2011	337
Minas Gerais	Uberlândia	MG14	Non-Bt	Dec. 2011	204
Bahia	Luís Eduardo Magalhães	BA26	Non-Bt	Dec. 2011	643
Second season 2011/2012					
Bahia	Barreiras	BA29	Non-Bt	Apr. 2012	615
Mato Grosso	Campo Verde	MT18	Non-Bt	Apr. 2012	505
Mato Grosso	Sinop	MT19	Non-Bt	Apr. 2012	568
Mato Grosso	Campo Novo do Parecis	MT20	Non-Bt	Apr. 2012	570
Goiás	Caiapônia	GO23	Non-Bt	May 2012	524
Mato Grosso do Sul	Dourados	MS13	Non-Bt	May 2012	228
São Paulo	Palmital	SP14	Non-Bt	May 2012	740
Paraná	Sabáudia	PR37	Non-Bt	May 2012	474
Paraná	Campo Mourão	PR38	Non-Bt	May 2012	601
Paraná	Palotina	PR39	Non-Bt	May 2012	435
Main season 2012/2013					
Rio Grande do Sul	São Paulo das Missões	RS12	TC1507	Oct. 2012	163
São Paulo	Casa Branca	SP15	Non-Bt	Nov. 2012	445
Paraná	Cascavel	PR40	Non-Bt	Nov. 2012	140
Paraná	Tibagi	PR41	Non-Bt	Nov. 2012	512
Bahia	São Desidério	BA32	Non-Bt	Dec. 2012	291
Goiás	Rio Verde	GO24	Non-Bt	Dec. 2012	636
Goiás	Flores de Goiás	GO26	Non-Bt	Jan. 2013	667
Minas Gerais	Araguari	MG16	Non-Bt	Jan. 2013	142
Rio Grande do Sul	Santo Ângelo	RS13	Non-Bt	Feb. 2013	473
Rio Grande do Sul	Não-Me-Toque	RS14	Non-Bt	Feb. 2013	593
Santa Catarina	Chapecó	SC1	Non-Bt	Feb. 2013	549
Second season 2012/2013					
Mato Grosso	Campo Verde	MT22	Non-Bt	Mar. 2013	377
Mato Grosso	Sorriso	MT23	Non-Bt	Mar. 2013	1015
Mato Grosso	Campo Verde	MS16	Non-Bt	Apr. 2013	587
Mato Grosso	Sapezal	MS17	Non-Bt	Apr. 2013	361
Mato Grosso do Sul	Chapadão do Sul	MT25	Non-Bt	Mar. 2013	335
Mato Grosso do Sul	Douradina	MT26	Non-Bt	Apr. 2013	467
Goiás	Montividiu	GO28	Non-Bt	Apr. 2013	520
Paraná	Palotina	PR46	Non-Bt	May 2013	240
Paraná	Rolândia	PR48	Non-Bt	May 2013	645

[a] Host (maize sampled).
[b] Number of sampled insects.

2.2.3 Baseline susceptibility

Bioassays were conducted with populations SUS, PR23, GO15, BA22, and MG12 (Table 2.1 and Figure 2.1A). The diet-overlay bioassay (MARÇON et al., 1999) was conducted using 128-well bioassay trays (BIO-ASSAY TRAY BIO-BA-128, Pitman, NJ, USA), with at least six replicates (16 larvae replicate) for each Cry1F protein concentration. Each well received 1 ml of artificial diet with a surface area of 1.5 cm^2. The Cry1F concentrations were logarithmically spaced over a range that enabled mortality and weight reduction from 10 to >95%, ranging from 0.2 up to 2,000 ng cm^{-2} of Cry1F protein, depending upon the population. For SUS population, the tested concentrations varied from 0.64 to 64 ng cm^{-2} of Cry1F protein. Cry1F protein (sample TSN104550, 35% Cry1F protein, available from Dow AgroSciences) was diluted in buffer 10mM potassium phosphate dibasic, followed by addition of 0.1% Triton X-100 to obtain a uniform distribution of the protein on the surface of the diet at a rate of 20 µl cm^{-2}, and allowed to dry. Individual neonate larvae were transferred with a fine paintbrush into each well, and the wells were closed with air-permeable lids. The trays were kept in a climate chamber at 27 ± 1°C, 60 ± 10% relative humidity and 14:10 h (L:D) photoperiod. Larval development and weight were measured at seven days after infestation for each replicate and concentration. Larvae that did not reach the second instar at this time were considered to have their molting inhibited, and considered dead. All alive larvae were weighed.

2.2.4 Susceptibility monitoring

The susceptibility monitoring to Cry1F was conducted in 43 populations of *S. frugiperda* (Table 2.1 and Figure 2.1). The diet-overlay bioassay was conducted by using 128-well bioassay trays, with nine replicates by concentration. In each replicate 112 larvae were tested with Cry1F protein and 16 larvae without Cry1F protein. The bioassay was performed with diagnostic concentrations, based on upper limit of the MIC_{99} values of the susceptible population and the joint analysis of four field populations used in the baseline susceptibility, respectively. The bioassay procedures were the same used to characterize the baseline susceptibility of *S. frugiperda* to Cry1F. Larval weight was not measured in monitoring.

2.2.5 Statistical analyses

The data from baseline susceptibility were analyzed to estimate the MIC_{50} (concentration that inhibits molting to second instar in 50% of individuals), EC_{50} (effective concentration that reduces weight gain by 50%), and MIC_{99} (concentration that inhibits molting to second instar in 99% of individuals). Absence of molting to second instar was subjected to Probit analysis (FINNEY, 1971) by using Polo Plus® software (ROBERTSON; PREISLER; RUSSELL, 2003). Weight from all survivors insects were analyzed with nonlinear regression to estimate the EC_{50} using the JMP SAS® (JMP, Version 10, SAS INSTITUTE INC., 2012). MIC_{50} and EC_{50} were considered significantly different among populations when their 95% confidence intervals did not overlap. The diagnostic concentrations were chosen as follows. Absence of molting to second instar from four field populations was pooled. The pooled data from field populations or SUS were analyzed by complementary log-log model (ROBERTSON; PREISLER, 1992) using SAS 9.1 (SAS INSTITUTE INC., 2004) to obtain MIC_{99} that was used in monitoring. The monitoring data were analyzed by estimating the 95% confidence intervals on the probability of success of survival in a binomial distribution by using the Probit parameterization. The analyses were performed using the function *binom.probit* from the package *binom* (DORAI-RAJ, 2009) in R 2.15.1 (R DEVELOPMENT CORE TEAM, 2012). Survival data at diagnostic concentrations were considered significantly different when their 95% confidence intervals did not overlap the 95% confidence intervals of field populations tested at same concentrations in baseline studies.

2.3 Results

2.3.1 Baseline susceptibility

Natural variability in the susceptibility to Cry1F in *S. frugiperda* populations in Brazil was ≈ 20-fold. Estimated MIC_{50} values ranged from 3.59 to 72.47 ng cm^{-2} of Cry1F protein. The MIC_{50} of SUS population was significantly lower than the values for the field populations. Among field populations of *S. frugiperda*, the lowest susceptibility to Cry1F protein was observed in population BA22 from Bahia. The

MIC_{50} of this population was significantly greater than the values for SUS, MG12, and PR23, but was not different from GO15. The slope was higher in populations with smaller MIC_{50} (SUS and MG12) which indicates a higher homogeneity in susceptibility to Cry1F from *S. frugiperda* larvae from these populations (Table 2.2). Estimated EC_{50} values from ranged from 0.80 to 1.74 ng cm^{-2} of Cry1F protein. Natural variability in susceptibility to Cry1F among *S. frugiperda* populations was only 2-fold. The EC_{50} values were higher in populations with smaller MIC_{50} (Table 2.2). The MIC_{99} for SUS population was 42.07 ng cm^{-2} of Cry1F protein (95% confidence interval between 21.03 and 195.70) and the concentration of 200 ng cm^{-2} of Cry1F protein was defined as one of the diagnostic concentrations. The MIC_{99} values estimated from the joint analysis of the four field populations was 1,268.00 ng cm^{-2} of Cry1F protein (95% confidence interval between 867.22 and 2,064.00), and the concentration of 2,000 ng cm^{-2} of Cry1F protein was defined as another diagnostic concentration for monitoring the susceptibility of *S. frugiperda* populations.

2.3.2 Susceptibility monitoring

Larval survival for monitoring the susceptibility was evaluated with two diagnostic concentrations for *S. frugiperda* populations sampled during the main and second season 2011/2012. A total of 19 populations were tested at 200 ng cm^{-2} of Cry1F protein (Table 2.3). Compared with the baseline populations, survival was significantly higher in five and significantly lower in eight populations. Survival for the same 19 populations was measured at 2,000 ng cm^{-2} of Cry1F protein, and survival was significantly higher than the baseline susceptibility data in four field populations. Monitoring with 2,000 ng cm^{-2} of Cry1F protein was more appropriate to detect changes in susceptibility among field populations.

A total of 43 populations were tested at 2,000 ng cm^{-2} of Cry1F protein during 2010/2011, 2011/2012 and 2012/2013 cropping seasons. Survival at 2,000 ng cm^{-2} of Cry1F protein increased significantly throughout crop seasons in populations from São Paulo, Santa Catarina, Rio Grande do Sul, Bahia, Mato Grosso, Goiás, Mato Grosso do Sul, and Paraná, but not in Minas Gerais. Highest survival (> 50%) was reached in populations from Bahia, Mato Grosso, Goiás, Mato Grosso do Sul, and Paraná. For example, in Bahia, the survival of *S. frugiperda* populations tested increased from 0.10% in second season 2010/2011 to 68.42% in second season

2011/2012. Survival did not increase significantly in *S. frugiperda* population sampled in Mato Grosso until tyhe second season 2011/2012, but four populations from this State had significantly higher survival than the baseline susceptibility data in second season 2012/2013. Survival increased from 0% in second season 2010/2011 to 93.05% in second season 2012/2013 in Mato Grosso.

Table 2.2 - Baseline susceptibility of *Spodoptera frugiperda* to Cry1F protein

Populations	n	Slope ± SE	MIC_{50} in ng cm^{-2} (95% CI)[a]	χ^2 (df)	n	EC_{50} in ng cm^{-2} (95% CI)[b]
SUS	672	1.98 ± 0.25	3.59 (2.75 – 4.50)	1.15 (3)	209	1.52 (1.35 – 1.74)
PR23	959	1.62 ± 0.16	26.90 (19.06 – 35.42)	3.28 (6)	491	0.93 (0.56 – 1.62)
GO15	800	1.11 ± 0.10	38.54 (26.44 – 52.88)	5.06 (6)	493	0.85 (0.52 – 1.37)
BA22	719	1.33 ± 0.12	72.47 (42.25 – 109.52)	5.85 (5)	592	0.80 (0.56 – 1.12)
MG12	767	2.14 ± 0.22	18.05 (11.44 – 25.63)	5.84 (4)	565	1.74 (1.24 – 2.47)

[a]Molt inhibition concentration (MIC) was considered larvae dead more that failed to molt to second instar.
[b]Effective concentration (EC), required to growth inhibition.

Table 2.3 - Survival of *S. frugiperda* in Cry1F protein at diagnostic concentrations from different locations and crop season in Brazil

Population	n	Percent Survival to 2nd Instar (95% CI)	n	Percent Survival to 2nd Instar (95% CI)
	200 ng cm^{-2}		2,000 ng cm^{-2}	
Second season 2010/2011				
MT16			1,008	0.00 (0.00 - 0.37)
PR27			1,008	0.69 (0.32 - 1.41)
BA23			1,008	0.10 (0.01 - 0.60)
Main season 2011/2012				
BA25	---	---	1,005	2.69 (1.83 - 3.85)
SP11	998	14.83 (12.73 - 17.14)	1,001	0.60 (0.26 - 1.28)
RS9	963	7.37 (5.86 - 9.17)*	1,003	0.30 (0.09 - 0.87)
RS10	1,000	8.20 (6.63 - 10.04)*	1,003	0.10 (0.01 - 0.60)
SC1	1,007	13.41 (11.41 - 15.62)	989	1.52 (0.90 - 2.46)
PR30	1,002	11.88 (9.99 - 14.00)	1,008	0.60 (0.26 - 1.28)
GO18	1,001	24.88 (22.28 - 27.63)*	1,005	8.76 (7.14 - 10.64)*
GO20	1,001	15.28 (13.16 - 17.62)	1,000	1.70 (1.04 - 2.68)
MG14	1,007	34.46 (31.57 - 37.44)*	1,003	1.69 (1.04 - 2.67)
BA26	1,005	22.99 (20.47 - 25.67)*	1,007	2.09 (1.35 - 3.14)
Second season 2011/2012				
BA29	960	74.90 (72.08 - 77.56)*	972	68.42 (65.44 - 71.28)*
MT18	996	4.72 (3.54 - 6.19)*	995	0.20 (0.05 - 0.74)
MT19	967	11.79 (9.88 - 13.95)	960	0.42 (0.15 - 1.05)
MT20	1,006	0.60 (0.26 - 1.28)*	1,006	0.10 (0.01 - 0.60)
GO23	1,005	11.44 (9.59 - 13.53)	1,006	0.20 (0.05 - 0.73)
MS13	1,006	6.66 (5.25 - 8.34)*	992	0.30 (0.09 - 0.88)
SP14	1,004	6.27 (4.91 - 7.92)*	1,005	3.78 (2.74 - 5.12)*
PR37	997	2.21 (1.44 - 3.29)*	987	0.30 (0.09 - 0.88)
PR38	981	4.69 (3.51 - 6.17)*	991	0.10 (0.01 - 0.61)
PR39	976	84.02 (81.61 - 86.21)*	975	81.33 (78.79 - 83.68)*
Main season 2012/2013				
RS12	---	---	1,003	68.20 (65.26 - 71.02)*
SP15	---	---	1,003	4.29 (3.17 - 5.70)*
PR40	---	---	1,008	78.08 (75.44 - 80.54)*
PR41	---	---	1,008	0.30 (0.09 - 0.87)
BA32	---	---	1,006	38.57 (35.60 - 41.61)*
GO24	---	---	1,008	60.91 (57.87 - 63.89)*
GO26	---	---	999	16.72 (14.51 - 19.13)*
MG16	---	---	1,008	0.00 (0.00 - 0.37)
RS13	---	---	960	19.17 (16.78 - 21.75)*
RS14	---	---	969	7.12 (5.64 - 8.89)*
SC2	---	---	980	4.18 (3.07 - 5.60)*
Second season 2012/2013				
MT22	---	---	917	92.15 (90.26 - 93.75)*
MT23	---	---	914	82.39 (79.81 - 84.74)*
MS16	---	---	874	70.71 (67.62 - 73.65)*
MS17	---	---	986	97.16 (95.95 - 98.05)*
MT25	---	---	949	93.05 (91.28 - 94.52)*
MT26	---	---	980	64.49 (61.45 - 67.44)*
GO28	---	---	982	59.16 (56.07 - 62.21)*
PR46	---	---	909	22.44 (19.82 - 25.25)*
PR48	---	---	1,006	87.18 (84.99 - 89.13)*

[a] Significantly different from field populations in baseline (PR23, GO15, BA22, and MG12). Survival for field populations were 14.20% (10.87 – 18.17) at 200 ng cm^{-2}, and 0.57% (0.13 – 2.05%) at 2,000 ng cm^{-2}.

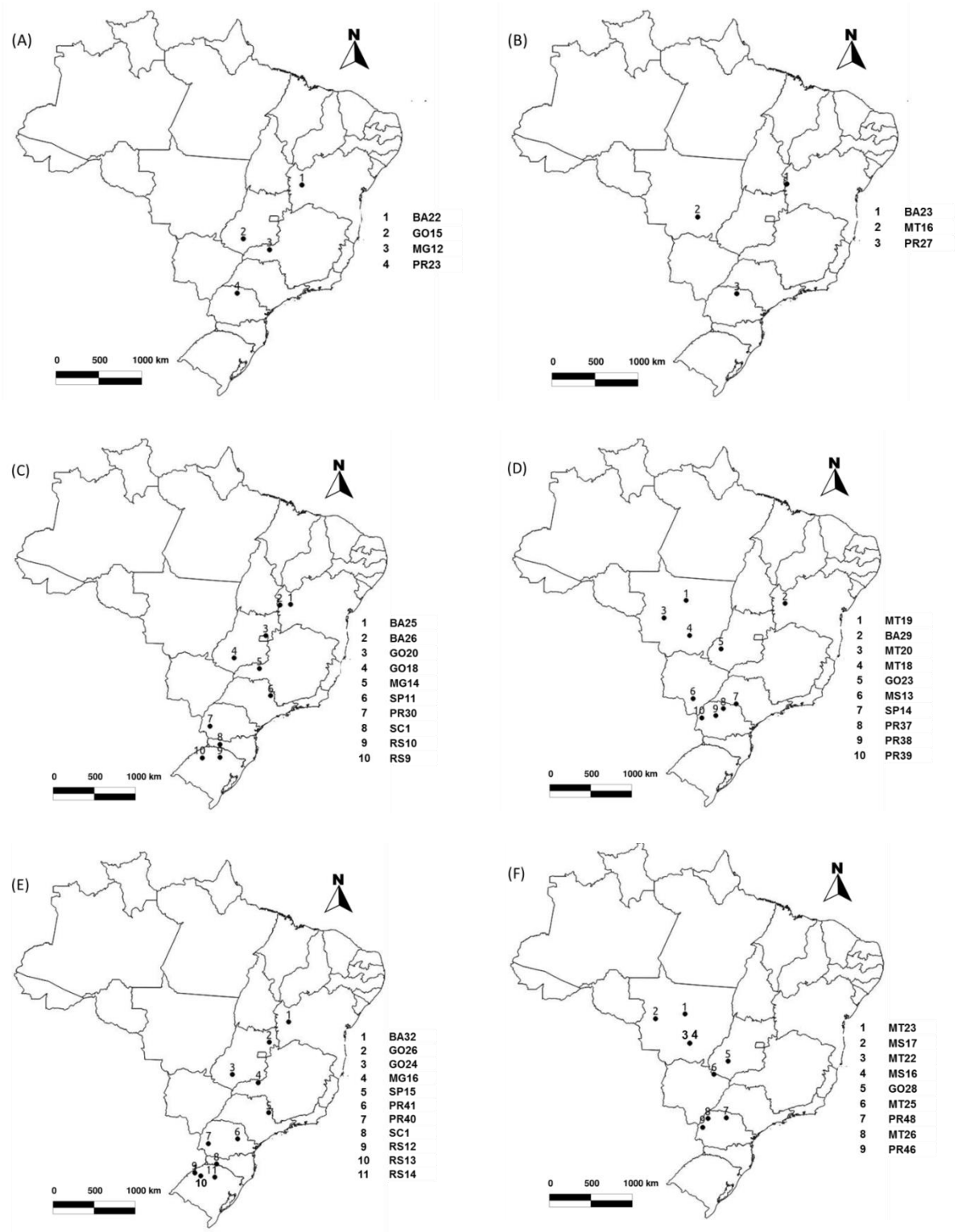

Figure 2.1 - Distribution of *S. frugiperda* populations used in baseline and susceptibility monitoring to Cry1F protein: (A) main season 2010/2011, (B) second season 2010/2011, (C) main season 2011/2012, (D) second season 2011/2012, (E) main season 2012/2013, and (F) second season 2012/2013

2.4 Discussion

The natural variation in the baseline to Cry1F protein in *S. frugiperda* populations from Brazil was ≈20-fold. A variation of ≈30-fold was also reported among populations of *S. frugiperda* to Cry1F protein in the U.S.A. (STORER et al., 2012). The MIC_{50} value of SUS population was only 3.59 ng cm^{-2} of Cry1F protein in this study. Other reported values of LC_{50}/MIC_{50} for susceptible populations of *S. frugiperda* to Cry1F were 0.14 (BLANCO et al., 2010), 36 (WAQUIL et al., 2004), 109 (LUO; BANKS; ADANG, 1999), 181 and 289 ng cm^{-2} of Cry1F protein (STORER et al., 2010). These differences may be associated with the insect generation, bioassay methods, mortality criteria and/or genetic variability of populations (LUO; BANKS; ADANG, 1999; MARÇON et al., 1999; WAQUIL et al., 2004; BLANCO et al., 2010; STORER et al., 2010). The lowest susceptibility to Cry1F in the baseline studies was detected in population from Bahia because of intensive agriculture system with use of other Bt maize, that is the MON810 event expressing *cry1Ab* gene prior to the approval of the event TC1507(CTNBIO, 2007; 2008). The possibility of cross-resistance between Cry1F and Cry1Ab in *S. frugiperda* has already been reported (STORER et al., 2010; HERNÁNDEZ-RODRÍGUEZ et al., 2013).

We defined 2,000 ng cm^{-2} of Cry1F protein as diagnostic concentration in diet-overlay bioassay for monitoring. The concentration of 200 ng cm^{-2} of Cry1F protein defined with data from SUS population was not appropriated for detecting potentially reistant individuals as there was significant survival of susceptible field populations at this concentration. When surviving larvae at 200 ng cm^{-2} of Cry1F protein were transferred into leaf tissues of TC1507, some larvae were not able to complete larval devolopement. On the other hand, all surviving larvae at the 2,000 ng cm^{-2} of Cry1F protein were able to develop in TC1507 event (unpublished data).

The significant difference in the susceptibility to Cry1F observed in *S. frugiperda* populations collected from different Brazilian States since 2011/12 (Table 2.2) is assumed to be a result of wide adoption of Bt maize in Brazil: Adoption reached ≈ 80% of the maize-cultivated area in Brazil in the 2012/2013 cropping season and high percentage of this area was grown with TC1507 maize (CÉLERES, 2013). A significant decrease in susceptibility to Cry1F was detected throughout time, especially in *S. frugiperda* populations from Bahia, Mato Grosso, Goiás, Mato Grosso

do Sul and Paraná by reaching high survivorship (> 50%) at diagnostic concentration of 2,000 ng cm^{-2} of Cry1F protein.

It is crucial to understand different cropping systems for the resistance risk analysis. The increase in survival was detected in different maize growing season, based on cropping systems of each location. We observed high survival (> 50%) in populations from Western Bahia and Western Paraná in second season 2011/2012. Populations from Mato Grosso, Goiás and Mato Grosso do Sul reached high survival one year later. High survival at the diagnostic concentration was not observed in populations collected in non-Bt maize from Minas Gerais, São Paulo, Santa Catarina and Rio Grande do Sul areas until the monitoring of susceptibility were conducted during the 2012/2013 cropping season.

The large and intensive maize production in Bahia and Paraná likely contributed to the earlier decrease in susceptibility to Cry1F in *S. frugiperda* populations. The cropping systems in Western Bahia are very intense with successive planting even during the winter season under irrigation. The largest maize production area in Brazil is located in Paraná State (CONAB, 2013). By contrast in Mato Grosso, maize is cultivated predominantly during the second season after the cultivation of soybean during the main season which is not an important host for *S. frugiperda*.

According to our data, significant decrease in susceptibility to Cry1F was detected in *S. frugiperda* throughout cropping seasons, especially in regions with intensive maize production in Brazil. Furthermore, the adoption of the refuge areas (10% refuge is recommended) has been low in most of the Brazilian farms. Therefore, it will be urgent to revise and implement more effective Insect Resistance Management measures to extend the durability of TC1507 event for controlling *S. frugiperda*. Bt maize lines that combine TC1507 with other Bt events to express multiple proteins such as TC1507 + MON810 (Cry1F + Cry1Ab) (CTNBIO, 2011) and TC1507 + MON89034 (Cry1F + Cry1A.105 + Cry2Ab2) (CTNBIO, 2010) have already been approved in Brazil. Such pyramided trait products are expected to increase the durability of Bt maize compared with single event products (STORER et al. 2012).

2.5 Conclusions

- There is a high natural variability in the susceptibility to Cry1F among *S. frugiperda* populations in Brazil.

- The concentration of 2,000 ng cm^{-2} of Cry1F protein in a diet-overlay bioassay is appropriate to detect the resistance of *S. frugiperda* to Cry1F.

- There is a significant decrease in the susceptibility to Cry1F in *S. frugiperda* populations throughout crop seasons (2011 to 2013) in Brazil, especially in regions with intensive maize production.

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3 FIELD-EVOLVED RESISTANCE TO CRY1F MAIZE BY *Spodoptera frugiperda* (J.E. SMITH) (LEPIDOPTERA: NOCTUIDAE) IN BRAZIL

Abstract

Evolution of resistance may be a potential limiting factor to continued use of Bt proteins in plants. In Brazil, the main target insect of Bt maize has been *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae). The Cry1F protein expressed in event TC1507 maize has been one of the most effective ways to control *S. frugiperda*. However, this species has a wide range of biological variability and has evolved resistance to Cry1F under field conditions in Puerto Rico. After reports of reduced effectiveness in areas of Brazil, research was undertaken to investigate if damage in Cry1F maize was caused by resistant *S. frugiperda*. Additional investigations into which methods can be used to define field-evolved resistance were also conducted. A population of *S. frugiperda* was collected from TC1507 maize fields with unexpected damage in Bahia state and divided into two subpopulations. One was subjected to further laboratory selection while the other was not subjected to artificial selection. Both sub-populations were able to survive on Cry1F maize as neonates and subsequently produce normal adults, the artificial selection enhancing the level of resistance. A comparison of bioassay methods indicated that survival of Cry1F-susceptible *S. frugiperda* on non-Bt maize was significantly higher in leaf than plant bioassays. Resistance ratio was higher in overlay bioassays than when Cry1F protein was incorporated into diet. Cry1F resistant *S. frugiperda* showed autosomal inheritance for alleles involved in resistance to Cry1F protein. Progeny of *S. frugiperda* collected in Cry1F maize fields that showed unexpected damage were able to survive on Cry1F maize plants under laboratory conditions from neonate to adult. The ability to survive on the plant showed a genetic basis indicating that the survivors were resistant to Cry1F. Leaf bioassays and diet-overlay bioassays are suitable for characterizing field-evolved resistance by *S. frugiperda*.

Keywords: Fall armyworm; *Bacillus thuringiensis;* Plant survival; Genetic basis; Detection methods

3.1 Introduction

Several genes from common soil bacterium *Bacillus thuringiensis* Berliner (Bt) have been introduced into crop plants to protect them from insect damage, and the *cry1F* gene, isolated from *B.t.* var. *aizawai* is one of the most widely used. Cry1F maize hybrids (event TC1507, Herculex® I Insect Protection) have being commercialized in the USA since 2003 (SIEBERT et al., 2008; STORER et al., 2012). In Brazil these hybrids were released in 2008 and commercially available for the 2009/2010 crop season (CTNBIO, 2008; STORER et al., 2012). Cotton varieties producing Cry1F and Cry1Ac (WideStrike™) were commercialized during 2005 in the USA and during 2009 in Brazil (US-EPA, 2005; CTNBIO, 2009). Maize and cotton with the *cry1F* gene provide high levels of control of fall armyworm, *Spodoptera*

frugiperda (J.E. Smith) (Lepidoptera: Noctuidae) (WAQUIL; VILELLA; FOSTER, 2002; SIEBERT et al. 2008). This species is native to the Americas and is distributed from Uruguay and northern Argentina to Florida and Texas in southern United States, including Central America and the Caribbean Islands (SPARKS, 1979; ASHLEY et al., 1989; NAGOSHI; MEAGHER, 2008). In summer, populations migrate into southern and northern temperate regions. In Brazil, *S. frugiperda* is the most important pest of maize and one of most important pests of cotton (CRUZ, 1995; BARROS et al., 2010).

Evolution of resistance in target insect pest populations can undermine the economic and environmental benefits of transgenic Bt crop plants. The continuous expression of *cry* genes in transgenic plants exerts strong selection for resistance in the targeted pest populations (McGAUGHEY; WHALON, 1992). Field resistance have been recorded in *Busseola fusca* (Füller) (Lepidoptera: Crambidae) to Cry1Ab maize in South Africa (VAN RENSBURG, 2007), *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) to Cry1Ac cotton in China (LIU et al., 2010), *S. frugiperda* to Cry1F maize in Puerto Rico (STORER et al., 2010), *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) to Cry1Ac cotton in India (DHURUA; GUJAR, 2011) and *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) to Cry3Bb1 maize in the USA (GASSMANN et al., 2011). The Cry1F resistant population of *S. frugiperda* from Puerto Rico was found to have autosomal, recessive resistance with limited cross-resistance to Cry1Ab and Cry1Ac, proteins that are also used in Bt maize and Bt cotton in Brazil. Evolution of resistance to Cry1F in Puerto Rico may have been due to the isolation of the island, tropical climate, year-round cultivation of maize with sequential plantings, extensive prior use of sprayable Bt's, high pest population and drought conditions 2006/2007, which reduced the availability of alternative hosts for *S. frugiperda* (STORER et al., 2010).

Similar to Puerto Rico, most of Brazilian agriculture is in a tropical climate, and in some regions, maize is grown in intensive system of production, where crops are planted all year without a break (STORER et al., 2012). In the Central-west and western Bahia regions in Brazil the winter season is dry and hot, but recently the use of irrigation has allowed maize production during the entire year without a break. This has enabled *S. frugiperda* to have continuous generations through the year in Bahia and exacerbated this pest problem (MARTINELLI et al., 2006; 2007). In southern Brazil, despite cool winters, rainfall allows crop production during the winter, which

again enables *S. frugiperda* to build up high population densities at the beginning of the summer season. These conditions that favor year-round use of Bt maize in Brazil and year round populations of *S. frugiperda* also are expected to favor the evolution of resistance to Bt maize in *S. frugiperda*. This study demonstrates that unexpected damage to TC1507 fields in Brazil are associated with field-evolved resistance to Cry1F protein in *S. frugiperda*. In addition, we verify that a leaf bioassay, a whole plant bioassay, a diet overlay bioassay, and a diet incorporation bioassay give comparable results and any can be used to document resistance in *S. frugiperda* to Cry1F protein.

3.2 Material and methods

3.2.1 Population sources and maintenance

Larval *S. frugiperda* were collected from Barreiras, Bahia, Brazil, after a report unexpected damage to TC1507 maize during early summer, 2011. The affected field was planted to hybrid 2B604HX (Dow AgroSciences) on 8 August 2011. About 30% of the plants confirmed to express Cry1F were severely damaged by *S. frugiperda*. Larvae were collected on 6 October 2011 from V_8 maize (RITCHIE; HANWAY; BENSON, 1993). The sample was taken ten days after the field was sprayed with methomyl at a rate of 172 g a.i. ha^{-1}. Maize plants were collected from different parts of the field that showed significant economic damage and were dissected to take the larvae. Larvae where shipped with Cry1F maize leaves to our laboratory in an insulated styrofoam box. The 94 surviving larvae were individually reared in plastic cups (50 ml) with artificial diet, as describe below. The adults (about 50% female) were used to start a laboratory colony; this population was designated BA25. A susceptible population designated SUS was obtained from EMBRAPA Milho e Sorgo, Sete Lagoas, Minas Gerais, Brazil, and has been maintained in laboratory since 1995. A field susceptible population designated Field-SUS was collected in Sabáudia, Paraná, Brazil, in May 2012 and it was started from 165 larvae and 309 pupae.

S. frugiperda larvae were reared on artificial diet (KASTEN; PRECETI; PARRA, 1978). Larvae were individually placed in 50 ml plastic cups with diet and sealed with an acrylic sheet. Larvae remained in the cups until pupation. Pupae were

placed in cylindrical PVC cages (10 cm diameter × 21 cm), lined with newsprint and closed at the top and bottom with Petri dishes (12 cm diameter × 1.5 cm), and the bottom Petri dish was lined with filter paper. After emergence adults were paired with about 20 pairs per cage. Adult food was a solution of 10% honey in a plastic cup (50 ml) plugged with water absorbent cotton. Eggs were collected every two days and stored in plastic containers (500 ml) containing filter paper moistened with distilled water. The neonate larvae were collected and used to maintain the population or conduct bioassays.

For maintaining the populations, eggs (climate chamber), larvae, pupae, and adults (climate-controlled rooms) were reared at 25 ± 1°C, 60 ± 10% relative humidity, and 14:10 h (L:D) photoperiod. Immature insects for all bioassays and selection experiments were kept in a climate chamber at 27 ± 1°C, 60 ± 10% relative humidity and 14:10 h (L:D) photoperiod. Adults were kept under similar conditions as the maintenance populations.

3.2.2 Production of resistant population by laboratory selection

The first lab generation of BA25 was separated into two populations, without and with selection, respectively designated BA25N and BA25R. BA25N was maintained on artificial diet without Cry1F protein during first generation and used in a bioassay during its second generation in laboratory. BA25R was selected for first two generations on TC1507 maize leaves (producing Cry1F) and for two additional generations with purified Cry1F protein as detailed below. Maize was planted in the field using 550 kg ha^{-1} of 4-14-8 (NPK). The hybrid 2B688 HX (TC1507, Dow AgroSciences) was planted in small plots at 80,000 seeds ha^{-1}. Every 15 days a new plot was planted, so that leaves between stages V_6 and V_9 (RITCHIE; HANWAY; BENSON, 1993) were always available for the assays and experiments. Nitrogen fertilization (urea) was made with 90 kg ha^{-1} in V_6. The whorl leaves were collected immediately before use, washed and chopped, discarding the sheath, collar and midrib.

BA25R was mass-selected first on TC1507 leaves and subsequently on diet with surface-applied Cry1F protein. In both selection methods, the number of plates used was that necessary to obtain at least 100 normal adults. In each of the F_1 and F_2 generations approximately 30 neonate larvae (≤24 hours) were released onto an

acrylic plate (5.6 cm diameter x 1 cm). Each plate received 7 ml of 2.5% agar solution, a disk of filter paper and leaf pieces of TC1507 maize and they were maintained in a climate chamber. At five days after inoculation (DAI), the surviving larvae were collected and isolated into plastic cups (100 ml), which contained 7 ml of 2.5% agar solution, filter paper and pieces of TC1507 maize leaves. TC1507 maize leaf pieces were changed in an interval of maximum three days until the larvae pupated. Pupae were collected, placed on trays with paper filter, and isolated using plastic cups (50 ml) until adult emergence. Adults were placed in cages, as described above.

Mass selection on artificial diet with purified protein Cry1F occurred during the F_3 and F_4 generations. Larvae were selected on the same acrylic plates used in leaf selection with 7 ml of artificial diet. The artificial diet was poured into the plates and the plates were tapped on a table to eliminate surface bubbles. Cry1F protein (lot TSN104550, 35% Cry1F protein, provided by Dow AgroSciences) was diluted in 10mM potassium phosphate dibasic, followed by addition of 0.1% Triton X-100 to obtain a uniform distribution of the protein on the surface of the diet at a rate of 20 µl cm^{-2}. Selection was performed with 11,200 ng cm^{-2} of Cry1F protein in the F_3 generation and 20,000 ng cm^{-2} in the F_4 generation. After drying the surface of the diet, 20 neonate larvae $plate^{-1}$were released. At seven DAI the larvae that molted to second instar were recovered and reared on diet without Cry1F until pupation. The pupae and adults were handled as previously described.

3.2.3 Plant and leaf bioassays

To measure phenotypic resistance to Cry1F maize plants in *S. frugiperda*, a whole plant bioassay was conducted with the TC1507 maize-derived and laboratory-selected BA25R population and the susceptible laboratory population SUS. Maize hybrids 2B688 (not producing Cry1F) and 2B688 HX (producing Cry1F) were grown in plastic pots (10 L) with a mixture of soil and organic compost (1:1) at 5 plants pot^{-1}. Nitrogen fertilization (urea) was made at V_6. When plants reached the V_8 stage, one neonate larvae was released with a fine artist's paintbrush on each maize whorl. Each insect population was released on ten pots of each maize hybrid. Ten days after infestation, surviving larvae were recovered and reared on the corresponding

maize leaves until pupation in laboratory. Survival at ten days after infestation, pupation (not deformed), and adult (not deformed) emergence were measured.

A laboratory leaf bioassay was conducted with BA25N, BA25R, and SUS using hybrid 2B688 HX (+Cry1F) and SUS using non-Bt hybrid 2B688. The hybrids were grown in the field. Each population was tested in 12-well acrylic plates containing 1 ml 2.5% agar, filter paper and one maize leaf disc 1.2 cm diameter. One neonate larvae was released with a fine artist's paintbrush into each well, 120 larvae from each population were used, and the plates were sealed with a plastic film and an acrylic cover. The larvae were recovered at five DAI and reared on the corresponding maize leaves until pupation. Survival at five DAI, pupation (not deformed), and adult (not deformed) emergence were measured.

3.2.4 Cry1F diet-overlay and diet-incorporation bioassays

Diet bioassays were conducted with populations BA25R, Field-SUS, and SUS using 128-well bioassay trays (BIO-ASSAY TRAY BIO-BA-128, Pitman, NJ, USA), with at least six replicates (16 larvae replicate^{-1}) for each Cry1F protein concentration. Cry1F was provided either overlayed on the diet surface or uniformly incorporated into the diet. Each well of the tray had 1 ml of artificial diet and a surface area of 1.5 cm^{-2}. The artificial diet was slowly poured into the wells and any surface bubbles were punctured and eliminated. The concentrations were logarithmically spaced over a range that enabled mortality and weight reduction of approximately 10 to 90%. BA25R was tested at concentrations up to 20,000 ng cm^{-2} and 100,000 ng ml^{-1} for the diet-overlay and diet-incorporation bioassays respectively. Field-SUS was tested at seven concentrations, ranging from 0.2 to 200 ng cm^{-2} and 32 to 5,600 ng ml^{-1}, respectively for the overlay and incorporation bioassays. SUS was tested at five concentrations, ranging from 0.64 to 112 ng cm^{-2}for the overlay bioassay, and at nine concentrations, ranging from 10 to 10,000 ng ml^{-1} for the incorporation bioassay. The overlay bioassay was similar to that used by Marçon et al. (1999) and concentrations were applied at 20 µl cm^{-2}, and allowed to dry. The incorporation bioassay concentrations were produced similar to the overlay bioassay, but without Triton X-100. Each concentration was produced with 4 ml of the diluted Cry1F mixed in 36 ml of artificial diet in 50 ml tubes. The tubes were kept in a water bath at 54 °C, homogenized in a mixer, poured into well sat a rate of 1 ml, and

allowed to cool. Controls were created by applying buffer with Triton X-100, or buffer alone, respectively in the overlay and incorporation bioassays. Individual neonate larvae were released with a fine artist's paintbrush into each well, and the wells were closed with air-permeable lids. Development and weight were measured at seven DAI for each replicate and concentration. Larvae that did not reach the second instar at this time were considered to have their molting inhibited, and presumably would eventually die.

3.2.5 Inheritance of resistance

To verify the genetic basis of resistance, several crosses were made. BA25R and SUS were mated in reciprocal crosses and in crosses within parental populations with at least 40 pairs per cross. Using the overlay bioassay, neonate larvae from each of the four crosses were tested for susceptibility to Cry1F. Crosses were tested at six concentrations, ranging from 0.64 to 200 ng cm^{-2}.

3.2.6 Statistical analyses

Plants and leaf bioassays were analyzed using maximum likelihood estimation of parameters for log-linear models and means were compared by planned contrasts (PROC CATMOD) (SAS INSTITUTE INC., 2004). For the plant bioassay, to test the hypothesis that BA25R was phenotypically resistant, the first contrast compared survival of BA25R on TC1507 maize with survival of SUS on TC1507 maize. To test the cost of resistance, the second contrast compared survival of BA25R on non-Bt maize with survival of SUS on non-Bt maize. For the leaf bioassay, to test the hypothesis that BA25R was phenotypically resistant, the contrast compared survival of BA25R on TC1507 maize with survival of SUS on TC1507 maize.

In the diet bioassays, absence of molting to second instar was subjected to Probit analysis (FINNEY, 1971) using Polo Plus®, version 1.0 (ROBERTSON; PREISLER; RUSSELL, 2003). Data were corrected by Abbott's formula (ABBOTT, 1925). The analyses determined the values of MIC_{50} (concentration that inhibits molting to second instar in 50% of individuals), slope, confidence intervals, and χ^2 significance tests. Weight data were analyzed with nonlinear regression to estimate the EC_{50} (effective concentration that reduces weight gain by 50%) using the JMP 10 (JMP, Version 10, SAS INSTITUTE INC. 2012). MIC_{50} and EC_{50} were considered

significantly different among treatments when their 95% confidence intervals did not overlap.

3.3 Results

3.3.1 Survival on plants and leaves

For the plant bioassay, there was significant deviance among the treatments (Table 3.1) for survival to ten d old larvae (χ^2 = 1051.29, p < 0.0001, 3 df), pupae (χ^2 = 1048.10, p < 0.0001, 3 df), and adult emergence (χ^2 = 1020.91, p < 0.0001, 3 df). BA25R was phenotypically resistant (Contrast 1, 1 df: larvae, χ^2 = 723.01, p < 0.0001; pupae, χ^2 = 702.11, p < 0.0001; adults, χ^2 = 639.87, p < 0.0001), and there was no survival cost of resistance (Contrast 2, 1 df: larvae, χ^2 = 0.04, p = 0.8326; pupae, χ^2 = 0.76, p = 0.3846; adults, χ^2 = 0.06, p = 0.8121). BA25R survived from neonate and produced normal adults on Cry1F maize.

For the leaf bioassay, there was significant deviance among the treatments (Table 3.1) for survival to five d old larvae (χ^2 = 2425.65, p < 0.0001, 3 df), pupae (χ^2 = 2787.39, p < 0.0001, 3 df), and adults (χ^2 = 2908.93, p < 0.0001, 3 df). BA25R was phenotypically resistant (Contrast 1, 1 df: larvae, χ^2 = 1990.39, p < 0.0001; pupae, χ^2 = 2017.02, p < 0.0001; adults, χ^2 = 1996.69, p < 0.0001). Survival of BA25R on TC1507 maize was slightly less than that of SUS on non-Bt maize (Table 3.1), supporting the results for the whole plant assay. Survival of BA25N was intermediate (Table 3.1), indicating that the original BA25 population was not fully resistant.

Both assays reliably detected phenotypic resistance in the BA25R population. No larvae of SUS survived on Cry1F maize, while both BA25N and BA25R had showed survival on TC1507 maize. Survival was 20-40% higher in the leaf bioassay than the whole plant bioassay for BA25R on Cry1F maize and SUS on non-Bt maize (Table 3.1). Because SUS survival on non-Bt maize was higher in leaf than the whole plant bioassay, the leaf bioassay may be more sensitive than the whole plant bioassay for detecting resistance.

Table 3.1 - Survival (% ± SE) of *Spodoptera frugiperda* in TC1507 event

Populations	Event	Larvae[a]	Pupae	Adults
Greenhouse (plant)				
BA25R	TC1507	44.0 ± 11.5	38.0 ± 10.5	28.0 ± 8.0
BA25R	Non-Bt	42.0 ± 9.6	32.0 ± 6.8	26.0 ± 6.7
SUS	TC1507	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
SUS	Non-Bt	44.0 ± 10.7	40.0 ± 8.9	24.0 ± 6.5
Laboratory (leaf)				
BA25R	TC1507	75.0± 3.3	60.8 ± 4.3	50.0± 4.5
BA25N	TC1507	55.0 ± 6.7	38.3 ± 7.4	34.2 ± 7.2
SUS	TC1507	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
SUS	Non-Bt	85.0 ± 3.9	70.8 ± 2.8	63.3 ± 3.6

[a]Survival at ten and five days after infestation in greenhouse and laboratory, respectively.

3.3.2 Concentration-response in overlay and incorporation bioassays

The concentrations used in both bioassays gave a similar range of molt inhibition (Figure 3.1), indicating that the statistical estimation of the slopes, MIC_{50}'s and EC_{50}'s will be reasonably precise. The diet-overlay bioassay (Figure 3.1A) discriminated between the two susceptible populations (SUS and Field-SUS), while the diet-incorporation bioassay did not (Figure 3.1B). The SUS population was slightly more sensitive to Cry1F than the field-SUS population, although this difference was not statistically significant. The overlay and incorporation bioassays gave qualitatively similar results (Table 3.2). In both assays, MIC slopes, MIC_{50}'s and EC_{50}'s, were not statistically different for the two susceptible populations. The diet-overlay bioassay required less Cry1F protein to estimate the MIC_{50} and EC_{50} values for the susceptible populations than the diet-incorporation assay.

The MIC_{50} and EC_{50} could not be accurately determined in BA25R using either method due to lack of concentration-response. The values for MIC_{50} exceeded the highest concentrations used in the bioassays (Table 3.2). Using a Cry1F stock solution of 1,000,000 ng ml^{-1}, it was possible produce a maximum concentration of 20,000 ng cm^{-2} in the overlay bioassay, and 100,000 ng ml^{-1} in the incorporation bioassay. At these highest concentrations, molt inhibition of BA25R did not exceed 50%. Hence the MIC_{50} must be greater than 20,000 ng cm^{-1} and 100,000 ng ml^{-1} for the overlay and incorporation bioassays and resistance ratios of at least 2.000 in the diet-overlay bioassay and at least 280 in the diet-incorporation bioassay. Although the EC_{50} in the overlay bioassay was estimated, it was not possible to determine a confidence limit.

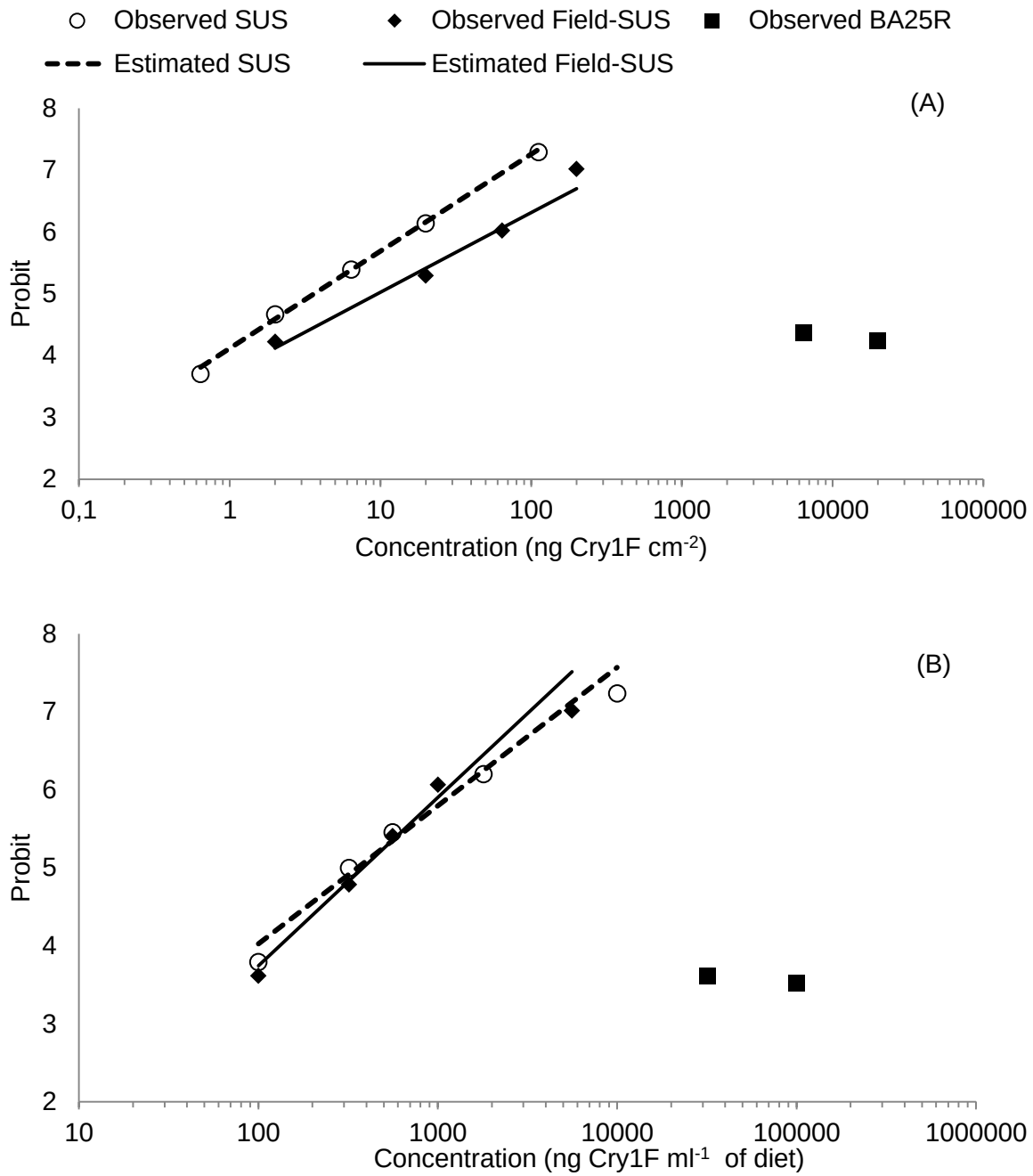

Figure 3.1 - Molting inhibition concentration (MIC) in (A) overlay and (B) incorporation bioassays of populations of *Spodoptera frugiperda* to Cry1F protein

3.3.3 Inheritance of resistance

Although the slope, MIC_{50}, and EC_{50} could not be estimated for BA25R in the diet-overlay bioassays, they were much higher than those estimated for SUS or the reciprocal crosses. The slopes, MIC_{50}'s, and EC_{50}'s in the reciprocal crosses were not statistically different from each other based on the overlapping 95% confidence

intervals. The MIC_{50} and EC_{50} values for the reciprocal crosses were not statistically different from the values for SUS with the exception of the EC_{50} for progeny of crosses of males from SUS and females from BA25R which was significantly higher in than for SUS, with a resistance ratio of 7.03 (Table 3.2).

3.4 Discussion

To prove that unexpected damage to a Bt crop is caused at least in part by field-evolved resistance, it is necessary to show: (1) previously the Bt crop provided economic control of the target pest population, (2) the target pest population later caused excessive damage to the Bt crop, (3) the pest has a resistant phenotype, i.e., individuals can survive from egg to adult feeding on the Bt crop at a greater proportion than susceptible insects, and (4) the resistant phenotype is genetically inherited.

TC1507 maize hybrids producing the Cry1F protein provided excellent economic control of *S. frugiperda* throughout Brazil (SIEBERT et al., 2008) since its introduction during 2009/2010. It is especially noteworthy that economic control in western Bahia was well documented. Recently *S. frugiperda* has caused economic damage to TC1507 maize hybrids in western Bahia. As noted in this paper, the BA25 population was collected from a TC1507 maize field in western Bahia during October 2011, and before collection the field was sprayed with methomyl to control economically damaging populations of *S. frugiperda*. Since that time, several additional TC1507 maize fields in western Bahia have been reported with economic damage caused by *S. frugiperda*.

To demonstrate the existence of phenotypically resistant *S. frugiperda*, we first subjected BA25 to selection by Cry1F in the laboratory to create BA25R, a population putatively homozygote resistant to Cry1F. Phenotypic resistance is demonstrated by the ability to survive on Bt plants from egg to pupa, and the adults must produce viable offspring (ANDOW, 2008). Here we assumed that all normal adults will produce viable offspring. We challenged neonates from BA25R with Cry1F maize plants in the greenhouse and with leaf tissue from Cry1F maize in laboratory during later larval stages. A field plant bioassay was not possible, because natural infestations of *S. frugiperda* are high, which would interfere with estimation of control

mortality, and larvae move readily among plants. Our results clearly show that BA25R *S. frugiperda* are phenotypically resistant to Cry1F maize (Table 3.1).

Before considering the genetic basis of resistance, we conducted experiments to compare bioassay methods and to develop an inexpensive, reliable bioassay. Although a whole plant bioassay provides a definitive proof of phenotypic resistance, for *S. frugiperda*, it takes up a large amount of valuable greenhouse space. A leaf bioassay is much easier, and can be conducted in small areas under controlled environmental conditions, which improves repeatability and precision. In addition, larval escape can be better managed. On the other hand, the concentration of protein could be affected after the leaves have been cut off the plant. Leaf bioassays have been used commonly for resistance monitoring (HUANG; LEONARD; ANDOW, 2007a; 2007b; HUANG et al., 2009; XU et al., 2009; HUANG et al., 2011), including studies on *S. frugiperda* on maize (STORER et al., 2012) or other insects on soybean (BERNARDI et al., 2012). Here we showed SUS survival on non-Bt maize was 1.7 to 2.6 higher in the leaf than the whole plant bioassay, and in both methods all SUS larvae died on TC1507 maize. We conclude that leaf bioassays are suitable to assess field-evolved resistance and the genetic basis of resistance in *S. frugiperda* to TC1507 maize.

We also conducted two diet-based bioassays, one where Cry1F is overlaid on the top of the diet (overlay bioassay), and another where Cry1F is incorporated into the diet (incorporation bioassay). Our results show that both bioassays can be used to detect resistance reliably (Table 4.2). Both diet-based bioassays provide qualitatively similar results as the whole plant and leaf tissue bioassays. The high resistance ratio shown in both diet-based bioassays agreed with the difference in survival of SUS and BA25R on Cry1F maize (Tables 3.1 and 3.2).

Table 3.2 - Bioassay methods in diet and inheritance of resistance of *Spodoptera frugiperda* to Cry1F protein

Populations	n	Slope ± SE	MIC_{50} (95% CI)[a]	χ^2 (df)	RR[c]	n	EC_{50} (95% CI)[b]	RR[c]
Overlay (ng cm^{-2})								
BA25R	1,022	---	>20,000.00	---	>5,524.86	271	5,263.05(not determined)	4,313.98
Field-SUS	676	1.29 ± 0.13	9.66 (3.02 - 19.63)	2.32 (2)	2.67	457	1.97 (0.96 - 3.87)	1.61
SUS	559	1.57 ± 0.15	3.62 (2.76 - 4.64)	0.55 (3)	1.00	259	1.22 (1.02 - 1.48)	1.00
♂BA25R x ♀SUS	396	1.54 ± 0.19	9.73 (3.42 - 21.48)	2.36 (2)	2.69	322	3.06 (1.92 - 5.31)	2.51
♂SUS x ♀BA25R	453	1.59 ± 0.18	25.46 (18.58 - 33.35)	1.14 (2)	7.03	336	1.69 (1.30 - 2.19)	1.38
Incorporation (ng ml^{-1} of diet)								
BA25R	1,528	---	>100,000.00	---	>280.71	1,253	>100,000.00	>2,326.66
Field-SUS	672	2.16 ± 0.21	383.24 (247.78 - 545.04)	5.43 (3)	1.08	428	83.44 (68.66 - 101.43)	1.94
SUS	622	1.77 ± 0.18	356.24 (281.59 - 440.35)	2.71 (3)	1.00	468	42.98 (29.25 - 63.93)	1.00

[a]Molt inhibition concentration (MIC) was considered larvae dead more that failed to molt to second instar.
[b]Effective concentration (EC), required to growth inhibition.
[c]Resistance Ratio (RR) (MIC_{50} or EC_{50} of tested population divided by MIC_{50} or EC_{50} of the SUS population).

The overlay bioassay was faster and used less Cry1F protein than the incorporation bioassay. To conduct baseline susceptibility (MIC) bioassay for SUS using the overlay bioassay with 96 larvae per concentration, we used 20.3 µg Cry1F protein, while for the incorporation bioassay, we used 1,226.9 µg Cry1F, 60 times more Cry1F. This is a greater difference than that reported by Siegfried et al. (2007) comparing the methods on another species. The overlay method has been criticized because surface irregularities on the diet could lead to a non-uniform exposure of larvae to Cry1F, resulting in inconsistent results (SIEGFRIED et al., 2007). Moreover, the Cry1F maize plant does not have Cry1F on its leaf surfaces, but Cry1F is expressed throughout the plant. The diet surface can be made very level by eliminating surface bubbles and consistent through the entire bioassay by reducing the drying rate of the diet by inoculating with neonates and closing the wells just after diet surface is dry. Unlike other maize stalk-boring insects, *S. frugiperda* feeds primarily on maize leaf tissue, which is virtually two-dimensional. In addition, studies comparing the overlay and incorporation showed no major differences between the methods (SAEGLITZ et al., 2006; SIEGFRIED et al., 2007), and the overlay method has been commonly used to assess the susceptibility of *S. frugiperda* to Cry1F (LUO; BANKS; ADANG, 1999; WAQUIL et al., 2004; BLANCO et al., 2010; STORER et al., 2010; 2012). Cry protein is an expensive component in these bioassays, so the overlay method should be preferred over the incorporation method for *S. frugiperda*.

We established the genetic basis of resistance by making reciprocal crosses between BA25R and SUS and crosses within both parental populations, and testing offspring in a concentration-response overlay bioassay (STORER et al., 2010). If resistance were not genetically determined, all crosses would have similar concentration-response curves. Clearly, expression of resistance has a large genetic component because the concentration response curve for the BA25R population is greatly different from the other crosses (Table 3.2). In addition, because the reciprocal crosses were similar (Table 3.2), resistance to Cry1F was autosomal. Resistance in *S. frugiperda* to Cry1F in Puerto Rico was also autosomal (STORER et al., 2010).

Our results showed that unexpected damage to TC1507 maize in Barreiras, Bahia, Brazil was caused by field-evolved resistance in *S. frugiperda*, a result similar to the field failures in Puerto Rico (STORER et al., 2010; 2012). Storer et al. (2012) suggested that conditions in Puerto Rico are similar to some areas of Brazil and that

field-evolved resistance in *S. frugiperda* to TC1507 maize might be fast in Brazil. Unexpected damage in Bahia occurred within three years after the introduction of TC1507 maize. Brazil and Puerto Rico share some characteristics that may have led to strong selection for resistance in *S. frugiperda*. The tropical climate coupled with irrigation during the dry season has enabled continuous maize cropping in Bahia during all months of the year. These conditions enable *S. frugiperda* to have more than 10 generations per year and a history of developing resistance to many classes of insecticides. In addition, there was widespread adoption of TC1507 maize in Bahia and minimal use of the recommended non-Bt refuges. Therefore, implementation of resistance management strategies is urgent to preserve this technology as well as the pyramided events with Cry1F and other Bt proteins in Brazil.

3.5 Conclusions

- Progeny of *S. frugiperda* collected in Cry1F maize in field failure had the ability to survive on the plant indicating that the survivors were resistant to Cry1F.

- Leaf bioassay and diet-overlay bioassay are suitable for characterizing field-evolved resistance to Cry1F in *S. frugiperda*.

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4 DOMINANCE OF CRY1F RESISTANCE ALLELE IN *Spodoptera frugiperda* (J.E. SMITH) (LEPIDOPTERA: NOCTUIDAE) FROM BRAZIL

Abstract

Dominance of resistance has been one of major parameters affecting the rate of evolution of resistance to Bt crops. If resistance is functionally recessive, then a Bt crop is high-dose. High-dose is the capacity of Bt crops to kill heterozygous insects and has been an essential component of the most successful strategy to manage resistance to these crops. Experiments were conducted to evaluate directly and indirectly if TC1507 event is high-dose to *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae). Some heterozygote neonate larvae were able to survive, complete development and emerge as normal adults on TC1507 leaves, but susceptible larvae could not survive for five days. The estimated dominance of resistance was significantly higher than zero; therefore the resistance to Cry1F expressed in TC1507 was not completely recessive. Dominance was estimated to be 0.15 ± 0.09. A 25 fold dilution of TC1507 maize leaf tissue was able to cause a maximum mortality of 37% and to inhibit growth of 82% at seven days after infestation. These values were significantly less than 99%. Resistance to Cry1F in TC1507 maize is incompletely recessive in *S. frugiperda*. TC1507 maize is not high-dose for *S. frugiperda*.

Keywords: High-dose; Fall armyworm; *Bacillus thuringiensis;* Resistance management; Recessive; TC1507

4.1 Introduction

Evolution of resistance is one of the major potential limitations to continued use of Bt crops, but there is a consensus that can be successfully managed. The primary objective of resistance management strategies is to delay and prevent field failures (ANDOW, 2008). The main resistance management strategy that has been used for transgenic crops that produce insecticidal proteins derived from the soil bacterium *Bacillus thuringiensis* (Bt crops) is the high-dose/refuge strategy, which is a combination of high-dose plants and refuge areas (SHELTON et al., 2000). To be effective refuges should be sufficiently large and near to the Bt crop so that they provide susceptible individuals to mate with resistant ones emerging from the Bt crop (McGAUGHEY; WHALON, 1992; SHELTON et al., 2000). In addition, refuges can be highly effective if the initial frequency of resistance alleles is low (ROUSH, 1994) and the Bt crop produces a “high-dose” of the Bt protein against the target insect population.

A plant expressing a high Bt protein concentration, does not necessarily express a high-dose (ANDOW, 2008). High-dose is defined as a protein

concentration in the plant capable of killing all or nearly all target insects that are heterozygous for resistance (TABASHNIK; CROFT, 1982; GOULD, 1998; TABASHNIK; GOULD; CARRIÈRE, 2004). This means that the protein concentration is sufficiently high so that resistance is functionally recessive (TAYLOR; GEORGHIOU, 1979; GOULD, 1998). For Bt plants, the higher the Cry protein concentration, the lower the fitness advantage of heterozygotes in relation to homozygous susceptible (CAPRIO; SUMERFORD; SIMS, 2000). Studies with models have shown that the high-dose is more effective in delaying resistance than low-dose (TABASHNIK; CROFT, 1982; TABASHNIK; GOULD; CARRIÈRE, 2004). Field experience with Bt crops supports generally this expectation (TABASHNIK; RENSBURG; CARRIÈRE, 2009).

High-dose and low dose concepts are based on the genetic concept of dominance (*D*). Dominance is determined by the fitness of the heterozygote in relation to the fitness of the two homozygotes (WRIGHT, 1929). In the insect resistance literature, dominance has been calculated using three different methods (BOURGUET; GENISSEL; RAYMOND, 2000). These differ based on the fitness being compared. D_{LC} is commonly calculated and compares the concentrations of insecticide or Bt protein at a fixed fitness, typically at the LC_{50} (STONE, 1968); technically this is not dominance because fitness are not compared. D_{ML} is calculated from survival rates at a given insecticide or Bt protein concentration (CURTIS; COOK; WOOD, 1978), and D_{WT} is calculated at the insecticide or Bt protein concentration that the insects would be exposed to in the field (BOURGUET; GENISSEL; RAYMOND, 2000). D_{WT} is one of the many possible values of D_{ML}, and is the value that is most relevant for the evolution of resistance. If the insecticide or Bt protein concentration varies in the field, D_{WT} may also vary. In any event, all of these values of *D* require a resistant population before they can be calculated. In the absence of a resistant population, *D* cannot be calculated and dose cannot be accurately determined.

When *D* cannot be calculated, dose must be predicted from operational definitions. US-EPA (1998) proposed several operational definitions of high-dose based on dominance of known resistance to Cry proteins in insects. Probably the most commonly used operational definition of high-dose is when the Cry protein concentration in the plant is 25-fold greater than that required to kill more than 99% of susceptible individuals (US-EPA, 1998). According with this concept, if the Bt plant

produced 25-fold the Bt concentration necessary to kill more than 99% of all susceptible insects, then $D_{WT} \approx 0$ and resistance is presumed to be recessive (BOURGUET; GENISSEL; RAYMOND, 2000). Later, Caprio; Sumerford and Sims (2000) reviewed a more extensive literature and suggested that a better operational definition is that the Bt plant must produce 50-fold the Bt concentration to kill more than 99% of all susceptible insects; however, this definition has rarely been used. One of the most common ways to test these operational definitions is to dilute Bt plant tissue 25-fold (or 50-fold) and determine if the diluted tissue kills more than 99% of susceptible larvae.

After the identification of field-evolved resistance in *Spodoptera frugiperda* (J. E. Smith) [Lepidoptera: Noctuidae] to transgenic Bt maize producing the Cry1F insecticidal protein (event TC1507) in Brazil (Chapter 3), we selected a population of resistant *S. frugiperda* which originated from a TC1507 maize field. In this paper, we use this resistant population to evaluate if the TC1507 in maize is high-dose for *S. frugiperda*. The TC1507 event was evaluated directly using heterozygous and homozygous insects in a leaf bioassay to estimate D_{WT}, and indirectly using a 25-fold tissue dilution in artificial diet with susceptible insects.

4.2 Material and methods

4.2.1 Population collections

A resistant population (BA25) was collected from Barreiras, Bahia, Brazil, after a report of a field failure on October 2011 (Chapter 3). Larvae of BA25 of the first generation in laboratory were divided into two populations, without and with selection in laboratory, respectively designated BA25N and BA25R. BA25N was maintained on artificial diet without Cry1F protein. BA25R was selected for first two generations on Cry1F maize leaves (TC1507) and for two more generations with purified Cry1F protein as describe in Chapter 3. A susceptible population (SUS) was obtained from EMBRAPA Milho e Sorgo, Sete Lagoas, Minas Gerais, Brazil, and has been maintained in laboratory since 1995.

4.2.2 Dominance in leaf bioassays

The hybrid 2B688 HX containing Cry1F (TC1507, Dow AgroSciences) was planted in the field at 80,000 seeds ha^{-1} and fertilized at 550 kg $ha^{-1}$with 4-14-8 (NPK). Every 15 days a new plot was planted, so that all bioassays were conducted with leaves between stages V_6 and V_9 (RITCHIE; HANWAY; BENSON, 1993). This is when *S. frugiperda* causes considerable economic damage to maize. Urea was top dressed at 90 kg ha^{-1} at V_6. Dominance of resistance was characterized in two leaf bioassays – the first with BA25N and the second with BA25R. SUS were mated with BA25N (S×N) and with BA25R (S×R) producing hybrids. Because resistance in BA25R is autosomal (Chapter 3), reciprocal crosses were not needed. At least 40 couples were used for each cross (S×N or S×R), including the three inbred crosses (SUS, BA25N or BA25R). Neonate larvae (<24hr) were used in bioassays.

Twelve larvae from each cross were evaluated separately in 12-well acrylic plates. Each well contained 1 ml of 2.5% agar solution, a filter paper disk and a Cry1F maize leaf disk cut with the aid of a metal punch. One neonate larva was placed with a fine artist's paintbrush in a well and the plates were sealed with plastic film and an acrylic cover. Each experiment was replicated 10 times. At five days after inoculation (DAI), the surviving larvae were collected and placed into plastic cups (100 ml), which contained 7 ml of 2.5% agar solution, filter paper and pieces of Cry1F maize leaves. Cry1F maize leaves were changed in an interval of maximum three days until pupation. Pupae were collected and kept in trays with filter paper and isolated in plastic cups (50 ml) until adult emergence. Larvae and pupae were kept in a climate chamber at 27 ± 1 °C, 60 ± 10% RH and 14:10 h (L:D) photoperiod. Survival at five DAI, pupation, and emergence of normal appearing adults were measured.

4.2.3 Tissue dilution bioassays

Maize was planted in two crop seasons on 20 October 2011 (main season) and on 4 February 2012 (second season). Hybrids from two companies were used 2B688 HX from Dow AgroSciences and 30F35 HX from Pioneer Hi-Bred, plus their non-Bt near-isogenic hybrids. Hybrids were planted in small plots at 80,000 seeds $ha^{-1}$and fertilized at 550 kg ha^{-1} with 4-14-8 (NPK). Urea was top dressed at 90 kg ha^{-1}at V_6 (RITCHIE; HANWAY; BENSON, 1993). Leaf tissue was collected at V_3, V_6 and V_9. The whorl leaves were collected washed, and the sheath, collar and midrib

discarded. The tissue was weighed (fresh weight) and stored at -80 °C. The frozen leaves were lyophilized to a constant weight and this weight was recorded (dry weight). Lyophilized tissue was crushed in blender, sieved (42 mesh of nylon sieve) to produce a fine powder, and stored at -80 °C. Before bioassays, the presence of the Cry1F protein in lyophilized tissue was tested with the QuickStix kit (lot 173621, EnviroLogic).

The bioassays were performed by adding lyophilized tissue to artificial diet (KASTEN JR.; PRECETI; PARRA, 1978). For each hybrid, crop season and maize stage lyophilized leaf powder was added to correspond to a 25-fold of dilution of fresh tissue in artificial diet. The 25-fold of dilution of fresh tissue, *TD*, was estimated with the formula

$$TD = \left(\frac{fw \times 0.04}{dw}\right) 1000$$

where *fw,* and *dw* are the weight of fresh (g) and dry (g) tissue, respectively. The *TD* is given in mg of *dw* by ml of artificial diet.

The leaf powder was mixed in 30 ml of artificial diet in Falcon tubes (50 ml) kept in a water bath at 54 °C. The diet was homogenized in a mixer, poured into wells at a rate of 1 ml, and allowed to cool. The bioassays were conducted in128-well trays (BIO-ASSAY TRAY BIO-BA-128, Pitman, NJ, USA). Half of each tray was used for a Cry1F hybrid and half for the non-Bt near-isogenic hybrid (control). These were replicated three times so that 192 SUS larvae were tested for each crop season (two seasons), hybrid (four hybrids) and maize stage (three stages). Individual SUS neonate larvae were placed into each well with a fine artist's paintbrush, and the wells were closed with air-permeable lids. Trays were kept in a climate chamber at 27 ± 1 °C, 60 ± 10% RH and 14:10 h (L:D) photoperiod. Development and weight were measured at seven DAI. Larvae that did not reach the second instar were considered dead.

4.2.4 Statistical analysis

Dominance, *D*, has been estimated commonly with the formula

$$D = \frac{w_{RS} - w_{SS}}{w_{RR} - w_{SS}}$$

where w_{SS}, w_{RS}, and w_{RR} are the fitness of the *SS*, *RS* and *RR* genotypes, respectively. For statistical analysis, this formula requires that fitness estimates are independent, but typically such data are not independent. In our case, fitness estimates were correlated because each replicate was conducted at the same time using similar materials in similar environments. Therefore we estimated dominance taking this covariance into account as follows. Let

$$x = w_{RS} - w_{SS} \text{and} \qquad y = \frac{1}{(w_{RR} - w_{SS})}$$

where the fitness (*w*'s) are estimated at field concentrations so that we can estimate D_{WT}. The expected value and variance for dominance is estimated from

$$E(D_{WT}) = E(xy) = \bar{x}\bar{y} + cov(x, y)$$
$$Var(D_{WT}) = Var(xy) = (\overline{x}^2\overline{y}^2) + cov(x^2y^2) + E(xy)^2$$

For our experiment, w_{RS} is the survival of the S×N or S×R larvae on Cry1F leaf tissue; w_{SS} is the survival of SUS larvae on Cry1F leaf tissue; and w_{RR} is the survival of either BA25N or BA25R larvae on Cry1F leaf tissue. In these experiments no SUS larva survived on Cry1F leaf tissue, therefore w_{SS} = 0 with no measured variance, and x and y simplified to

$$x = w_{RS} \text{and} \qquad y = \frac{1}{w_{RR}}$$

Values of D_{WT} were tested against the hypothesis that D_{WT} = 0 using the estimated expected values and variances with a t-test. In tissue dilution bioassays TC1507 would be considered high-dose if more than 99% of susceptible larvae died at 25-fold dilution (US-EPA, 1998). The weight data were converted to growth inhibition in relation to the non Bt isogenic hybrid. Dead larvae had a zero weight. The growth inhibition data were analyzed as a two-way RCB ANOVA with replicate

as the block and maize hybrids (2B688 HX and 30F35), and maize growth stage (V_3, V_6 and V_9) as the two factors. Means were separated with Turkey's HSD test at 5% probability of error. Mortality was analyzed with a log-linear model with replicate, maize hybrid and maize stage as factors. The crop season (main season and second season) were considered different experiments, and the analyses were done using the R 2.15.1 program (R CORE TEAM, 2012).

4.3 Results

4.3.1 Dominance (D_{WT}) in leaf bioassays

Dominance of *S. frugiperda* resistance was measured on two populations derived from BA25. Survival of BA25N larvae to adults was 38.7%, while comparable survival of BA25R was 59.2% on Cry1F maize tissue. Some of the heterozygous S×N and S×R larvae were able to complete development on TC1507 leaf tissue and to produce normal adults. Survival of S×N was 9.2% and survival of S×R was 8.3%.No SUS larvae survived on the Cry1F leaf tissue; all died by five DAI (Figure 4.1).

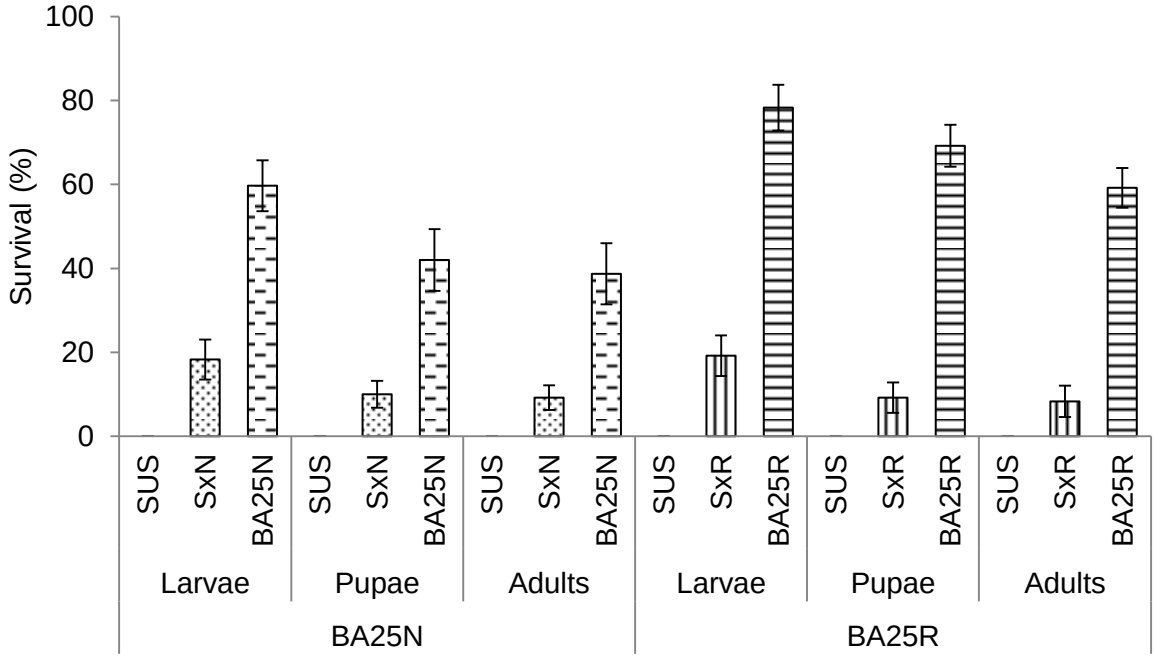

Figure 4.1 - Survival (% ± SE) of *Spodoptera frugiperda* on V_6-V_9 TC1507 event in leaf bioassay

D_{WT} was calculated for BA25N and BA25R. D_{WT} ranged from 0.14 (survival to adults of BA25R) to 0.35 (5-d survival of larvae of BA25N) and in all cases was significantly greater than zero; therefore resistance to Cry1F expressed in TC1507 was not completely recessive (Table 4.1). It was consistently, although non-significantly, higher for larvae than for pupae or adults, and was consistently and non-significantly higher for BA25N than BA25R.

Table 4.1 - Dominance of relative fitness (D_{WT} ± SE) of resistant *Spodoptera frugiperda* on V_6-V_9 TC1507 event in leaf bioassay

Populations	Larvae[a]	Pupae	Adults
BA25N	0.35 ± 0.19	0.22 ± 0.12	0.22 ± 0.12
BA25R	0.25 ± 0.13	0.14 ± 0.09	0.15 ± 0.09

[a]five days after infestation.

4.3.2 Tissue dilution bioassays

Twenty-five fold dilution of TC1507 maize tissue showed activity against susceptible larvae of *S. frugiperda* (Figure 4.2). In no case, however, did the activity come close to approaching the 99% necessary to consider TC1507 to be high dose. When incorporated into artificial diet at a 25-fold dilution, mortality (dead plus alive first instar larvae) of susceptible *S. frugiperda* after seven days ranged from 12% to 37% for both crop seasons, both Cry1F maize hybrids and three maize stages. The maximum mortality was observed in the 2B688 hybrid during the second season at the V_3 stage (Figure 4.2 – (A) and (B)). There were no significant differences in survival related to season, hybrid, or maize growth stage.

The diluted, lyophilized TC1507 tissue showed high growth inhibition of susceptible insects. Growth inhibition ranged from 44% to 82% for both crop seasons, both Cry1F maize hybrids and three maize stages. Even if we would assume that all larvae with inhibited growth ultimately die, mortality of susceptible *S. frugiperda* on diluted TC1507 tissue was significantly lower than 99%. The maximum values of growth inhibition were observed in V_9 stage in main crop season in both maize hybrids. The average weight of larvae was 12.10 milligrams for non-Bt isogenic hybrid and 4.15 milligrams for Bt hybrids, therefore the average of growth inhibition was around 66% (Figure 4.2 – (C) and (D)).

Growth inhibition in main season was significantly different among maize stages (F = 23.74, p < 0.0001, 2 df). Stage V_9 was significantly higher than V_3 (p < 0.0001, 1 df) and V_6 (p < 0.0001, 1 df). Maize hybrid and the interaction (maize x stage) were not significantly different during the main season. In the second season, maize stage was again significantly different (F = 7.28, p = 0.0016, 2 df), and the interaction of maize hybrid x stage was also significant (F = 8.99, p= 0.0004, 2 df). Stage V_9 was significantly higher than V_3 (p = 0.0333, 1 df) and V_6 (p = 0.0013, 1 df).

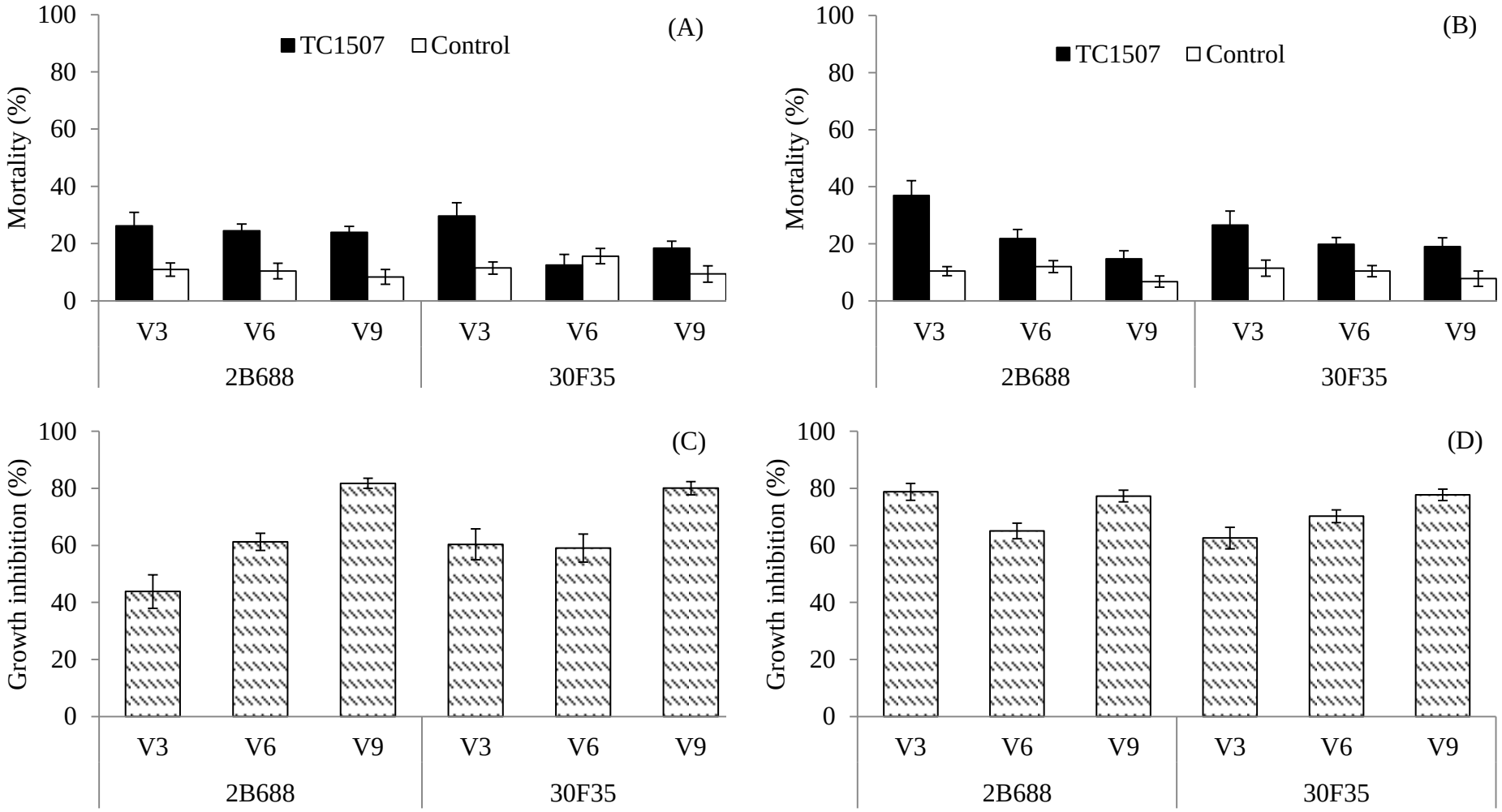

Figure 4.2 - Mortality (A – main season, and B second season) and growth inhibition (C – main season, and D second season) (% ± SE) of *Spodoptera frugiperda* at 25-fold dilution in artificial diet of fresh leaf tissue of Cry1F (TC1507) maize hybrids

4.4 Discussion

We found that the Cry1F R allele in *S. frugiperda* from Bahia is not completely recessive on TC1507 maize when compared to a susceptible laboratory population, because values of D_{WT} were significantly greater than zero. This was caused by the higher fitness of heterozygotes on Cry1F maize than that of the susceptible population. The susceptible larvae did not survive on TC1507 leaf tissue, but around 9% of heterozygotes survived. Dominance was estimated to be 0.15 ± 0.09, which is large enough that TC1507 would not be considered high-dose (STORER et al., 2010; GHIMIRE et al., 2011). However, it is possible that this estimate of dominance was high because of potential hybrid vigor from the outcrossing of SUS and BA25R/N. However, inbreeding depression is not known in *S. frugiperda*, so this only a hypothesis. It is also possible that this estimate of dominance was high because the SUS population is not related to the BA25 population. The estimates of dominance are phenotypic based, not genotypic. If the SUS population is more susceptible to Cry1F, or performs weaker on maize tissue, than unselected field populations, the fitness of SUS will be under-estimated by this population and therefore dominance values over-estimated. The relative fitness of the susceptible laboratory population and susceptible field populations were not measured. Even so, we conclude that Cry1F in TC1507 maize is probably not high-dose against *S. frugiperda*.

The estimated dominance was similar to that obtained with *S. frugiperda* resistant to Cry1F TC1507 maize from Puerto Rico, although D_{ML} was estimated (STORER et al., 2010). According to the concentration considered, D_{ML} can vary from 0 to 1 (CURTIS; COOK; WOOD, 1978; TAYLOR; GEORGHIOU, 1979; FERRÉ; VAN RIE, 2002). D_{ML} was inversely related to the protein concentration in *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) resistant to Cry1Ac (TABASHNIK et al., 2002). Some authors have proposed to measure dominance based on series of concentrations (TABASHNIK; GOULD; CARRIÈRE, 2004). However, estimation of dominance should focus more on D_{WT}, which is more important for projecting the evolution of resistance (BOURGUET; GENISSEL; RAYMOND, 2000). D_{WT} varied from 0.19 to 0.25 in a leaf tissue bioassay for *Diatraea saccharalis* (Fabricius) (Lepidoptera: Crambidae) resistant to Cry1Ab; therefore the maize events with *cry1Ab* gene were not considered high-dose (GHIMIRE et al., 2011). In Puerto Rico, *S. frugiperda* resistant to Cry1F had D_{ML}= 0.14 at 3,330 ng cm^{-2} (STORER et al.,

2010). The authors used this Cry1F concentration, because it caused similar levels of mortality of *S. frugiperda* as was seen under field conditions.

Incompletely recessive dominance to Cry proteins has been explained by two main causes, haploinsufficiency (SARJAN et al., 2009) and presence of modifiers (BOURGUET; GENISSEL; RAYMOND, 2000; SARJAN et al., 2009). Haploinsufficiency is reduced protein synthesis when the locus is in heterozygosis and, therefore it results an abnormal phenotype (DEUTSCHBAUER et al., 2005). In insect resistance it means that just one copy of the allele of susceptibility is not enough to generate completely susceptible phenotypes (SARJAN et al., 2009). Modifiers can be genes of metabolic tolerance mechanisms or regions in the genome that regulate synthesis of major resistant genes (BOURGUET et al., 1997; SARJAN et al., 2009). Metabolic tolerance mechanisms are caused by dominant multi-gene traits and account from low to medium resistance and possibly are not connected to the major resistance gene (SARJAN et al., 2009), but can affect the dominance.

The data from the tissue dilution bioassays confirmed that TC1507 is a non-high-dose event for *S. frugiperda*. Low mortality was observed at 25-fold dilution, and although growth inhibition was significantly higher, this could not give 99% mortality. Susceptible survival in the 25-fold dilution tissue bioassay was 76.8%, while heterozygous survival in the Cry1F leaf bioassay was 8.3% for the same hybrid and maize stage. Clearly, since larvae did not have 99% mortality at a 25-fold dilution of leaf tissue, larvae would also not have 99% mortality on a 50-fold dilution, so both the US-EPA (1998) and Caprio; Sumerford and Sims (2000) operational definitions for high-dose were not met. Using the 25-fold dilution concept, the Cry1Ac soybean events TIC107 and MON 87701 × MON 89788 were apparently high-dose for *Anticarsia gemmatalis* Hübner (Lepidoptera: Noctuidae), but were not high dose for *Pseudoplusia includens* (Walker) (Lepidoptera: Noctuidae) (MacRAE et al., 2005; BERNARDI et al., 2012).

One potential limitation to these operational definitions is the possibility of variation among cultivars/hybrids, plant growth stage, and various environmental conditions (US-EPA, 1998). We tested two hybrids from different companies at three growth stages in two environments. Although mortality was not significantly affected by any of these factors, growth inhibition was significantly affected by maize growth stage or an interaction between growth stage and hybrid. Had the observed mortality

been close to 99%, this variation could affect whether the operational definition of high-dose would have been met.

One possible reason for the rapid occurrence of field-evolved resistance to Cry1F TC1507 maize in *S. frugiperda* in Puerto Rico and Brazil could be that TC1507 is a low-dose event (HARDKE et al., 2011; Chapter 3). This was confirmed for western Bahia, Brazil in this paper. We showed that event TC1507 is not high-dose for *S. frugiperda*, because the heterozygotes can survive and develop on Cry1F leaf tissue and produce normal adults. Models have shown that low dose events are expected to have more rapid resistance evolution than high-dose events (TABASHNIK; CROFT, 1982; TABASHNIK; GOULD; CARRIÈRE, 2004). Strategies to delay resistance to Cry1F maize in *S. frugiperda* are based on the high-dose plus refuge strategy in Brazil. Field failures of Cry1F TC1507 maize caused by *S. frugiperda* in Puerto Rico and Brazil (STORER et al., 2010; Chapter 3) should alert us that the useful life of single trait TC1507 maize may be short. Additional or alternative resistance management strategies, such as the replacement of single-trait Bt maize with pyramided Bt maize that produces multiple proteins targeting the same insect pests, should be implemented, not just in Brazil, but wherever this technology is in use and *S. frugiperda* is the major pest. For example, maize containing the TC1507 event and MON 89034 event has recently been launched in Brazil and Argentina. These hybrids produce Cry1A.105 and Cry2Ab2 in addition to Cry1F. Each of these proteins is active against *S. frugiperda*. In the absence of high levels of cross resistance, such pyramided-trait products are expected to be much more durable than single trait products (STORER et al., 2012).

4.5 Conclusions

- TC1507 maize is not high-dose event for *S. frugiperda*.

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5 COMPLEMENTATION TEST FOR ALLELISM IN CRY1F RESISTANT *Spodoptera frugiperda* (J.E. SMITH) (LEPIDOPTERA: NOCTUIDAE) POPULATIONS FROM BRAZIL

Abstract

Field-evolved resistance to Bt plants has been recorded in many countries, insect species and Bt proteins, and resistance has been found to be usually monogenic, autosomal, and from recessive to incompletely recessive alleles. However, it has not been determined if resistance allele were at same locus in the field-evolved resistance populations. Field-evolved resistance was already reported to *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) in Puerto Rico and Brazil. Here we propose to test if Cry1F resistance in *S. frugiperda* populations from different states of Brazil shares the same locus. Bioassays were conducted with eight resistant populations and one susceptible population. We first measured the dominance of resistance at 2,000 ng cm^{-2} of Cry1F protein in a diet overlay bioassay using two resistant populations. The hybrids between susceptible and two resistant populations were all dead at seven days after infestation with 2,000 ng cm^{-2} of Cry1F protein in the overlay bioassay. Therefore, resistance in these two resistant populations was recessive at this Cry1F concentration; in other words this concentration was discriminatory. In complementation tests one resistant population was cross with the other seven resistant populations. F_1 larvae from each cross had the same survival with or without 2,000 ng cm^{-2} of Cry1F protein, demonstrating that they are all complementary. The results from the complementation tests showed geographically widespread resistance to Cry1F in *S. frugiperda* in Brazil and strongly suggested that Cry1F resistance alleles in all eight populations were at the same locus.

Keywords: Resistance locus; Fall armyworm; *Bacillus thuringiensis;* Genetic basis; Allele

5.1 Introduction

Transgenic plants with genes from the bacterium *Bacillus thuringiensis* Berliner (Bt crops) have been grown since 1996 (JAMES, 2010), but the evolution of resistance has been a major concern in the continued use of Bt proteins expressed in plants (McGAUGHEY; WHALON, 1992). Despite that insect resistance management (IRM) strategies have been used to delay the evolution of resistance in Bt crops, field-evolved resistance has been recorded in several countries, insect species and Bt proteins (VAN RENSBURG, 2007; LIU et al., 2010; STORER et al., 2010; DHURUA; GUJAR, 2011; GASSMANN et al., 2011; Chapter 3). Resistance

management strategies can be more accurately designed if the genetic basis of resistance to Bt crops is better understood (GOULD, 1998; FERRÉ; RIE, 2002). Resistance to Cry proteins has been found to be inherited as monogenic (TABASHNIK et al., 1997a; MAHON et al., 2007; PEREIRA; STORER; SIEGFRIED, 2008), usually autosomal (MAHON et al., 2007; PEREIRA; STORER; SIEGFRIED, 2008; STORER et al., 2010; Chapter 3), recessive to incompletely recessive alleles (MAHON et al., 2007; PEREIRA; STORER; SIEGFRIED, 2008; STORER et al., 2010; PETZOLD-MAXWELL et al., 2012; ZHANG et al., 2012; Chapter 4). The genetic basis of field-evolved resistance has been characterized in populations of *Helicoverpa armigera* (Hübner) (ZHANG et al., 2012), *Diabrotica virgifera* virgifera LeConte (PETZOLD-MAXWELL et al., 2012), and *Spodoptera frugiperda* (J.E. Smith) (STORER et al., 2010; Chapter 3; Chapter 4). However, it has not been determined if resistance alleles were at the same locus in different field-derived resistance populations.

Complementation tests are used to determine if independently derived alleles are located at the same locus for different populations (LEWIS, 1951). For resistance to Bt proteins, complementation tests have been used on several laboratory derived colonies. In *Plutella xylostella* (Linnaeus) resistance to Cry1Aa, Cry1Ab, Cry1Ac, and Cry1F proteins were located at the same locus in two populations and that allele probably conferred resistance to all of the proteins. A third resistant population was allelic, but conferred resistance only to Cry1Ab (TABASHNIK et al., 1997b). In *Pectinophora gossypiella* (Saunders) three populations were allelic for resistance to Cry1Ac (TABASHNIK et al., 2004; FABRICK; TABASHNIK, 2012). A recent work selected two resistant populations of *Helicoverpa punctigera* (Wallengren) to Vip3A, and these populations were allelic (MAHON; DOWNES; JAMES, 2012). In Australia ten populations of *H. armigera* selected for Cry2Ab resistance were allelic (MAHON; OLSEN; DOWNES, 2008; MAHON et al., 2010). Another work with *H. armigera* in Australia with two populations selected to Vip3A resistance shared the locus of resistance (MAHON; DOWNES; JAMES, 2012). In China, ten populations of *H. armigera* were selected for resistance to Cry1Ac; eight populations were allelic at a cadherin locus and the other two populations were not allelic. One population had at least one non-recessive allele, and the other population had a high resistance ratio but in different loci (ZHANG et al., 2012).

Cry1F maize (TC1507, Herculex® I Insect Protection) has been grown in Brazil to protect against *S. frugiperda* since the 2009/10 crop season (STORER et al., 2012). Resistance to Cry1F maize has already been documented in this species in Puerto Rico (STORER et al., 2010) and Brazil (Chapter 3). The genetic basis of resistance was partially characterized, and both populations had monogenic, autosomal and incompletely recessive resistance (STORER et al., 2010; Chapter 4). The species migrates annually from the southern United States to central and eastern USA and Canada (NAGOSHI; MEAGHER, 2008). There is genetic heterogeneity within the species that results in a phenotypic variability (CLARK et al. 2007; NAGOSHI; MEAGHER, 2008), such as the maize and rice biotypes (PASHLEY, 1986; BUSATO et al., 2004). Based on this, we expected to find considerable genetic variability for resistance in *S. frugiperda*. Variation in resistance could involve different loci or multiple resistance alleles at the same locus. Here we propose to test if Cry1F resistant *S. frugiperda* from different states of Brazil share the same resistance locus.

5.2 Material and methods

5.2.1 Geographic source of populations

The bioassays were conducted with eight resistant populations of *S. frugiperda* (BA25R, BA27, MS11, BA29, MT19, GO23, PR39, and RS12) and one susceptible (SUS) population. BA25R was sampled in Barreiras, Bahia, Brazil, after a report of Cry1F maize field failure in October 2011. The BA25R population was confirmed in the laboratory as resistant to Cry1F protein (Chapter 3). The populations BA27 and RS12 were sampled in Cry1F maize after failuresunexpected field damage was reported in São Desidério, Bahia, Brazil in January 2012, and in São Paulo das Missões, Rio Grande do Sul, Brazil in October 2012, respectively. The remaining five populations, MS11, BA29, MT19, GO23, and PR39 were sampled from non-Bt maize fields. MS11 was sampled in São Gabriel do Oeste, Mato Grosso do Sul, Brazil. BA29 was sampled in Barreiras, Bahia, Brazil five months after a field failure report (BA25). MT19 was selected from populations sampled in Sinop and Campo Novo dos Parecis, Mato Grosso, Brazil. GO23 was sampled in Caiapônia, Goiás, Brazil, and PR39 was sampled in Palotina, Parana, Brazil. The susceptible population (SUS)

was obtained from EMBRAPA Milho e Sorgo, Sete Lagoas, Minas Gerais, Brazil, and has been maintained in the laboratory since 1995 (Table 5.1).

Table 5.1 - Populations of *Spodoptera frugiperda* used in complementation test

Code	Location	Maize[a]	Date	Detection[b]	N
SUS	Sete Lagoas/MG	---	1995	---	---
BA25	Barreiras/BA	TC1507	Oct. 2011	Phenotypic	94
BA27	São Desidério/BA	TC1507	Jan. 2012	F_2 screen	480
MS11	São Gabriel do Oeste/MS	Non-Bt	Mar. 2012	F_2 screen	486
BA29	Barreiras/BA	Non-Bt	Apr. 2012	Phenotypic	615
MT19	Sinop/MT	Non-Bt	Apr. 2012	F_2 screen	568
	Campo Novo dos Parecis/MT	Non-Bt	Apr. 2012	F_2 screen	570
GO23	Caiapônia/GO	Non-Bt	May 2012	F_2 screen	524
PR39	Palotina/PR	Non-Bt	May 2012	Phenotypic	435
RS12	São Paulo das Missões/RS	TC1507	Oct. 2012	Phenotypic	163

[a] Host (maize sampled)
[b] Method used to detect resistance.

5.2.2 Isolation and identification of populations

As described by Chapter 3, the population BA25R was selected in the laboratory at two selection intensities; the last selection was made with 20,000 ng cm^{-2} of Cry1F protein. Resistance was detected in the remaining populations with phenotypic screen or F_2 screen (ANDOW; ALSTAD, 1998). BA29, PR39, and RS12 were detected using a phenotypic screen, and BA27, MS11, MT19, and GO23 were detected with an F_2 screen. During detection and selection, eggs, pupae, and adults were reared at 25 ± 1 °C, 60 ± 10% relative humidity, and 14:10 h (L:D) photoperiod. Larvae were kept in climate chamber at 27 ± 1 °C, 60 ± 10% relative humidity and 14:10 h (L:D) photoperiod.

In the phenotypic screen, field-collected larvae were individually placed in 50 ml plastic cups with 10 ml of artificial diet (KASTEN JÚNIOR; PRECETI; PARRA, 1978) and sealed with an acrylic sheet, and remained in the cups until pupation. Pupae were placed in cylindrical PVC cages (10 cm diameter × 21 cm), lined with newsprint and closed at the top and bottom with Petri dishes (12 cm diameter × 1.5 cm), and the bottom Petri dish was lined with filter paper. After emergence adults were paired with about 20 pairs per cage, and food was provided by solution of 10% honey in a plastic cup (50 ml) clogged with water absorbent cotton. F_1 eggs were collected every three days by switching adults to new containers, and stored in the containers with filter paper moistened with distilled water. The F_1 neonate larvae (<24

h) were inoculated on 20 ml of artificial diet in plastic cups (100 ml), and at the second instar, larvae were placed individually in plastic cups (50 ml) with 10 ml of artificial diet and sealed with an acrylic sheet, and remained in the cups until pupation. These procedures were repeated with F_1 pupae and adults and F_2 eggs.

F_2 generation neonate larvae were used in the phenotypic screen using an overlay bioassay (MARÇON et al., 1999) conducted in 128-well bioassay trays (BIO-ASSAY TRAY BIO-BA-128, Pitman, NJ, USA), with nine replicates. Each replicate tested 112 larvae with Cry1F protein and 16 larvae without Cry1F protein. Cry1F protein (sample TSN104550, 35% Cry1F protein, available from Dow AgroSciences) was diluted in buffer (10mM potassium phosphate dibasic), followed by addition of 0.1% Triton X-100 to obtain a uniform distribution of the protein on the surface of the diet at a rate of 20 µl cm^{-2}. The bioassay was performed with 2,000 ng cm^{-2} of Cry1F protein. Each well of the tray had 1 ml of artificial diet and a surface area of 1.5 cm^{-2}. The artificial diet was slowly poured into the wells and any surface bubbles were punctured and eliminated. Individual neonate larvae were released with a fine artist's paintbrush into each well, and the wells were closed with air-permeable lids. Larvae that did not reach the second instar at 7 days after infestation (DAI) were considered to have their molting inhibited, and presumably would eventually die.

In the F_2 screen, field-collected larvae were individually reared as described for the phenotypic method. Pupae were collected and kept in trays with filter paper, and isolated in plastic cups (50 ml) until adult emergence. Adults were pair-mated to produce isofemale lines (one couple) in 500 ml plastic containers. Eggs were collected every two days and neonates were handled as described above. Five second instar larvae were transferred on 45 ml of artificial diet in plastic cups (100 ml). Each isofemale line was inoculated into 22 plastic cups (110 second instars). Rearing methods otherwise followed those described for the phenotypic screen. F_2 neonate larvae were screened using the overlay method as described above. After the diet surface dried, 20 F_2 neonates were released on each plate. For each F_2 line, 120 neonates were tested. Larvae that did not reach the second instar at 7 DAI were considered to have their molting inhibited, and presumably would eventually die.

Selection to establish homozygous resistant lines started with the larvae that reached the second instar in the phenotypic and F_2 screen screens. Selection was conducted with the diet overlay method at 20,000 ng cm^{-2} of Cry1F protein. Insects recovered from different isofemale lines but the same geographic population were

pooled. The number of insects tested was that necessary to obtain at least 100 normal adults for each geographic population. At 7 DAI the larvae that molted to second instar were recovered and reared in a diet without Cry1F protein until pupation. The pupae and adults were handled as previously described.

5.2.3 Effective dominance

Effective dominance was estimated for resistant populations BA25R and RS12. Reciprocal crosses were made with these two resistant populations and SUS. In addition, BA25R, RS12, and SUS intra-population crosses were tested. Neonate larvae from each cross were tested individually in four 128-well trays; 112 wells contained Cry1F at 2,000 ng cm^{-2} and 16 wells were controls with no Cry1F. The Cry1F concentration was chosen to render resistance fully recessive, which allows for unambiguous interpretation of the results of the complementation tests. Bioassays were conducted as described above. Assays were conducted in a climate chamber at 27 ± 1 °C, 60 ± 10% relative humidity and 14:10 h (L:D) photoperiod. Survival was measured at 7 DAI, and larvae that did not reach the second instar were considered to have their molting inhibited, and presumably would eventually die. Dominance and its standard error were estimated using the statistical method in Chapter 4. The calculated dominance is effective dominance (D_{ML}) (CURTIS; COOK; WOOD, 1978; BOURGUET; GENISSEL; RAYMOND, 2000).

5.2.4 Complementation test bioassays

We previously confirmed that BA25R carried a single autosomal Cry1F resistance allele (Chapter 3), therefore it was used as the standard resistant population. BA25R was mated in reciprocal crosses with all the resistant populations (Table 5.1). Reciprocal crosses were done with at least 30 insect pairs in each cross. Neonate progeny were subjected to mortality bioassays without or with Cry1F protein at 2,000 ng cm^{-2}, as described above. If alleles for Cry1F resistance in two populations were at the same locus, then the progeny would be phenotypically resistant, and if the alleles in two populations were at different loci without epistasis, then the progeny would be phenotypically susceptible (TABASHNIK et al., 1998;

TABASHNIK et al., 2004). The data were analyzed using the MLEs of parameters for log-linear models (PROC CATMOD) (SAS INSTITUTE INC, 2004).

5.3 Results

5.3.1 Effective dominance

Larvae from SUS and the hybrids BA25R x SUS, and RS12 x SUS were all dead at 7 DAI when exposed to 2,000 ng cm^{-2} of Cry1F protein. Larvae from the two resistant populations survived at 2,000 ng cm^{-2} of Cry1F protein at rates similar to the controls. Mortality in controls was <11% for all crosses. Dominance was calculated as D_{ML} = 0.0±0.0 for BA25R and RS12. Both resistant populations were confirmed to be phenotypically totally recessive at 2,000 ng cm^{-2} of Cry1F protein in the overlay bioassay (Table 5.2).

Table 5.2 - Survival (% ± SE) of Cry1F resistant *Spodoptera frugiperda* populations from Brazil in complementation test

Cross	2,000 ng of Cry1F cm^{-2}	Without Cry1F	Statistic
	Dominance		D_{ML}[a]
BA25R x BA25R	94.2 ± 1.7	96.9 ± 1.8	0.0 ± 0.0
BA25R x SUS	0.0 ± 0.0	93.8 ± 2.6	
RS12 x RS12	85.1 ± 1.7	92.2 ± 3.9	0.0 ± 0.0
RS12 x SUS	0.0 ± 0.0	89.1 ± 6.4	
SUS x SUS	0.0 ± 0.0	93.3 ± 3.8	---
	Complementation test		*P* value[b]
BA27 x BA25R	98.7 ± 0.3	98.4 ± 1.6	0.6682
BA29 x BA25R	93.9 ± 1.8	90.6 ± 3.1	0.2771
MT19 x BA25R	95.5 ± 1.4	93.8 ± 2.6	0.4918
GO23 x BA25R	93.8 ± 2.8	100.0 ± 0.0	0.1340
MS11 x BA25R	92.0 ± 4.1	95.2 ± 1.6	0.4090
PR39 x BA25R	97.5 ± 0.9	93.8 ± 4.4	0.0793
RS12 x BA25R	96.6 ± 0.2	98.4 ± 1.6	0.5735

[a] Effective dominance at discriminating dose estimated according to Chapter 4.
[b] log-linear models.

5.3.2 Complementation test

The cross BA27 x BA25R had the highest survival on Cry1F protein, and MS11 x BA25R had the lowest survival. The difference between the lowest and the highest survival was 6.5% in Cry1F protein. In all of the complementation crosses, there was no statistical difference in survival with or without Cry1F protein at 2,000 ng

cm^{-2} in the overlay bioassay. F_1 larvae from all seven complementation crosses were resistant to Cry1F protein. These results reject the hypothesis that any of the resistance alleles in all of the populations were at different loci with no epistasis, and suggests strongly that the resistance alleles are allelic. Resistance alleles in all eight resistant populations probably share the locus (Table 5.2).

5.4 Discussion

Before conducting the complementation tests, the first step was determine the dominance of resistance because results from complementation tests are most easily interpreted when the trait of interest is fully recessive (LEWIS, 1951). In two previously published works, resistance to Cry1F in *S. frugiperda* was incompletely recessive. Populations from Puerto Rico showed D_{ML} = 0.14 at 3,330 ng cm^{-2} (STORER et al., 2010); however different bioassay conditions were used than in the present paper. We previously showed that dominance in BA25R based on survival on Cry1F maize was D_{WT} = 0.15 (Chapter 4). Dominance can vary depending on the trait and environment. D_{WT} is the dominance value that will drive resistance evolution in the field, while D_{ML} will vary with the concentration of Cry1F (BOURGUET; GENISSEL; RAYMOND, 2000). Dominance (D_{ML}) in *P. gossypiella* resistant to Cry1Ac was inversely the protein concentration tested (TABASHNIK et al., 2002). We found that resistance was fully recessive in a diet overlay bioassay with 2,000 ng cm^{-2} Cry1F. In others words, 2,000 ng cm^{-2} Cry1F was a discriminatory concentration.

The results from the complementation tests strongly suggested that the Cry1F resistance alleles in all eight populations were at same locus in *S. frugiperda* from Brazil. Because the resistance alleles are probably allelic, F_1 screens may be appropriate for resistance monitoring in the future (YUE et al., 2008; MAHON et al., 2010; MAHON; DOWNES; JAMES, 2012). Resistance alleles have been from the same locus in many insect species selected for resistance to a Bt protein, such as *P. xylostella* (TABASHNIK et al., 1997b), *P. gossypiella* (TABASHNIK et al., 2004; FABRICK; TABASHNIK, 2012), *H. punctigera* (MAHON; DOWNES; JAMES, 2012), and *H. armigera* (MAHON; OLSEN; DOWNES, 2008; MAHON et al., 2010; ZHANG et al., 2012). In some cases the allele conferred resistance for more than one Bt proteins (TABASHNIK et al., 1997b). In one published case, resistance alleles to a

single Bt protein in different populations were not at the same locus (ZHANG et al., 2012).

BA25R was sampled in Barreiras, Bahia, in the northeast region of Brazil during October 2011 in a field of Cry1F maize with significant damage from *S. frugiperda*. One year later, during October 2012, RS12 was sampled in São Paulo das Missões, Rio Grande do Sul at the border with Argentina in another field of Cry1F maize with significant damage from *S. frugiperda*. The distance between these two places is more than 2,000 km in a straight line (Figure 5.1), and Cry1F maize was first introduced during the 2009/2010 cropping season, so resistance emerged in Bahia in third cropping season after introduction, and in Rio Grande do Sul in fourth cropping season after introduction.

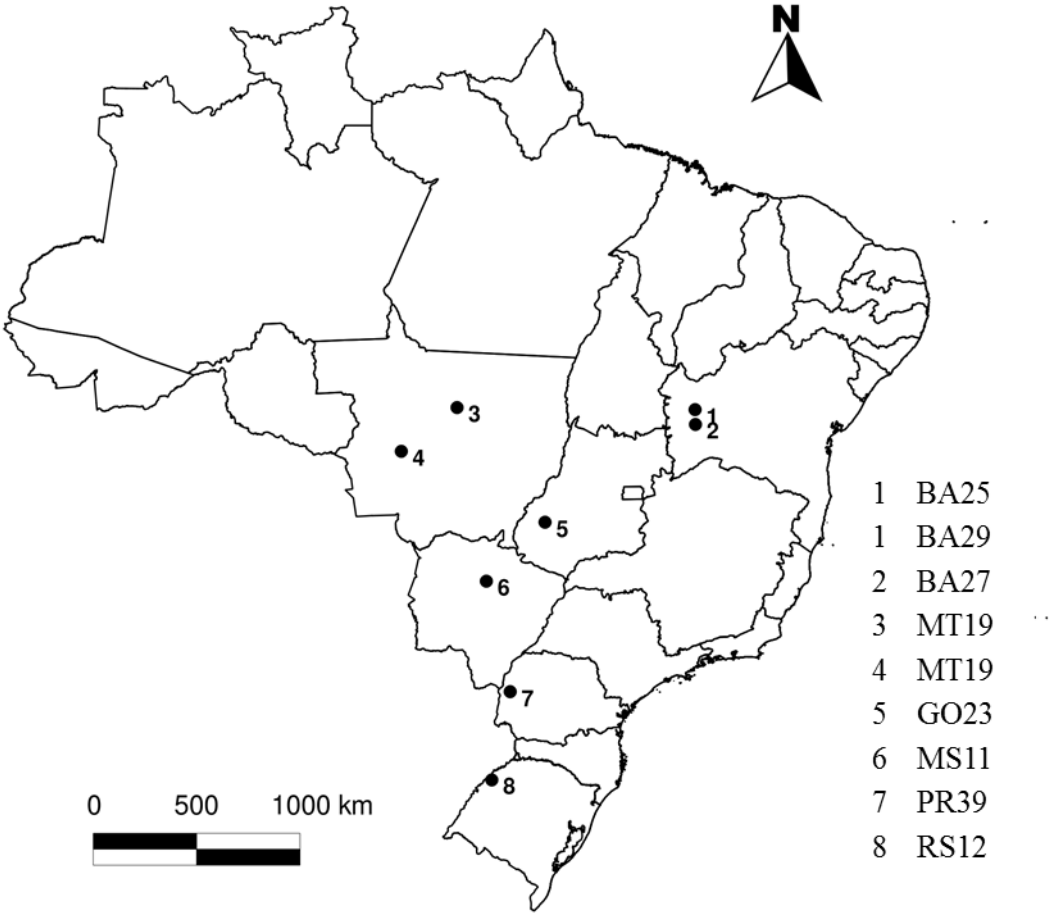

Figure 5.1 - Distribution of populations of *Spodoptera frugiperda* used in complementation test

Why do populations from distant regions share allelic resistance alleles? Possible hypotheses are: (1) extensive recent gene flow and introgression of a single resistance allele selected after the introduction of Cry1F, and (2) gene flow and introgression of a single resistance allele prior the introduction of Cry1F or geographic variation for resistance alleles at the same locus. The first hypothesis is the less probable because despite the migratory potential of *S. frugiperda* (NAGOSHI; MEAGHER, 2008), gene flow and introgression are unlikely to be so extensive to spread a resistance allele across 2,000 km in a single cropping season. One of the other hypotheses, or both acting together, could explain the observed pattern. Expectations rely on the frequency and type of mutations (MCKENZIE; BATTERHAM, 1994). If the mutations are exceedingly rare, a single mutational event might occur and the resulting resistance conferring allele might subsequently spread by migration. A scenario like this was proposed by Raymond et al. (1991) for the worldwide distribution of a single allele to organophosphates resistance in the mosquito *Culex pipiens* (Diptera: Culicidae). If the mutations are highly conpopulationed (FFRENCH-CONSTANT et al., 1993), although common, similar or identical mutations might arise independently in different populations.

Gene flow between the *S. frugiperda* populations from northern to southern Brazil could spread one or several mutant alleles at the Cry1F resistance locus before the introduction of TC1507 maize. Previous investigations on *S. frugiperda* movement in Brazil imply that this may not be the main explanation for the widespread emergence of resistance (MARTINELLI et al., 2006; 2007). Many studies have been conducted to understand the migration of *S. frugiperda* in the United States, sometimes with inconsistent results (NAGOSHI; MEAGHER, 2008). *S. frugiperda* is a non-diapausing insect and cannot survive winters in most of the USA (SPARKS, 1979; NAGOSHI; MEAGHER, 2008), but its migration capacity allows it to colonize crops in Canada every year (NAGOSHI; MEAGHER; JENKINS, 2010). Migration behavior in temperate climates is influenced by the short crop season, but in tropical climates, such as Brazil, crops are planted over a period of several months in sequential system so that *S. frugiperda* has host plants throughout the whole year in all regions, reducing the adaptive value of migration. Many different Cry1F alleles at the same locus in different populations could be a reason the observed wide distribution. The results here showed that Cry1F resistance alleles are geographically widespread in *S. frugiperda* in Brazil and that resistance alleles are allelic. However,

the presence of resistance alleles in *S. frugiperda* populations is not always associated with unexpected field damage or field-resistant populations.

5.5 Conclusion

- Cry1F resistance alleles from geographically distinct *S. frugiperda* populations in Brazil share the same locus.

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6 FREQUENCY OF CRY1F RESISTANCE ALLELES IN *Spodoptera frugiperda* (J.E. SMITH) (LEPIDOPTERA: NOCTUIDAE) IN BRAZIL

Abstract

The frequency of resistance alleles is a major factor influencing the rate of evolution of resistance. Although several methods have been proposed to estimate the frequency of resistance alleles, the F_2 screen is the most efficient when resistance is nearly recessive and the resistance allele is at low frequency. This method concentrates a resistance allele in an isofemale line, where it can be detected in the homozygous state. Here we develop a F_2 screen procedure for *Spodoptera frugiperda* (J.E. Smith), and estimate the frequency of resistance allele to Cry1F protein in *S. frugiperda* in Brazil. It was possible to establish 953 isofemale lines in the P_0 generation, and 587 isofemale lines produced eggs in the F_2 generation, and of these 517 isofemale lines were screened in Cry1F protein in overlay bioassay. Populations from Bahia showed the highest frequency of Cry1F resistance allele. The frequency in Bahia was 0.192 with 95% confidence interval (CI) between 0.163 and 0.220. The lowest frequency was in populations from Paraná with 0.042 and 95% CI between 0.025 and 0.063. The frequency of Cry1F resistance allele in Brazil was 0.088 with 95% CI between 0.077 and 0.100. F_2 screen protocol was able to produce enough F_2 larvae to estimate the frequency of resistance allele. Cry1F resistance alleles were not rare, and found at frequencies which may compromise the useful life of TC1507 event in Brazil in the absence of improved resistance management strategies.

Keywords: Resistance: Fall armyworm; *Bacillus thuringiensis;* Evolution; Monitoring

6.1 Introduction

Spodoptera frugiperda (J.E. Smith) (Lepidoptera: Noctuidae) is the most important pest of maize in Brazil (CRUZ, 1995). *S. frugiperda* is a native pest in tropical and subtropical regions, where it can survive during the winter (SPARKS, 1979; ASHLEY et al., 1989; NAGOSHI; MEAGHER, 2008). *S. frugiperda* has many host plants, including cotton, peanuts, rice, oats, potatoes, sugarcane, beans, maize, wheat, sorghum, soybeans, and many species of pasture grasses and weeds (SILVA et al., 1968). Recent intensification of agriculture has allowed many these plants to persist during the entire year in Brazil, which has enabled *S. frugiperda* to reach high population densities and consequently challenging all available pest management strategies. The main strategy for *S. frugiperda* control in maize was insecticides in Brazil (CRUZ, 1995), but high and multiple infestations this species resulted in low

efficacy (SILVA, 1999), in part caused by insecticide resistance (DIEZ-RODRIGUEZ; OMOTO, 2001).

Transgenic maize with a *cry1Ab* gene (MON810) from the bacterium *Bacillus thuringiensis* Berliner were released in 2007 in Brazil (CTNBIO, 2007), but low efficacy was observed against *S. frugiperda* (FERNANDES et al., 2003). TC1507 with a *cry1F* gene was released in 2008 and became commercially available for the 2009/2010 crop season in Brazil (CTNBIO, 2008; STORER et al., 2012). TC1507 maize showed high efficacy against *S. frugiperda* (WAQUIL; VILELLA; FOSTER, 2002; SIEBERT et al., 2008), but resistance has already been reported in Puerto Rico and Brazil (STORER et al., 2010; Chapter 3). The major successful strategy for resistance management in Bt crops is the high-dose/refuge strategy (GOULD, 1998; HUANG; ANDOW; BUSCHMAN, 2011; TABASHNIK; BRÉVAULT; CARRIÈRE, 2013). This strategy requires a large enough refuge (COMINS, 1977), recessive resistance (GEORGHIOU; TAYLOR, 1977), and a low initial frequency of the resistance allele (ROUSH, 1994).

The frequency of the resistance allele is a key element to predict the rate of evolution of resistance (COMINS, 1977; IVES; ANDOW, 2002). One method that can detect recessive resistance alleles at low frequency is an F_2 screen (ANDOW; ALSTAD, 1998). This method is based on maintenance of genetic variation in isofemale lines, and concentrating resistance alleles in homozygote genotypes by inbreeding, which can be detected by bioassay. An isofemale line is produced with a male and a female from the field. In the F_1 generation their offspring will be mated together, and the F_2 progenies will be screened using a discriminating concentration of Bt protein, insecticide or Bt plant. Although the method is labor intensive and requires a good rearing facility to maintain a large number of isofemale lines, it is far more efficient than phenotypic screens of field-collected insects or their mass-breeding progeny for detecting recessive resistance alleles (ANDOW; IVES, 2002). This method has been used in routine monitoring programs in Australia (MAHON; OLSEN; DOWNES, 2008; MAHON et al., 2010; MAHON; DOWNES; JAMES, 2012) and France (BOURGUET et al., 2003), and is very useful to estimate the resistance allele frequency to evaluate the potential usefulness of the high-dose/refuge strategy (ANDOW; ALSTAD, 1998; SIEGFRIED et al., 2007).

Several studies have used an F_2 screen to estimate the frequency of resistance alleles in many species of insects and against several Bt proteins in

different countries. The frequency of resistance alleles was estimated in *Diatraea saccharalis* (Fabricius) (Lepidoptera: Crambidae), *Ostrinia nubilalis* Hübner (Lepidoptera: Crambidae), *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), and *Helicoverpa punctigera* (Wallengren) (Lepidoptera: Noctuidae). These studies were conducted in the U.S.A., France, Australia, and China. The estimated frequencies have ranged from <0.00092 to 0.052 (ANDOW et al., 2000; BOURGUET et al., 2003; HUANG; LEONARD; ANDOW, 2007b; DOWNES; MAHON; OLSEN, 2007; HUANG et al., 2008; HUANG et al., 2009; XU et al., 2009; MAHON et al., 2010; HUANG et al., 2011; MAHON; DOWNES; JAMES, 2012; ZHANG et al., 2012). The frequency of resistance alleles in *S. frugiperda* has not been published for any Bt proteins, despite its importance to Brazilian agriculture and occurrence of resistance in Puerto Rico. Here we develop a F_2 screen procedure for *S. frugiperda*, and use it to estimate the frequency of resistance alleles for Cry1F protein in *S. frugiperda* in several populations in Brazil.

6.2 Material and methods

6.2.1 Insect samples

The frequency of Cry1F-resistance alleles was estimated for populations from12 locations across five states of Brazil. Populations were sampled from January until June 2012. BA27 was sampled from a field suffering control failure of TC1507 maize in São Desidério, Bahia, and the remaining populations were from non-Bt maize. Populations were sampled in major regions of maize production in Brazil. BA27 and BA31 were sampled in Bahia, in northeastern Brazil. MT19 and MT20 were sampled in Mato Grosso, GO22 and GO23 were sampled in Goiás, MS11, MS12, and MS13 were sampled in Mato Grosso do Sul in the central-west Brazil. PR34, PR37, and PR38 were sampled in Paraná in the south (Table 6.1 and Figure 6.1).

6.2.2 Isofemale lines

Field larvae were individually placed in 50 ml plastic cups with artificial diet (KASTEN; PRECETI; PARRA, 1978), and sealed with an acrylic sheet, and remained

in the cups until pupation. Pupae were collected and kept in trays with filter paper, and isolated in plastic cups (50 ml) until adult emergence. Adults were mated to produce isofemale lines (one couple) in 500 ml plastic cups. Adult food was a solution of 10% honey in acrylic cell culture dishes (35 mm diameter x 10 mm, BD Falcon™) soaked in water absorbent cotton. Every two days, the plastic cup (500 ml) was replaced for each isofemale line and the eggs were maintained in this container with filter paper moistened with distilled water until larvae eclosion.

The neonate larvae (< 24 hours) were inoculated in plastic cups (100 ml) with 20 ml of artificial diet, and at second instar, five larvae were placed in plastic cups with 30 ml of artificial diet. Each isofemale line was prepared with 22 plastic cups. Pupae were placed in cylindrical PVC cages (10 cm diameter x 21 cm), lined with newsprint and closed at the top and bottom with Petri dishes (12 cm diameter x 1.5 cm), and the bottom Petri dish was lined with filter paper. Adult food was a solution of 10% honey in a plastic cup (50 ml) clogged with water absorbent cotton. Eggs were collected every three days and stored in plastic containers (500 ml) containing filter paper moistened with distilled water. All stages were kept in climate-controlled rooms at 25 ± 1 °C, 60 ± 10% relative humidity, and 14:10 h (L:D) photoperiod.

6.2.3 Screening bioassays

The F_2 generation neonate larvae were used in diet-overlay bioassays that were performed in acrylic plates (5.6 cm diameter x 1 cm) with 7 ml of artificial diet (Chapter 5). The artificial diet was poured into the plates and after some seconds the plates were tapped on a table to eliminate surface bubbles. Cry1F protein (sample TSN104550, 35% Cry1F protein, available from Dow AgroSciences) was diluted in buffer, 10mM potassium phosphate dibasic, with 0.1% Triton X-100 to obtain a uniform distribution of the protein on the surface of the diet at a rate of 20 µl solution cm^{-2}. Screening was performed with 2,000 ng Cry1F cm^{-2} (Chapter 5). After the surface of the diet dried, 20 neonate larvae $plate^{-1}$ were released. The number of insects tested was 120 insect per isofemale line. Acrylic plates were kept in climate chamber at 27 ± 1 °C, 60 ± 10% relative humidity and 14:10 h (L:D) photoperiod. Survival was measured at seven days after infestation, and larvae that did not reach the second instar were considered to have their molting inhibited, and presumably would eventually die.

Table 6.1 - Populations of *Spodoptera frugiperda* and results of F_2 screen procedure

State	Location	Code	Maize[a]	Date	n[b]	P_0 generation		F_1 generation	
						Couple[c]	Egg[d]	Adult[e]	Egg[f]
Bahia	São Desidério	BA27	TC1507	Jan. 2012	480	116	97	31.3	97
	Luís Eduardo Magalhães	BA31	Non-Bt	June 2012	500	83	69	44.7	59
Mato Grosso	Sinop	MT19	Non-Bt	Apr. 2012	568	84	59	25.2	59
	Campo Novo dos Parecis	MT20	Non-Bt	Apr. 2012	570	87	36	32.7	31
Góias	Montividiu	GO22	Non-Bt	Mar. 2012	550	119	92	27.3	75
	Caiapônia	GO23	Non-Bt	May 2012	524	124	57	33.3	56
Mato Grosso do Sul	São Gabriel do Oeste	MS11	Non-Bt	Mar. 2012	486	101	52	30.0	52
	Chapadão do Sul	MS12	Non-Bt	May 2012	150	50	26	36.4	20
	Dourados	MS13	Non-Bt	May 2012	228	33	27	30.7	27
Paraná	Sabáudia	PR34	Non-Bt	Feb. 2012	248	37	29	24.3	27
	Sabáudia	PR37	Non-Bt	May 2012	474	73	46	34.7	46
	Campo Mourão	PR38	Non-Bt	May 2012	601	46	38	37.1	38

[a] Host (maize sampled).
[b] Number of sampled insect (larvae and pupae).
[c] Number of couple mated in P_0 generation (field generation).
[d] Number of couple (isofemale line) with egg in P_0 generation (field generation).
[e] Average number of normal adults by isofemale line in F_1 generation (first lab generation).
[f] Number of couple with eggs in F_1 generation (first lab generation).

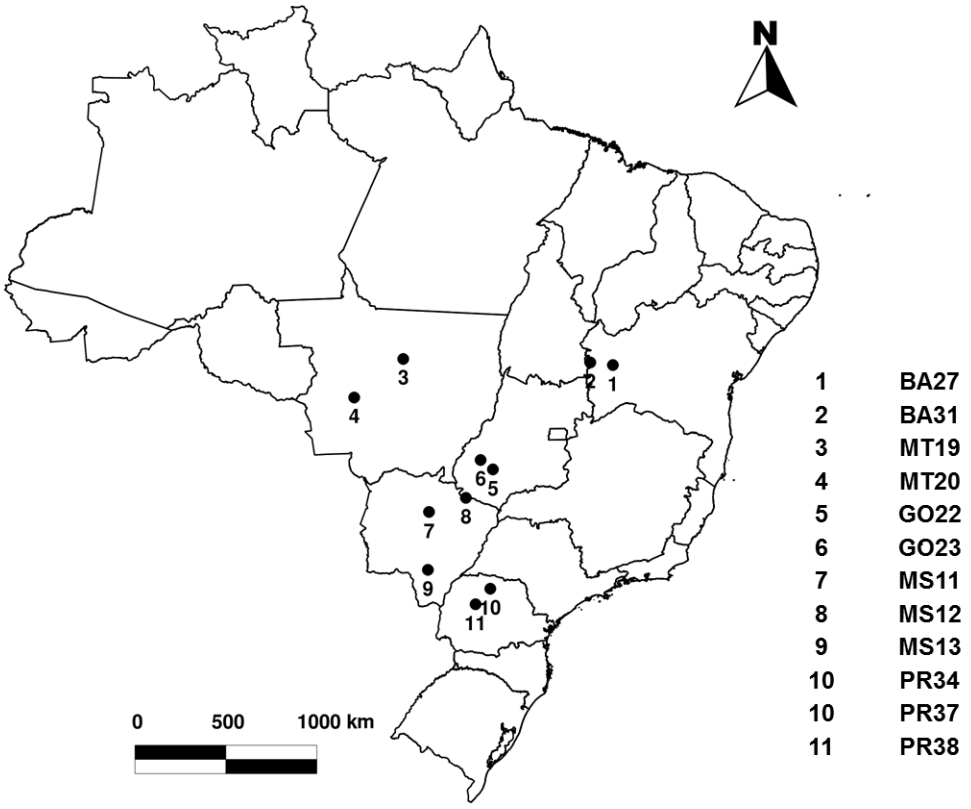

Figure 6.1 - Distribution of populations of *Spodoptera frugiperda* used in F_2 screen

6.2.4 Statistic analyses

Each isofemale line carries four gametic haplotypes, two from the male parent and two from the female parent. If resistance is monogenic and one parent was heterozygous, the expected survival in F_2 generation would be 6.25% (ANDOW; ALSTAD, 1998). On average each isofemale line was 33 F_1 adults and 50% of female, and it were screened 7.2 F_2 neonates/F_1 female. With these values, the probability of not detecting a resistance allele would be < 0.034 (STODOLA; ANDOW, 2004).

It is likely that when many positive lines are found that some of these lines have more than one resistance allele. For example, suppose 50 lines are screened and 20 are positive. The probability that some of these positive lines have more than one resistance allele is equal to 1- probability that all of the lines have only one resistance allele. The probability that all of the lines have only one resistance allele is $\prod_{a=1}^{20} \frac{50-(a-1)}{50}$ = 0.012023, so the probability that some lines have more than one resistance allele is 0.987977.

We are developing methods for classifying lines with multiple resistance alleles from the empirical data, but here we provide a probabilistic method for estimating the number of resistance alleles in a sample of positive lines.

The estimation of resistance allele frequency from an F_2 screen comprises two parts: a Bayesian model relating the number of positive lines to a frequency of positive lines, and a genetic model relating the frequency of positive lines to the frequency of recessive resistance alleles (ANDOW; BENTUR, 2010). In this application, the Bayesian model remains the same as given in Andow and Alstad (1998) with modifications by Stodola and Andow (2004), but the genetic model is more complex, because in previous models it was assumed that each positive line had only one resistance allele. This assumption is valid when the resistance allele frequency is low, but for *S. frugiperda*, it is likely that this assumption is not valid.

Let n = the number of F_2 lines screened, S = the number of lines testing positive, and a = the total number of recessive resistance alleles in the n lines, where $a > 0$ and $a \geq S$, and p = the probability that there are a resistance alleles given S and n. The inequalities on a mean that there is always at least one resistance allele found and there must be at least as many resistance alleles as there are positive lines.

The earlier genetic model (ANDOW; ALSTAD, 1998) was quite simple: $1 - P = (1 - p_R)^4$, where P is the frequency of positive lines, and p_R is the resistance allele frequency, and when p_R is very small, this simplifies to $\mathrm{E}[p_R] = \mathrm{E}[P]/4$. The Bayesian model is $\mathrm{E}[P] = \frac{S+1}{n+2}$, which is derived from the beta distribution. Now we elaborate this genetic model to calculate an expected resistance allele frequency as follows:

$$\mathrm{E}[p_R] = \frac{1}{\sum_{a=S}^{4S} p(a|S,n)} \mathrm{E}[P] \sum_{a=S}^{4S} \frac{a}{4S} p(a|S,n). \qquad [1]$$

The total number of loci in the S positive lines is $4S$, so $a/4S$ is the frequency of resistance in the positive lines, and $p(a|S,n)$ is the probability weight for this frequency. The number of alleles is at least S and at most $4S$, which determine the limits of the summation. The term in the denominator normalizes the p, so that the total probability is 1. When p_R is very small and $a = S$, equation [1] simplifies to $\mathrm{E}[p_R] = \mathrm{E}[P]/4$. A recursion for p is

$$p(a|S,n) = \frac{n-S+1}{n} p(a-1|S-1,n) + \frac{S}{n} p(a-1|S,n). \qquad [2]$$

The right hand side of the equation describes the two ways to get *a* alleles in *S* positive lines. The first is that there are *a*-1 alleles in *S*-1 positive lines, and the next allele is in a new line. The second is that there are *a*-1 alleles in *S* positive lines and the next allele is in one of the positive lines. Note that $p(a|0,n) = 0$, which means that the probability that *a* > 1 alleles are present when no lines are positive = 0, and $p(S-1|S,n)$ = 0, which means that the probability that there are fewer resistance alleles than there are positive lines = 0.

The boundary conditions for the recursion are $p(a|1,n) = \frac{1}{n}p(a-1|1,n) = \left(\frac{1}{n}\right)^{a-1}$, and $p(S|S,n) = \frac{n-S+1}{n}p(S-1|S-1,n) = \prod_{a=1}^{S}\frac{n-a+1}{n}$. Equation [2] can be calculated for a given *S* and *n*. The results can be substituted into equation [1] to estimate the expected resistance allele frequency. The 95% CIs for *P* can be calculated directly from the beta distribution (ANDOW; ALSTAD, 1998), and these values can be substituted into equation [1] for E[*P*] to calculate the 95% CIs for the resistance allele frequency. A program was written in Mathematica 8 that calculates all of these values. When individual estimates are compared statistically, we used the joint probability density function for the two estimates as described in Wenes et al. (2006).

6.3 Results

6.3.1 F_2 screen procedure

The total number of larvae sampled was 5,379, and it was possible to establish 953 isofemale lines in P_0 generation (field generation). Most of isofemale lines were lost at this time. A total of 325 isofemale lines were unable to produce eggs or lost during adult handling. Despite the cannibalistic behavior of *S. frugiperda*, larvae were reared successfully with five larvae per container. Around 30% of the neonates produced normal F_1 adults. A total of 587 isofemale lines produced eggs in F_1 generation, and from these 517 isofemale lines were screened in the F_2 (Table 6.1).

6.3.2 Frequency of Cry1F resistance alleles

Resistance was detected in all populations of *S. frugiperda* sampled, except for MS13 (Table 6.2). Estimated resistance allele frequencies varied from 0.009 (MS13) to 0.227 (BA31). Populations from western Bahia showed highest frequency of Cry1F resistance. There was a higher frequency of resistance in the second season in Bahia. BA27 was sampled during the main season in 2012 from TC1507 maize that was economically damaged by *S. frugiperda*, and the resistance allele frequency was 0.157. BA31 was sampled from non-Bt maize during the second season and the resistance allele frequency was higher than BA27. This may indicate that the resistance allele frequency had increased from 0.157 in January to 0.277 in June 2012. At Sabáudia, Paraná, two samples were taken. The first was during the main cropping season during February 2012 (PR34), and the second during the second season during May 2012 (PR37). The resistance frequency increased significantly (p=0.0307) from 0.028 in February to 0.091 in May, 2012.

Populations were pooled by state (Table 6.2) and differences among states were analyzed. Bahia had the highest frequency of Cry1F resistance alleles, 0.192 (95% CI: 0.163, 0.220), and this was significantly higher than any of the other states (Table 6.3). Although populations from Mato Grosso do Sul had the second highest frequency, they were not significantly greater than Mato Grosso or Goiás. Populations from Paraná had the lowest frequency of Cry1F resistance, and almost significantly lower than Mato Grosso do Sul (p = 0.0213, Bonferroni corrected p-crit = 0.005) and not significantly different from Mato Grosso or Goiás.

6.4 Discussion

To conduct an F_2 screen with *S. frugiperda*, some initial decisions were necessary to improve the efficiency of the screen. The first decision was about sampling of field insects. The first F_2 screen was conducted on *O. nubilalis* (ANDOW et al., 1998), which sampled mated adult females in field because this was the most efficient method for acquiring P_0 adults. A later version was started with field-collected larvae (HUANG; LEONARD; ANDOW, 2007a). We started the F_2 screen with *S. frugiperda* larvae because *S. frugiperda* larvae are easy to sample and easy to send to the laboratory. We received larvae in artificial diet that were sampled three

to four days earlier, which is not possible with adults. For these reasons, we started isofemale lines with larvae.

Table 6.2 - Frequency of resistant Cry1F allele in *Spodoptera frugiperda* from Brazil estimated by F_2 screen method (ANDOW; ALSTAD, 1998)

State	Population	Date	Tested lines	Positive lines	Frequency	95% CI
Bahia	BA27	Jan. 2012	95	44	0.157	(0.124 – 0.190)
	BA31	June 2012	51	34	0.277	(0.222 – 0.327)
	Subtotal	---	146	78	0.192	(0.163 – 0.220)
Mato Grosso	MT19	Apr. 2012	22	8	0.120	(0.063 - 0.183)
	MT20	Apr. 2012	32	3	0.031	(0.009 - 0.065)
	Subtotal	---	54	11	0.061	(0.033 - 0.093)
Goiás	GO22	Mar. 2012	70	18	0.077	(0.049 - 0.108)
	GO23	May 2012	53	6	0.034	(0.014 - 0.061)
	Subtotal	---	123	24	0.056	(0.038 - 0.077)
Mato Grosso do Sul	MS11	Mar. 2012	49	18	0.118	(0.078 - 0.160)
	MS12	May 2012	18	7	0.131	(0.066 - 0.202)
	MS13	May 2012	26	0	0.009	(0.000 - 0.027)
	Subtotal	---	93	25	0.080	(0.055 - 0.108)
Paraná	PR34	Feb. 2012	26	2	0.028	(0.006 - 0.064)
	PR37	May 2012	41	12	0.091	(0.053 - 0.134)
	PR38	May 2012	34	1	0.014	(0.002 - 0.038)
	Subtotal	---	101	15	0.042	(0.025 - 0.063)
Brazil	Total	---	517	153	0.088	(0.077 – 0.100)

The second decision was how to minimize cost and labor to complete the screening of an isofemale line (STODOLA; ANDOW, 2004). While the F_2 screen is an effective method to detect rare recessive resistance alleles and estimate their frequency (ANDOW, IVES, 2002), the cost and labor per line must be minimized. In our experiment, the cost was reduced by increasing the number of products screened for each isofemale line (here we show only Cry1F data), and by rearing *S. frugiperda* in groups of larvae in the F_1 generation. Despite the cannibalism by this species, we optimized the number of larvae reared per container so that we typically had 32 normal F_1 adults per isofemale line. In *D. saccharalis* an average of 41 F_1 pupae by isofemale line were considered enough to use in F_2 screen (HUANG; LEONARD; ANDOW, 2007a). In addition, the cost of the F_2 screen is higher when more isofemale lines are lost prior to screening. Most of the isofemale lines were lost in the P_0 mating, and because the highest cost is rearing the F_1 larvae, loss of isofemale lines did not affect the total cost of the screen very much.

Table 6.3 - Pairwise comparison of the estimated resistance allele frequencies in each of the sampled States, giving p-values for the hypothesis that the two frequencies are the same. Significantly different values for Bonferroni corrected p-values are in bold

States	Bahia	Mato Grosso	Goiás	Mato Grosso do Sul
Mato Grosso	**2.17E-05**	---		
Goiás	**6.45E-09**	0.383	---	
Mato Grosso do Sul	**3.73E-05**	0.224	0.0983	---
Paraná	**1.20E-10**	0.164	0.198	0.0213

The third decision was the screening method. The three major screening methods are the plant (ANDOW et al., 1998; BOURGUET et al., 2003), excised leaves (HUANG; LEONARD; ANDOW, 2007a), and a diet bioassay (ZHAO et al., 2002; BOURGUET et al., 2003). The plant bioassay is limited by greenhouse space or the period of time the plant can be grown in the field. A leaf bioassay uses less space (HUANG; LEONARD; ANDOW, 2007a), but it is still necessary to grow plants, and furthermore this method requires frequent changing of the leaves, which increases the labor (Chapter 3). A diet bioassay is limited by the availability of purified and quantified Cry protein, and the determination of a diagnostic concentration (HUANG; LEONARD; ANDOW, 2007a). In our experiment, the plant bioassay was not used because the greenhouse and field space was limited, and the leaf bioassay was not used because of the intensive labor in handling leaves. We used a diet bioassay because purified, quantified Cry1F was readily available, and a diet overlay bioassay was well characterized for detecting resistance in *S. frugiperda* (Chapter 3), and 2,000 ng cm^{-2} was determined to be a discriminatory concentration (Chapter 5).

We tested 517 isofemale lines in five months. Our results showed frequencies of Cry1F resistance alleles that were much higher than any other published studies (ANDOW et al., 2000; BOURGUET et al., 2003; HUANG; LEONARD; ANDOW, 2007b; DOWNES; MAHON; OLSEN, 2007; HUANG et al., 2008; HUANG et al., 2009; XU et al., 2009; MAHON et al., 2010; HUANG et al., 2011; MAHON; DOWNES; JAMES, 2012; ZHANG et al., 2012). The highest resistance allele frequency in the previous literature was 0.027 to Vip3A in Australia (MAHON; DOWNES; JAMES, 2012), and 0.052 to Cry1Ac in northern China (ZHANG et al., 2012) both in *H. armigera.* This species is from Noctuidae family, as well as *S. frugiperda*. Species from the Crambidae showed much lower resistance allele

frequencies (ANDOW et al., 2000; BOURGUET et al., 2003; HUANG; LEONARD; ANDOW, 2007b; HUANG et al., 2008; HUANG et al., 2009; HUANG et al., 2011).

The populations from Bahia had a higher resistance allele frequency than any other state. Brazilian agriculture is an intensive system of crop production. Western Bahia has recently become an important maize production region in Brazil because of the proliferation of irrigated maize during the dry season. The summer period is hot and wet, and the winter period is hot and dry. Irrigation during the winter has allowed expansion of maize production during this period, resulting in three maize crops per year in Bahia and continual availability of maize for *S. frugiperda* all year. In Mato Grosso the climate and latitude are almost the same as in Bahia, but the cropping system is different. The main crop during the summer season in Mato Grosso, Goiás and Mato Grosso do Sul is mainly soybean, which is not an important host for *S. frugiperda*. Therefore the lower resistance frequency in Mato Grosso, Goiás and Mato Grosso do Sul than Bahia may be related by two main factors. The lower population of *S. frugiperda* during the second season when maize is grown and irrigation system during dry period that enables host plants during the dry season. In Paraná there are two maize seasons, but the subtropical weather with cold winter gives lower infestations especially in main season.

The resistance allele frequency increased more than three-fold in three months from the main to the second season in Paraná and almost two-fold in five months in Bahia. Maize is grown in two seasons in these two states. The large and continuously cropped areas with Bt maize in these states probably contributed to this result. In both crop seasons, more than 80% of the maize area was planted to Bt maize in Paraná and around 50% in Bahia (CÉLERES, 2012), and most of this was TC1507 maize hybrids. Refuge areas were not used in most of Bt maize fields (personal communications). The continuous crop and high temperature enabled *S. frugiperda* to have at least three generations from the first to the second season in Paraná and more than that in Bahia, based on laboratory development experiments (GIOLO et al., 2002; BUSSATO et al., 2004). Therefore, the high adoption of TC1507, the low or insignificant refuge areas, and many generations of *S. frugiperda* resulted in high selection pressure and rapid increase in the frequency of Cry1F resistance alleles in these two states. It will be necessary to test additional samples from the same location in later cropping seasons to determine whether the increase in frequency is stable and sustained over years.

Many reasons could account for the observed Cry1F resistance allele frequencies only three years after commercial release of the TC1507 event in Brazil. The first is that the TC1507 event has been widely grown in Brazil. In the 2011/2012 crop season, Bt maize reached around 70% of the maize area in Brazil (CÉLERES, 2012). Most of non-Bt maize fields are in small farms where maize is grown for subsistence with low technology. Therefore, in regions with intensive maize production, Bt maize comprises much more than 70% of maize area. The major pest problem with *S. frugiperda,* low efficacy of insecticides (SILVA, 1999), and high efficacy of the TC1507 event (SIEBERT et al., 2008) enabled high adoption of TC1507 in a short period of time. The second reason probably is the low use of refuge areas. This created a situation where most of resistant insects mated with other resistant insects, speeding up the evolutionary process. The third reason is that TC1507 is not high-dose against *S. frugiperda*. Some heterozygous *S. frugiperda* can survive and develop on this maize event (Chapter 4). The resistance allele frequency is expected to increase more rapidly in less-than-high-dose situations in the presence of a refuge (TABASHNIK; CROFT, 1982; TABASHNIK; GOULD; CARRIÈRE, 2004). The fourth reason may be that the initial frequency of Cry1F resistance at the time TC1507 was commercially released in Brazil was not low. For example, the Vip3A resistance allele in *H. armigera* and *H. punctigera* was not rare prior to the commercial release of Vip3A cotton in Australia (MAHON; DOWNES; JAMES, 2012). The fifth reason is the possibility of cross resistance for Cry1F and Cry1Ab in *S. frugiperda* (STORER et al., 2010). Cry1Ab events were released some years earlier than the Cry1F event (CTNBIO, 2007), and may have contributed to the evolution of resistance to Cry1F protein (STORER et al., 2012). Regardless of exact cause or causes, the present elevated allele frequency of resistance to Cry1F protein may compromise the use the TC1507 in Brazil within a few years. After five years without TC1507 in Puerto Rico, the frequency of the Cry1F resistance allele remains high (STORER et al., 2012). The results shown here suggest that field failures could happen in next cropping season in Brazil. The Cry1F resistance allele was not rare in any region, therefore continued use of Cry1F-producing maize without adequate refuge could lead to field failures at locations throughout the regions that were sampled here. Additional or alternative resistance management tools are needed to prolong the durability of Cry1F maize, such as pyramiding Cry1F with other Bt toxins to which there is little or no cross resistance, the introduction of more robust refuge

strategies, the adoption of alternative pest management strategies, and avoidance of Cry1F maize in one or more of the maize cropping cycles each year.

6.5 Conclusions

- The frequency of Cry1F allele is high in Brazil.

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