MARCELO MENDES RABELO

BT TOXINS AND INSECTICIDES AGAINST NOCTUID PESTS: SUSCEPTIBILITY, PARENTAL EFFECTS, AND GROWTH POTENTIAL

Thesis submitted to the Universidade Federal de Viçosa in partial fulfillment of the requirements of the Entomology Graduate Program for the degree of *Doctor Scientiae*.

Advisor: Eliseu José Guedes Pereira
Co-advisor: Silvana Vieira de Paula-Moraes

VIÇOSA - MINAS GERAIS
2020

Ficha catalográfica elaborada pela Biblioteca Central da Universidade Federal de Viçosa - Campus Viçosa

T
Rabelo, Marcelo Mendes, 1991-
R114b Bt toxins and insecticides against noctuid pests :
2020 susceptibility, parental effects, and growth potential / Marcelo
Mendes Rabelo. – Viçosa, MG, 2020.
90 f. : il. (algumas color.) ; 29 cm.

Orientador: Eliseu José Guedes Pereira.
Tese (doutorado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. Algodão - Resistência a doenças e pragas. 2. Aptidão.
3. Tabelas de vida. 4. *Bacillus thuringiensis*. 5. *Spodoptera* spp.
I. Universidade Federal de Viçosa. Departamento de
Entomologia. Programa de Pós-Graduação em Entomologia.
II. Título.

CDD 22. ed. 633.5197

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APPROVED: April 30, 2020.

Assent:

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I dedicate this dissertation to god and my family.

ACKNOWLEDGMENTS

I would like to thank my advisors Eliseu Jose Guedes Pereira and Silvana Vieira de Paula-Moraes, who have shared intellectual support, assistance, suggestions, and friendship that have contributed to my personal and professional life.

I also would like to acknowledge the entomology team at WFREC and at UFV for all the support to make this dissertation possible.

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001, USDA NIFA CPPM EIP project #005649, USDA NIFA hatch project # 005643, and Florida Peanut Checkoff funds. Grants and fellowships were also provided by the FAPEMIG and CNPq Agencies.

ABSTRACT

RABELO, Marcelo Mendes, D.Sc., Universidade Federal de Viçosa, April, 2020. **Bt toxins and insecticides against noctuid pests: susceptibility, parental effects, and growth potential.** Adviser: Eliseu José Guedes Pereira. Co-adviser: Silvana Vieira de Paula Moraes.

Lepidopteran has around 120 thousand species described, and all of them are phytophagous. Several species, mostly Noctuidae damage row crops cultivated across the south and north Americas. Historically, lepidopteran pests have been managed with the use of synthetic insecticides, and plants expressing *Bacillus thuringiensis* toxins. This thesis has reported the effect of Bt toxins and insecticides against noctuid pests from Brazil and the United States. In Brazil, the susceptibility *Spodoptera cosmioides* (Walker), to three Cry toxins and its life-history traits feeding on dual-gene Bt cotton and an artificial diet containing sublethal concentrations of Cry1Ac was documented. In concentration-response bioassays, *S. cosmioides* larvae showed low susceptibility to Cry toxins. The decreasing rank of toxicity was Cry1F, Cry2Aa, and Cry1Ac. Foliage of dual-toxin Bt cotton varieties (Cry1Ac+Cry1F and Cry1Ab+Cry2Ae) caused higher larval mortality than Cry1Ac-expressing cotton. *Spodoptera cosmioides* showed reduced larval weight when growing on the Cry1Ac-treated diet, yet they reached adulthood and produced offspring. Interestingly, these offspring when grown on the control diet showed reduced weight gain associated with the toxin exposure of the previous generation, indicating a parental effect of the exposure to Cy1Ac. The reduced larval weight was recovered in later instars, and there was no significant change in the population fitness of the parental *S. cosmioides* or their offspring. In the United States, *Helicoverpa zea* (Boddie) life-history traits were determined for individuals of three field populations from a region where this pest likely overwinter. Triple-gene Bt cotton cultivars expressing Cry and Vip3Aa toxins killed 100% of the larvae in all populations tested. In contrast, dual-gene Bt cotton expressing Cry1Ac+Cry1F and Cry1Ac+Cry2Ab2 allowed population growth with the intrinsic rate of population growth (r_m) 38% lower than on non-Bt cotton. The insects feeding on Bt cotton plants expressing Cry1Ac+Cry2Ab2, Cry1Ac+Cry1F, and Cry1Ab+Cry2Ae exhibited reduced larval weight, survival rate, and increased development time. Additionally, fitness parameters varied significantly among the insect populations, even on non-Bt cotton plants, likely because of their different genetic background and/or previous Bt-toxin exposure. The susceptibility of eight noctuid species from the Florida Panhandle to bifenthrin (pyrethroid) and chlorantraniliprole (diamide) was also determined. Larvae from field and laboratory populations were exposed to commercial insecticide formulations using the leaf-dip method in concentration-mortality bioassays. The field populations of *H. zea*, *S. frugiperda* (Smith), *S. eridania* (Stoll), *S. exigua* (Hubner), and *Chloridea virescens* (Fabricius) had reduced susceptibility to bifenthrin compared with the laboratory populations, while there was no reduced susceptibility in *Agrotis ipsilon* (Hufnagel). The susceptibility to chlorantraniliprole was similar between the field and laboratory populations studied, except for *S. exigua* that exhibited 630-fold resistance to the diamide. The probit regression equations indicated that the larval mortality of *S. exigua* and *S. frugiperda* populations was less than 80% with bifenthrin at the concentration equivalent to the label rate. Likewise, the estimated mortality of *S. exigua* larvae with chlorantraniliprole at the label rate concentration was less than 80%. This study advances our understanding of potential responses of target and nontarget pest species exposed to Bt toxins and insecticides, which help to design pest management programs for lepidopteran species in the Americas.

Keywords: Bioassay. *Bacillus thuringiensis*. *Spodoptera* spp.

RESUMO

RABELO, Marcelo Mendes, D.Sc., Universidade Federal de Viçosa, abril de 2020. **Toxinas Bt e inseticidas contra pragas noctuid: suscetibilidade, efeitos parentais e potencial de crescimento.** Orientador: Eliseu José Guedes Pereira. Coorientadora: Silvana Vieira de Paula-Moraes.

Lepidoptera possui cerca de 120 mil espécies descritas e todas são fitófagas. Várias espécies, principalmente Noctuidae, danificam plantacoes na América do sul e norte. Historicamente, as lepidópteros pragas têm sido manejados com o uso de inseticidas sintéticos e plantas que expressam as toxinas de *Bacillus thuringiensis*. Esta tese relata o efeito de toxinas Bt e inseticidas contra noctuid pragas do Brasil e dos Estados Unidos. No Brasil, foi documentada a suscetibilidade de *Spodoptera cosmioides* (Walker) a três toxinas Cry e suas características de história de vida alimentadas com algodão Bt e uma dieta artificial contendo concentrações subletais de Cry1Ac. Nos bioensaios de concentração-resposta, as larvas de *S. cosmioides* apresentaram baixa suscetibilidade às toxinas Cry. A classificação decrescente de toxicidade foi Cry1F, Cry2Aa e Cry1Ac. A folhagem das variedades de algodão com duas toxinas Bt (Cry1Ac+Cry1F e Cry1Ab+Cry2Ae) causou maior mortalidade larval do que o algodão que expressa Cry1Ac. *Spodoptera cosmioides* mostrou peso larval reduzido ao crescer na dieta tratada com Cry1Ac, mas atingiu a idade adulta e produziu descendentes. Curiosamente, essas descendentes foram criados na dieta controle e mostraram ganho de peso reduzido associado à exposição a toxina da geração anterior, indicando um efeito parental da exposição ao Cy1Ac. O peso larval reduzido foi recuperado em instares posteriores, e não houve mudança significativa na aptidão de *S. cosmioides* pais ou de seus descendentes. Nos Estados Unidos, os traços da história de vida de *Helicoverpa zea* (Boddie) foram determinados para indivíduos de três populações de campo de uma região onde essa praga provavelmente passava o inverno. As cultivares de algodão Bt com três genes que expressam as toxinas Cry e Vip3Aa mataram 100% das larvas em todas as populações testadas. Em contraste, o algodão Bt de dois genes que expressa Cry1Ac+Cry1F e Cry1Ac+Cry2Ab2 permitiu o crescimento populacional com a taxa intrínseca de crescimento populacional (r_m) 38% menor que no algodão não Bt. Os insetos que se alimentavam de plantas de algodão Bt expressando Cry1Ac+Cry2Ab2, Cry1Ac+Cry1F e Cry1Ab+Cry2Ae exibiram peso larval e taxa de sobrevivência reduzidos e aumento no tempo de desenvolvimento. Além disso, os parâmetros de aptidão variaram significativamente entre as populações de insetos, mesmo em plantas de algodão que não são Bt, provavelmente por causa de suas diferencas em carga genética e/ou exposição anterior à toxina Bt. Também foi determinada a suscetibilidade de oito espécies de noctuid do Panhandle da Flórida à bifentrina (piretróide) e clorantraniliprol (diamida). Larvas de populações de campo e de laboratório foram expostas a formulações comerciais de inseticidas usando o método de imersão em folhas em bioensaios de mortalidade-reposta. As populações de campo de *H. zea*, *S. frugiperda* (Smith), *S. eridania* (Stoll), *S. exigua* (Hubner) e *Chloridea virescens* (Fabricius) aprezentaram reduzida suscetibilidade à bifentrina em comparação com as populações de laboratório, enquanto nao houve susceptibilidade reduzida em *Agrotis ipsilon* (Hufnagel). A suscetibilidade ao clorantraniliprol foi semelhante entre as populações de campo e de laboratório estudadas, com exceção de *S. exigua* que exibiiu resistência de 630 vezes à diamida. As equações de regressão probit indicaram que a mortalidade larval das populações de *S. exigua* e *S. frugiperda* foi inferior a 80% com bifentrina na concentração equivalente à taxa de campo. Da mesma forma, a mortalidade estimada de larvas de *S. exigua* com clorantraniliprole na concentração da taxa de campo foi inferior a 80%. Este trabalho avança nosso entendimento das possíveis respostas de espécies-alvo e não-alvo de pragas expostas a inseticidas e toxinas Bt, que ajudam a projetar programas de manejo de pragas para espécies de lepidópteros nas Américas.

Palavras-chave: Bioensaio. *Bacillus thuringiensis*. *Spodoptera* spp.

CONTENTS

INTRODUCTION

Synthetic insecticides and genetically engineered insect-resistant plants expressing *Bacillus thuringiensis* (Bt) toxins are valuable tools for pest management in agroecosystems worldwide (Fernandez-Cornejo et al. 2011, Koch et al. 2015). Lepidoptera has around 120 thousand species described, and 100% are phytophagous (Scoble 1992). Several species from Noctuidae family caused economic impact in agriculture, and their managemen have been based on the adoption of insecticides, including pyrethroids, diamides, carbamates, and organophosphates (Suckling et al. 2017). In 1996, Bt corn and Bt cotton varieties expressing a single-gene of *B. thurigiensis* toxins were introduced commercially in the United States (U.S.), followed by several other countries in South America, including Brazil (CTNBio 2005, ISAAA 2017).

In the past two decades, the number of Bt traits in corn and cotton, and their adoption have increased (ISAAA 2018). Currently, Bt corn and Bt cotton are widely adopted worldwide. Since 2013, Bt soybean has been adopted in several countries with the exception of the U.S. The commercial Bt traits currently available in corn and cotton express one, two, or three Bt toxins, targeting one or multiple lepidopteran pests (Carrière et al. 2015). Bt crops have reduced the foliar insecticides use for their target pests of *Heliotinae*, *Spodopotera, Pyraustinae* and *Plusinae* complex (Hutchison et al. 2010, Naranjo 2011).

Insecticide use has also changed considerably over the past five decades. Wamfull insecticides had the registration canceled in several countries (Buffington and McDonald 2006), and new classes of insecticides with lower mammalian toxicity, such as Diamides, were introduced on the market with high efficacy against multiple lepidopteran pests (Teixeira and Andaloro 2013). However, oldest classes of insecticides (i.e., pyrethroids, neonicotinoids, and carbamates) have been still adopted to manage lepidopteran pests in Bt and non-Bt crops (Housset and Dickmann 2009).

Resistance of lepidopteran pests to insecticides and Bt toxins is a global challenging in crop protection, which threats the continued success of these control measures (Teixeira and Andaloro 2013, Nelson and Alves 2014, Nauen and Steinbach 2016). Resistance to insecticides and Bt toxins may lead control failure and the need for additional sprayings, rising operational costs, and overexposing nontarget insects, human, and the environment (Suckling et al. 2017, Reisig and Kurtz 2018, Reisig et al. 2019).

High-dose Bt traits associated with adoption of refuge are a demanded strategy for resistance management delaying evolution of target pests (Andow 2008). This strategy is based on the idea that most of the rare resistant pests surviving on Bt crops will mate with abundant susceptible pests from nearby refuges of host plants without Bt toxins (Tabashnik et al. 2013). This approach referred to as the "high-dose refuge strategy" because in high dose events, Bt plants should kill all or nearly all of the hybrid progeny (Tabashnik et al. 2009). With long-time use of Bt crops, however, target pests, such as *Helicoverpa zea,* responded actively by evolving resistance to the Bt crops, which some Bt traits are listed as non-high dose for this pest (Luttrell and Jackson 2012). In case of insecticide resistance, a recommended strategy is the rotation of mode of action. Though this strategy is central, operational factors can also delay the insecticide resistance, including adequate spray coverage, sufficient field rates, correct application timing.

Target and nontarget lepidopteran pests should have their susceptibility to insecticides and Bt traits monitored continuously due to changes in agricultural practices, and their rapid adaptation to new environments (Huang 2006, Siegfried et al. 2007, Sivasupramaniam et al. 2007). Thus, the objective of this study was to improve the knowledge in toxicological responses of target and nontarget lepidopteran pests from the Florida Panhandle (U.S.) and Minas Gerais (Brazil) to insecticides and Bt toxins.

The Florida Panhandle region is located in the Gulf Coastal Plain of the southeastern U.S., in an ecological transition zone between temperate and subtropical climates, where lepidopteran pests can overwinter, disperse throughout the growing season (Raulston et al. 1986, Morey et al. 2012), and carry-over source of Bt and insecticide resistance between seasons (EPA 2019). While Brazil has one of the large high input agricultural area adopting Bt crops and use of insecticides.

The study is divided into three chapters;

- Chapter I had the objective of documenting the susceptibility of *Spodoptera cosmioides* (Walker) (black armyworm) to three Bt toxins, Cry1Ac, Cry1F and Cry2Aa. Life-history traits were measured in larvae feeding on Cry1Ac, Cry1Ac + Cry1F, and Cry1Ab + Cry2Ae Bt cotton, and artificial diet treated with Cry1Ac. Outbreaks of *Spodoptera cosmioides* have been often reported in soybean and cotton crops in South America (Bueno et al. 2013), in areas with large areas cultivated with Bt cultivars. Native to the American continent, *S. cosmioides* is a polyphagous pest and was listed as a non-target pest of the first-generation of Bt cultivars expressing Cry 1Ac in cotton and soybean, released in Brazil in 2005 and 2013, respectively (CTNBio 2019). This research has been published in the Journal of Economic Entomology (doi: 10.1093/jee/toaa051).

- Chapters II had the objective of documenting the effects of dual and triple Bt toxins expressed in Bt cotton cultivars on the life-history traits and demographic performance of *Helicoverpa zea* (Boddie). This is a major agricultural noctuid pest widely distributed across the Americas and responsible for annual losses in several crops such as cotton, soybean, sweet corn, and peanut (Jackson et al. 2008). Cases of resistance have been performed to many Bt toxins and insecticides (Abd-Elghafar et al. 1993, Head et al. 2010, Luttrell and Jackson 2012). This research was submitted to Pest Management Science and received recommendation for publication after appropriate revisions.

- In Chapter III, the objective was to document the susceptibility of *H. zea*, *Chloridea virescens* (Fabricius), *S. frugiperda* (Smith), *S. eridania* (Stoll), *S. exigua* (Hubner), *S. ornithogalli* (Guenee), *S. latifascia* (Walker), and *Agrotis ipsilon* (Hufnagel) to bifenthrin (pyrethroid) and chlorantraniliprole (diamide) insecticides. *Spodoptera frugiperda* and *A. ipsilon* impact in corn yield as much as 45% and 20%, respectively (Hruska and Gladstone 1988, Oloumi-Sadeghi et al. 1992). *Spodoptera exigua* and *S. eridania* are two of the three major North American pests in this genus (Mitchell and Tumlinson 1994). Before the widespread adoption of Bt cotton, *H. zea* and *C. virescens* were the most severe pests throughout the U.S. Cotton Belt with annual estimated losses of $1 billion in cotton alone (Blanco 2012, Luttrell and Jackson 2012). This research is in final preparation for publication in a high-quality journal.

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CHAPTER I - ABSTRACT

RABELO, Marcelo Mendes,D.Sc., Universidade Federal de Viçosa, April, 2020. **Like parents, like offspring? Susceptibility to Bt toxins, development on dual-gene Bt cotton, and parental effect of Cry1Ac on a non-target lepidopteran pest**. Adviser: Eliseu Jose Guedes Pereira. Co-adviser: Silvana Vieira de Paula Moraes.

An important step to devise appropriate pest management strategies for armyworms in *Bacillus thuringiensis* Berliner (Bt) crops is to determine the lethal, sublethal, and parental effects of Bt toxins on target and non-target pest species. Here we documented the susceptibility of black armyworm, *Spodoptera cosmioides* (Walker), to three Cry toxins and its life-history traits feeding on dual-toxin Bt cotton and an artificial diet containing sublethal concentrations of Cry1Ac. In concentration-response bioassays, black armyworm larvae showed low susceptibility to Cry toxins, with 853 ng/cm^2 as the lowest value estimated for the median lethal concentration (LC_{50}). The decreasing rank of toxicity was Cry1F, Cry2Aa, and Cry1Ac. Foliage of dual-toxin Bt cotton varieties (Cry1Ac + Cry1F and Cry1Ab+Cry2Ae) caused higher larval mortality than Cry1Ac-expressing cotton. Black armyworms showed reduced larval weight when growing on the Cry1Ac-treated diet, yet they reached adulthood and produced offspring. Interestingly, these larvae were grown on the control diet and showed reduced weight gain associated with the toxin exposure of the previous generation, indicating a parental effect of the exposure to Cy1Ac. The reduced larval weight was recovered in later instars, and there was no significant change in the population fitness of the parental armyworms or their offspring. To our knowledge, this is the first study documenting the parental effects of Bt toxins in insects. These results advance our understanding of potential responses of non-target species when exposed to Bt toxins and contribute to design pest management programs.

Keywords: *Spodoptera cosmioides.* Tolerance. Insecticidal Bt toxins. Secondary pest.

1. INTRODUCTION

Genetically engineered crops expressing *Bacillus thuringiensis* toxins (Bt crops) have promoted efficient control of target pests and more productive and safer agriculture (Cattaneo et al. 2006, Naranjo 2010, Sanahuja et al. 2011, Koch et al. 2015). However, there is a concern that secondary or non-target pest species may be favored by the large-scale adoption of Bt crop varieties, changing the pest status of some phytophagous insect species (Wu and Guo 2005, Catangui and Berg 2006, Arpaia 2010, Zhao et al. 2011, Catarino et al. 2015). In South America, outbreaks of the *Spodoptera cosmioides* (Walker) (Lepidoptera: Noctuidae), black armyworm, have been often reported in soybean and cotton crops, in which there is a large-scale adoption of Bt cultivars (Bernardi et al. 2014, Lutz et al. 2018, Murúa et al. 2018).

Native to the American continent, the polyphagous black armyworm was not listed as a target pest of the first-generation Bt cultivars of cotton and soybean (containing one Cry1Ac-protein gene) released in Brazil in 2005 and 2013, respectively (CTNBio 2019), although new Bt soybean technologies expressing Cry1Ac + Cry1F seem to have some plant resistance effects on the larvae (Marques et al. 2016). The black armyworm used to be referred to as *Spodoptera latifascia* (Walker), which is distributed in North and Central but not in South America, and therefore should be considered a synonym of *S. latifascia* in some of the early reports (Silvain and Lalanne-Cassou 1997). The black armyworm can be identified appropriately based on morpho-physiological traits and sexual pheromone composition (Silvain and Lalanne-Cassou 1997, Zenker et al. 2007).

Black armyworms are voracious, large-size larvae (50-60 mm when fully grown). The larvae have a high potential of defoliation estimated to be twice as much as the foliar consumption of other lepidopteran pests in cotton and soybean (de Freitas Bueno et al. 2011). A relatively high tolerance to the Cry1Ac Bt toxin has been reported for this species (Bernardi et al. 2014), with previous studies reporting Cry1Ac soybean plants causing insignificant mortality of the black armyworms (Bernardi et al. 2014, Marques et al. 2017). In most soybean producing regions in Brazil, where there is a large-scale adoption of Cry1Ac Bt soybean to control mainly plusines and heliothines (over 20 million ha) (James 2018), the black armyworm is currently considered a primary lepidopteran pest (Bueno et al. 2012). Although Bt cotton cultivars expressing one or two Cry toxins have been adopted in South America, currently there is no precise information available about their potential efficiency to manage black armyworm infestations. Most Cry Bt cotton technologies provide poor control of the complex of *Spodoptera* larvae (e.g., *S. frugiperda*, *S. exigua*, *S. eridania,* and *S. cosmioides*) (Adamczyk et al. 2008, Santos et al. 2009). Therefore, applications of synthetic insecticides are often needed, which increase crop production costs, limit the benefits promoted by the Bt technology (Catarino et al. 2015), and raise concerns regarding the long-term sustainability of pest technologies and their ecological effects (Pemsl et al. 2005, Catarino et al. 2015, Romeis et al. 2019).

An understanding of the lethal and sublethal effects of Bt crops on target and non-target insects is especially important to manage pest species of low inherent susceptibility to Bt toxins (Poppy 2000, Carriere et al. 2010). While lethal effects directly reduce the population levels of a given species, sublethal effects may lead to a range of indirect changes in the insect community, including interactions with other species and eventual pest outbreaks (Marques et al. 2018). Insect exposure to synthetic or phytochemical xenobiotics is known to induce detoxification enzymes (e.g., P450 monooxygenases), which can contribute to metabolic resistance to insecticides (Yu 1983, Bantz et al. 2018). The same concept holds for genetically modified plants expressing insecticidal proteins like the Bt toxins. These toxins, when ingested by a susceptible insect, are variable types and level of activity of proteolytic enzymes (Pardo-Lopez et al. 2013, Jurat-Fuentes and Crickmore 2017). Any change in this process due to a pre-exposure to the toxin may influence the insect susceptibility to a given Bt toxin in future exposures. Although the sublethal effects of Bt toxins are known to change life-history traits (Storer et al. 2001, Bernardi et al. 2014, Sousa et al. 2016, Campos et al. 2019), the processes that underlie the sublethal effects and their inheritance remain poorly understood. Toxicant-induced sublethal effects can be transferred from one generation to another as a parental legacy (effect) of the previous exposure to a stressor (Youngson and Whitelaw 2008, Brevik et al. 2018).

Parental effects occur when the phenotype of an individual is affected by the phenotype or environmental experiences of its parents (Donelson et al. 2009). Such alterations in the phenotype may confer an advantage or disadvantage under a similar future environment (Woestmann and Saastamoinen 2016). In the past few years, parental effects have been studied in the context of insect food quality, temperature, age, and population density, but not under the influence of toxicant stress such as that caused by Bt-toxin exposure (Woestmann and Saastamoinen 2016). Parental effects can disturb insect reproduction, development time, and diapause (Woestmann and Saastamoinen 2016), potentially changing the correspondence between offspring genotype and phenotype and driving evolutionary processes (Carrière 1992). Determining the influence of the parental environmental experiences (e.g., exposure to Bt toxin) on the life-history traits of black armyworm is an important step to understand their consequences on insects of low-inherent susceptibility to Bt toxins.

This study was designed to investigate the toxicity of purified Cry toxins, the effects of Bt cotton cultivars on black armyworm and the offspring performance where there was parental exposure to Cry1Ac. Overall, the black armyworm showed low susceptibility to Cry toxins as well as Bt cotton cultivars. In addition, there was a parental effect of Cry1Ac, a novel finding that contributes to our understanding of the influence of Bt crops on field populations of non-target insect species, particularly noctuid pests of low inherent susceptibility to Bt toxins.

2. MATERIALS AND METHODS

2.1.Insect collection and rearing

A population of black armyworm was established in 2016 from gravid moths captured in UV light traps placed in an experimental field in the main campus of the Federal University of Viçosa, Minas Gerais, Brazil. In the collection site, several crops were grown, including beans, soybean, cotton, and vegetable crops. The moths collected were brought to the laboratory, transferred to polyvinylchloride cages (40 cm high x 30 cm in Φ), and fed with a solution of 10% sugar and 5% ascorbic acid. The inner walls of the cages were covered with sulfite paper as an oviposition substrate. The eggs were collected every two days for 10 days and stored in plastic bags until hatching. A large group of neonates (> 1000) were transferred to 500-ml plastic cups containing an artificial diet based on white beans (Greene et al. 1976). Third instarswere placed in 16-well polyvinyl chloride (PVC) trays (Advent of Brazil, Diadema, SP) (one larva/well) with the artificial diet until pupation. The insects were held at 26 ± 2 °C, relative humidity of 70 ± 10 % and a photoperiod of 14: 10 h (L:D).

2.2.Source of cotton leaf tissues

Cotton cultivars used in the larval feeding study were field grown using crop management practices to obtain optimum growth. New seeds were sowed every seven days to have leaves taken from the plants at the same growth stage (Roberts 2010) for the assays. The plants were irrigated daily and fertilized at planting and in the vegetative stage with 100 kg. ha^{-1} NPK (4-14-8). Weed control was performed manually and no insecticides were applied. The cotton cultivars used were Bollgard - NUOPAL RR (Monsanto do Brasil, São Paulo, SP, Brazil), expressing the Cry1Ac toxin, Widestrike - FM 975 (Dow Agrosciences, São Paulo, SP, Brazil), expressing Cry1Ac + Cry1F toxins, and TwinLink - EB 0250 (Bayer CropScience, São Paulo, SP, Brazil), expressing Cry1Ab + Cry2Ae toxins. Each Bt cotton cultivar was compared in parallel with its non-Bt control isogenic cultivar: NUOPAL isoline and FM 933. The data for the near non-Bt isogenic cultivar of TwinLink were composed of the average of the other two isogenic cultivars (NUOPAL isoline, FM 933) due to the absence of a near isogenic cultivar for this cultivar in the laboratory.

2.3.Susceptibility to Cry toxins

The toxicity of the Cry1Fa, Cry2Aa, and Cry1Ac Bt toxins was determined to neonates of the black armyworm. The toxins were obtained from the laboratory of Dr. Marianne P. Carey (Case Western Reserve University, OH). The Cry1Ac and Cry1Fa toxins were trypsin-activated and purified on HPLC while Cry2Aa protoxin was used, all of which provided in the lyophilized form. Stocks of these toxins were maintained at -80º C until use. Aiming solubilization of the insecticidal proteins just before the bioassays, specific buffers were used for Cry1 toxins (50 mM CAPS, pH 11, 2 mM DTT) and Cry2 protoxin (25 mM CAPS; pH 10.3, 1 M benzamidine-HCl,
0.1 mM EDTA, and 0.2 mM DTT).

Increasing concentrations of the insecticidal proteins were applied on the same artificial diet used for rearing in 128-well plastic trays (each well 16 mm Φ x 16 mm height; CD International, Pitman, NJ). The dilutions were prepared in 0.1% Triton-X 100 to obtain a uniform distribution on the surface of the diet. The control treatment consisted of an artificial diet treated with 0.1% Triton-X 100. One neonate (< 24 hold) was placed to each well using a fine camelhair-paint brush, and mortality and larval weight were evaluated after seven days of exposure. The larvae that failed to grow beyond the first instar of development or their weight was less than 0.1 mg were considered dead (Marçon et al. 1999). The Cry toxin bioassays were replicated two-four times on different dates. Bioassay trays were kept in a growth chamber at 27 ± 2ºC, relative humidity 70 ± 10% and photoperiod of 14: 10 h (L:D).

2.4.Life-history traits on Bt cotton

Characteristics of the life history of black armyworm feeding on Bt and non-Bt cotton cultivars were documented using fresh leaves taken from the upper plant canopy in the growth stage of first bloom (Roberts 2010). The leaves were arranged in 16-well PVC trays (Advent of Brazil, Diadema, SP) according to treatment (i.e., cotton cultivars expressing Cry1Ac, Cry1Ac + Cry1F, Cry1Ab + Cry2Ae, and respective isolines). One 24-hour neonate was transferred to each well, and the leaves were replaced daily until pupation. The trays were held at 27 ± 2ºC, relative humidity of 70 ± 10% and a photoperiod of 14: 10 h (L:D). The experiment was conducted in a completely randomized design with 32 replicates per cotton cultivar (1 larva per replication). The daily survival rates, development time until pupation, larval, and pupal weight were recorded.

2.5.Parental effects of Cry1Ac

The sublethal effect of the Cry1Ac toxin on black armyworm larvae was assessed during the parental and its F_1 generation. An experiment with the larvae of parental generation was performed using Cry1Ac concentrations of 0, 13, 25, 50, 100, 200 ng.cm^{-2} applied on the surface of the artificial diet as previously described. These concentrations were well below the LC_{50} of the black armyworm based on the results of the susceptibility bioassay previously conducted.

After seven days of larval (parental) exposure to Cry1Ac, the larvae were weighed and transferred to the 16-well PVC trays containing diet without toxin. After 14 days, the larval weight was recorded again. The pupal weight (< 24 h after pupation) and gender were also determined. The pupae were placed in polyethylene cups (5 cm high x 7.5 cm Φ) lined with moist paper to avoid desiccation. 'Honeymoon' mating cages (30 cm high x 20 cm Φ) containing three pairs were set up. Adults were fed a water solution with sugar (10%) and ascorbic acid (5%). The eggs were collected daily on the sulfite paper placed on the inner walls of the cage and transferred to transparent plastic bags until hatching to record the number of neonates produced.

To determine the parental effect in the offspring, groups of neonates (F_1) from each of the Cry1Ac concentrations tested were individualized and reared following the same procedures used for the parents, except that there was no toxin exposure. After 7 and 14 days on a diet, the larvae were weighed and reared to adulthood using the procedures previously described for the parental generation. Survival to adulthood and age that females started reproduction were determined as for the previous generation. The life table parameters were estimated, including the intrinsic rate of population increase (r_m, which is the rate of population increase per day) and net reproductive rate (R_0, which is the rate of a multiplication per generation). Three or four cages each with three pairs couples of black armyworm moths were used. For both parents and offspring, the experiment was conducted in a completely randomized using n = 64 larvae (4 replicates of 16 larvae).

2.6.Statistical analyses

Data from the susceptibility bioassays were analyzed by using the probit model (Finney, 1971) in PoloPlus (Robertson et al. 2007) to estimate median lethal concentration values (LC_{50}), effective concentration to reduce 50% of larval growth (EC_{50}), with their 95% confidence limits (95% CL). Relative toxicity ratios based on mortality and growth inhibition and their respective 95% confidence intervals were determined using the Cry1Fa toxin as reference for all comparisons (Wheeler et al. 2006).

Life history data met the assumptions of the analysis of variance and were compared using the F test ($P < 0.05$). Survival curves for the larvae on Bt and non-Bt isogenic cotton leaves were analyzed using Kaplan-Meier estimators and log-rank chi-square tests ($P < 0.05$). All the analyses were performed using the SAS statistic program (SAS 2011).

To examine the parental effects, the larval weight was analyzed using linear regression. The criteria for selection of the most appropriate model were the significance of the regression coefficients using the t-test ($P < 0.05$) and the magnitude of the determination coefficients. The slope of the regression curve of parents and offspring were compared using analysis of covariance. The distribution of the residuals was normal and homoscedastic. This analysis was performed using the statistical program R-software.

The larval weight of parents at 7 and 14 days feeding on 0 and 200 $ng.cm^{-2}$ of Cry1Ac were compared using the F test ($P < 0.05$). The population growth parameters (R_0, r_m) were determined using the SAS code developed by Maia et al. (2000) and the variances associated with the estimates were obtained by the Jackknife method (Efron 1982, Meyer et al. 1986). This procedure allows the construction of confidence intervals for the estimated parameters and comparisons using t-tests and respective P values. This analysis was done using the statistical program SAS 9.4 (SAS 2011).

3. RESULTS

3.1.Susceptibility to Cry toxins

The probit model showed a suitable fit ($P > 0.05$) to the results of the bioassays with the Cry toxins on artificial diet as indicated by the lack-of-fit chi-square test (Table 1). The concentrations of Cry1Fa and Cry2Aa that caused 50% larval mortality (LC_{50}) were 853.4 and 1132.1 ng.cm^{-2}, respectively, while the LC_{50} for the larvae exposed to Cry1Ac toxin was greater than 10000 ng.cm^{-2}. Black armyworm larvae had greater growth inhibition when exposed to Cry1Fa (effective concentration to reduce 50% of larval growth, EC_{50} = 9.4 ng.cm^{-2}). The EC_{50} for the toxins Cry2Aa and Cry1Ac were 689.5 and 358.9 ng.cm^{-2}, respectively. The relative toxicity values using LC_{50} showed that Cry1Fa and Cry2Aa have similar toxicity to black armyworm larvae based on mortality, while Cry1Ac is at least 11.7 times less toxic than Cry1Fa. For the relative toxicity, values obtained using EC_{50} as the response variable, Cry1Ac and Cry2Aa were 72 and 38 times less toxic to the larvae than was Cry1Fa.

3.2.Life-history traits on Bt cotton

No difference was detected in larval survival rates, larval and pupal weight, and larval development time between black armyworm larvae feeding on Cry1Ac cotton and non-Bt isoline (Fig. 1a, 2a). Contrasting results were observed when black armyworm fed on the foliage of Cry1Ac + Cry1F or Cry1Ab + Cry2Ae cotton cultivars (Fig. 1b, c and Fig. 2b, c). Insects reared on the Cry1Ac + Cry1F cotton leaves exhibited reduced larval weight and did not complete the development with no pupae obtained. The insects reared on the Cry1Ab + Cry2Ae cultivar exhibited reduced larval and pupal weight and survival of 20%; the larvae had longer developmental times compared to those on non-Bt cotton isogenic cultivars.

3.3.Cry1Ac-mediated sublethal and parental effects

The larval weight in the F_1 offspring in the absence of Cry1Ac had the same effect of the exposure to the toxin in the larva from the parental generation (Fig. 3). F_1 larvae unexposed to Cry1Ac showed a response similar to that shown by their parents when exposed to the toxin in the larval stage. Growth inhibition in the parents exposed to the highest concentration of Cry1Ac tested (200 ng.cm^{-2}) was 60% compared to control, and unexposed offspring had growth inhibition of 30% compared to the control. The linear regression model describing the response of the parental generation exposed to Cry1Ac and the model describing the response of F_1 generation, which was not exposed to the Bt toxin, were not significantly different ($F_{5, 36} = 0.91$, $P = 0.48$).

After seven days of exposure to a low concentration of Cry1Ac (200 ng.cm^{-2}), the larval weight of the parental generation was reduced compared to the control. However, when the individuals reached the end of larval development under toxin exposure (14 days), their larval weight was no longer reduced relative to the control (Fig. 4 a). The net reproductive rate (R_0) and the intrinsic rate of population increase (r_m) of the black armyworm did not vary between parents exposed and not exposed to the sublethal Cry1Ac concentration on the diet (Fig. 4b, c) and control. In addition, the demographic performance of the progeny of parents exposed to Cry1Ac was not different ($P > 0.05$) from that of the progeny of parents not exposed to the toxin (Fig. 4b, c).

4. DISCUSSION

Concentration-response bioassays showed that black armyworm larvae have relatively low susceptibility to Cry toxins produced in the Bt cotton cultivars tested. The LC_{50} values obtained were greater than 850 ng.cm^{-2}, which are 10-100 times higher compared to other species of high susceptibility to these toxins (Tan et al. 2011, Pardo-Lopez et al. 2013, Bel et al. 2017). Several *Spodoptera* species have low-inherent susceptibility to Cry Bt toxins (Hernández-Martínez et al. 2008, Santos et al. 2009, Lu et al. 2013, Bernardi et al. 2014, Palma et al. 2014) and mechanistically, it is likely due to the toxin affinity to protein receptors or its proteolysis in the midgut of black armyworm larvae (Pardo-Lopez et al. 2013). The most potent of the Cry toxins tested in this study was Cry1F, a toxin that initially had high efficacy against the fall armyworm (*S. frugiperda*) (Storer et al. 2010, Santos-Amaya et al. 2017) and was widely deployed in Bt corn hybrids in the Americas.

In addition, our results showed that the combination of Cry1Ac with Cry1F in Bt cotton increased the efficacy to black armyworm when compared to Bt cotton expressing only the Cry1Ac toxin. In bioassays performed in this study, Cry1F and Cry2A toxins had relatively higher toxicity to the larvae; hence, they are likely to be responsible for the increased lethal effect of the dual-toxin Bt cotton cultivars. Because the Cry1Ab toxin has greater than 78% similarity to Cry1Ac in the aminoacid sequence (de Maagd et al. 2001, Pardo-Lopez et al. 2013), it is unlikely that Cry1Ab improved the control efficacy of

Cry1Ab+Cry2Ae Bt cotton cultivars to black armyworm larvae. Dual-toxin cotton expressing Cry1A and Cry2A should not be considered pyramids for resistance management of black armyworm because Cry1A is unlikely to be effective to the larvae.

Nevertheless, these dual-toxin Bt cotton technologies may still be useful for integrated pest management of black armyworm. For example, in our results, the larvae feeding on cotton Cry1Ac + Cry2Ab cotton took 10 days to reach 50% mortality, which is quite a long time compared to other susceptible noctuid species for which 100% mortality is attained before the third day of exposure to Cry1Ac and/or Cry2Ab (Sorgatto et al. 2015, Rodrigues-Silva et al. 2019). The relatively low larval weight and delayed development time of black armyworm larvae feeding on cotton Cry1Ab + Cry2Ae suggest that in the field they will be exposed longer to mortality risks, increasing their likelihood of death by natural enemies or reducing larval competitive abilities (Sansone and Smith 2001, Sousa et al. 2016). In addition, individuals initially smaller may not have a disadvantage during important life history events, such as maturity and reproduction (Le Galliard et al. 2005). This can be a positive factor in the management of black armyworm (Bentivenha et al. 2017), although changes in larval behavior need to consider. This is critical for Bt cotton, which shows reduced expression of Cry1 and Cry2 toxins in reproductive structures and other parts during senescence (Adamczyk Jr et al. 2001, Willrich Siebert et al. 2009). Black armyworm larvae may move to plant parts with less expression of Bt toxin as observed for *H. zea* (Braswell et al. 2019). Thus, it is important to investigate how the temporal and spatial variation in Bt toxin expression in cotton plants affects the on-plant larval movement and feeding behavior (Pannuti et al. 2015) of black armyworm larvae and its consequences for pest management.

Currently, several countries in South America have adopted Bt soybean expressing Cry1Ac and Bt cotton expressing Cry1Ac, Cry1Ab, Cry1Fa, Cry2Ab, and Cry2Ae. A new Cry1Ac + Cry1F Bt soybean (event DAS-81419-2) was recently approved and should provide control of black armyworm and other lepidopterans (Marques et al. 2016). Additionally, in the near future, a third-generation of Bt cotton expressing Cry and Vip toxins is expected to be available for the control of lepidopteran pests (Carrière et al. 2015, Santos-Amaya et al. 2015). However, published reports on the effects of Vip cotton on black armyworm are not yet available, and attention should be given when deploying these new Bt technologies

Exposure of black armyworm larvae to low concentrations of Cry1Ac reduced the parental larval weight and consequently promoted a parental effect, as reflected by the weight reduction in early-instar offspring, which were not exposed to the toxin (Fig. 3). These results indicate that sublethal exposure to Bt toxins can change the phenotype of black armyworm in more than one generation. If such offspring phenotypes had inherited Cry1Ac resistance gene(s), one would expect to notice their advantage (i.e., increased larval weight) in a second exposure to a Bt toxin (Woestmann and Saastamoinen 2016). Thus, an alternative hypothesis is needed, perhaps an epigenetic change that modified the pattern of gene expression without changing the underlying DNA sequence (Youngson and Whitelaw 2008, Brevik et al. 2018). As for xenobiotics and environmental stressors, such as synthetic insecticides (Brevik et al. 2018), Bt toxins may also induce epigenetic modification, a topic that deserves further investigation. Epigenetic modifications are fundamentally important to cellular differentiation, developmental plasticity and are passed across cell divisions to the offspring (Carrière 1994, Donelson et al. 2009). These epigenetic changes can persist for at least several generations (parental effect) in a way to their offspring to be able to better tolerate stress (Youngson and Whitelaw 2008, Glastad et al. 2019). The sublethal exposure to Cry1Ac and the parental effect did not modify the net reproductive rate (R_0) or intrinsic rate of population increase (r_m) in parents or offspring (Fig. 4 b, c). The parental larval weight was recovered after transferring the larvae to a Bt-toxin free diet (Fig. 4 a) or when the F_1 larvae were fed a diet containing a low concentration of the toxin throughout development. Therefore, feeding on a high-quality food source may recover the reduced larval weight in the parents and the moderate initial reduction in larval weight of the offspring (Woestmann and Saastamoinen 2016). Such recovery was sufficient so that reproduction was not impacted. This indicates that the quality of the offspring growth environment may alter the magnitude of the parental effects. These effects may have greater significance under the chronic exposure of black armyworm to Bt toxins in the field due to the mortality risks that the larvae are exposed to (Deans et al. 2016).

The low-inherent susceptibility of black armyworm to Cry toxins reported in the present study combined with the potential reduction of insecticide sprays for the target lepidopteran pest complex in Bt fields of soybean and cotton may open a new ecological niche increasing the risk that black armyworms could emerge as a primary pest in Bt cotton. In regions such as in the south of U.S and tropical areas in Central and South America, which the abundance of food sources and high adoption of Bt crops may afford fast changes in the pest status of the multivoltine black armyworm. In addition, the polyphagia nature of the larvae may facilitate their growth on alternative host plants such as some weeds present in cotton and soybean fields, potentially favoring their adaptation in agroecosystems and man-modified landscapes. The adoption of an IPM represents the framework that should be adopted in Bt crops to deal with pest species not targeted by the technology. Thus, sampling plans and economic thresholds for Bt soybean and Bt cotton are needed for a sound IPM program for the black armyworm (Bueno et al. 2013). One challenge may be the lack of registered synthetic insecticides for black armyworm control (Lutz et al. 2018), and thus new studies should be conducted to address these issues.

In summary, the Cry1Ac-induced parental effect in the black armyworm larva advances our understanding of the consequences of the exposure of insect species of low-inherent susceptibility to Bt toxins. The results of this study could also contribute to improving the black armyworm management in Bt crops. The low susceptibility of black armyworm larvae to Cry toxins and some Bt cotton cultivars emphasizes that the Bt technology should be used within an integrated pest management framework to properly manage populations of black armyworm.

5. ACKNOWLEDGMENTS

We thank Dr. Miguel Soria for helping to obtain seed samples of the cotton cultivars used. This study was financed in part by the CAPES Foundation (Finance code 001; Brazilian Ministry of Education) and the National Council of Scientific and Technological Development (CNPq; Brazilian Ministry of Science and Technology).

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7. TABLES AND FIGURES

Table 1. Susceptibility of black armyworm larvae (*Spodoptera cosmioides*) exposed to the Cry1Fa, Cry2Aa, and Cry1Ac toxins from *Bacillus thuringiensis* based on mortality and growth inhibition after seven days of exposure.

Variable	Toxin	N[a]	Slope ± SE	---- LC$_{50}$ (95% CL)[b] ----	χ^2	*P*	Relative toxicity[d]
Mortality	Cry1Fa	473	2.13 ± 0.36	853.4 (454.9 - 1289.6)	6.18	0.28	1
	Cry2Aa	512	1.66 ± 0.19	1132.1 (795.4 - 1497.4)	3.80	0.57	1.4 (0.9 – 2.1)
	Cry1Ac	192	nc[e]	> 10000	nc	-	> 11.7
				---- EC$_{50}$ (95% CL)[c] ----			
Growth Inhibition	Cry1Fa	505	1.26 ± 0.10	9.4 (6.2 - 13.2)	5.05	0.40	1
	Cry2Aa	512	1.86 ± 0.20	689.5 (488.3 - 1046.0)	6.67	0.57	73.2 (47.1 – 113.8)
	Cry1Ac	192	0.72 ± 0.07	358.9 (139.3 - 609.5)	8.90	0.11	38.1 (23.8 – 60.8)

Concentration values are in nanograms per centimeter square (ng.cm^{-2}) of the toxin on the surface of the artificial diet. [a]N, Number of insects tested in the bioassays. [b]LC$_{50}$, Lethal concentration to cause 50% mortality and its 95% confidence limits. EC$_{50}$, Effective concentration to cause 50% larval growth inhibition its 95% confidence limits. [d]Relative toxicity = LC$_{50}$ or EC$_{50}$ of the toxin ÷ LC$_{50}$ or EC$_{50}$ of Cry1Fa with its respective 95% confidence limits; it indicates how many times a particular toxin is less toxic than the most potent one (Cry1F). [e]nc, Not calculated because of a lack of significant response even at the highest concentration tested.

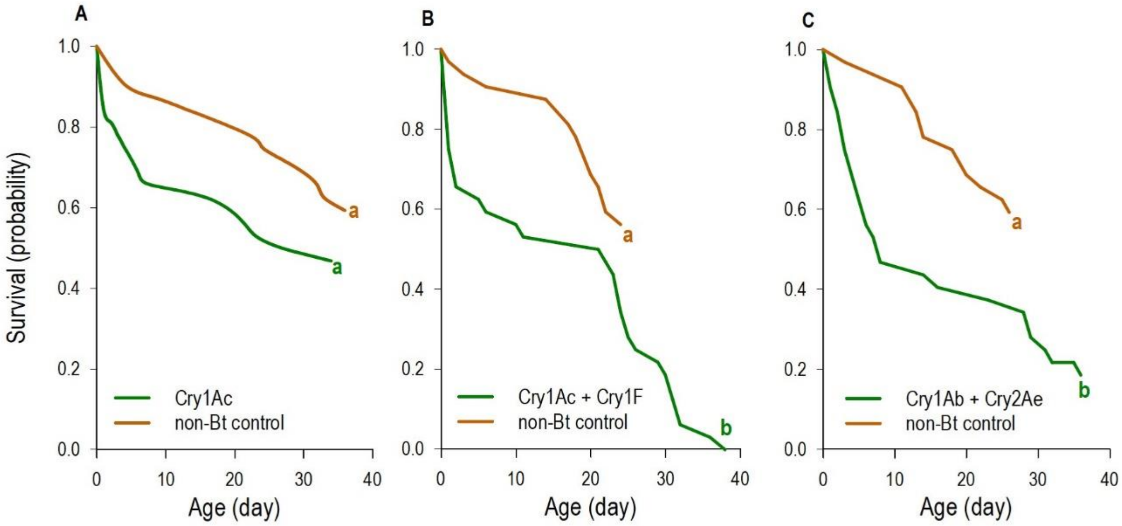

Figure 1. Survival of black armyworm larvae (*Spodoptera cosmioides*) on Bt and non-Bt cotton cultivars. (a) Cry1Ac (b) Cry1Ac + Cry1F and (c) Cry1Ab + Cry2Ae. Effect was measured as time to death and summarized as survival curves estimated by the Kaplan-Meier method. Survival curves followed by different letters are significantly different by the log-rank chi-square test ($P < 0.05$).

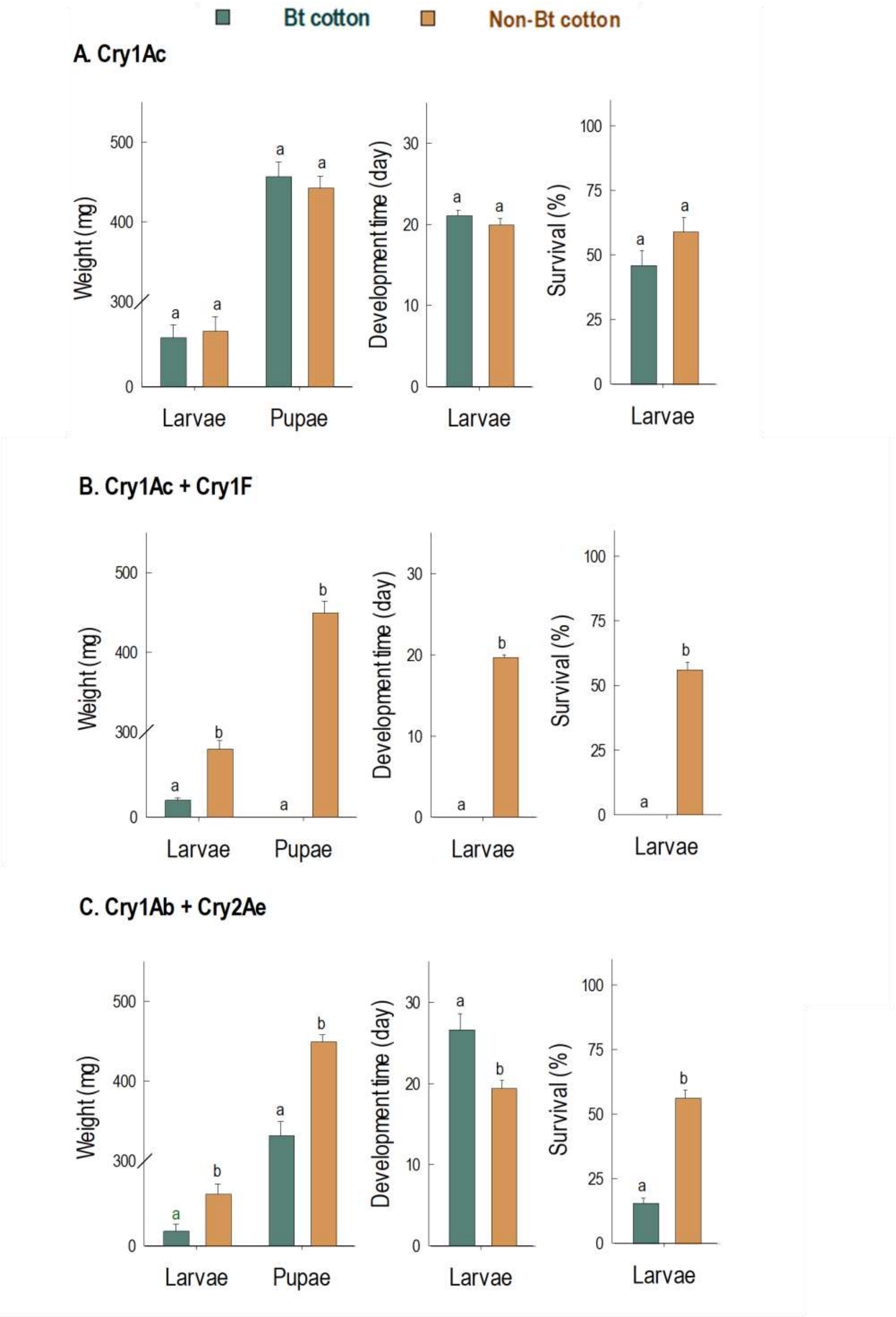

Figure 2. Life-history traits of black armyworm larvae (*Spodoptera cosmioides*) on Bt and non-Bt cotton cultivars expressing (a) Cry1Ac (b) Cry1Ac + Cry1F and (c) Cry1Ab + Cry2Ae. Larval weight was recorded at seven days of age. Development time and survival represent the time from neonate to pupa stage. Data are means ± standard error. Pairs of bars with the same letters are not significantly different (ANOVA, $P < 0.05$).

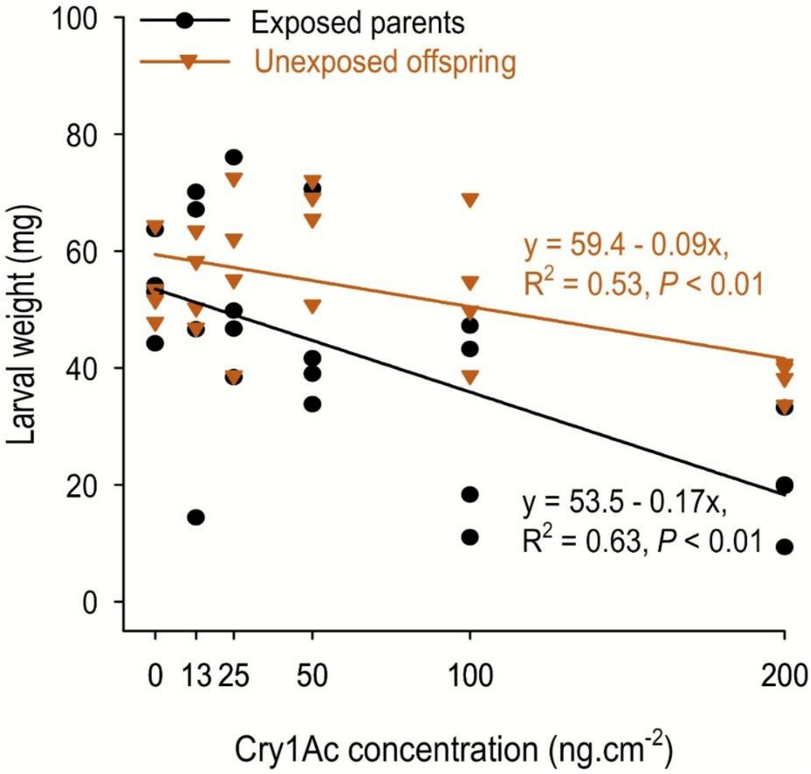

Figure 3. Parental effect in the larval weight of black armyworm larvae (*Spodoptera cosmioides*) induced by exposure to a Bt toxin**.** Larvae of the parental generation were exposed to increasing concentrations of Cry1Ac for seven days and reared to adulthood; the larval weight of the offspring was recorded under no toxin exposure and showed a trend similar to that of the parental generation. Each symbol represents the mean larval weight of 16 individuals, and the lines represents the linear regression curve fitted to the data of each generation. The slopes of the two regression lines were not significantly different (ANCOVA, $P = 0.48$).

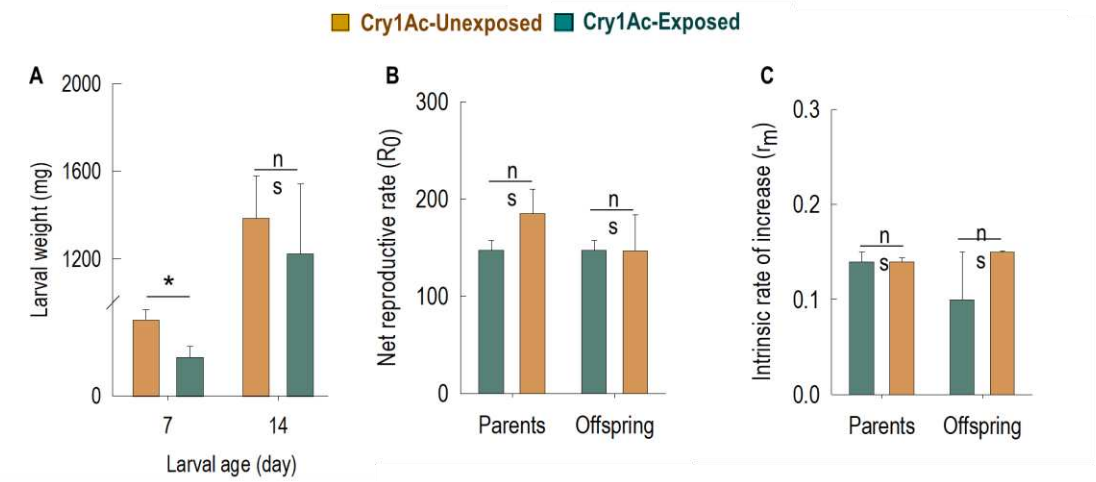

Figure 4. Recovery of the reduced larval weight in black armyworm (*Spodoptera cosmioides*) exposed to Cry1Ac Bt toxin and reproductive parameters of parents and offspring exposed on unexposed to Cry1Ac toxin. (A) Larval weight of parents (means ± standard errors, n = 128) after seven days of toxin exposure to purified 200 ng.cm^2 of Cry1Ac in the artificial diet for seven days during the parental generation. (B) Net reproductive rate (R_0) (i.e., population multiplication rate per generation). (C) Intrinsic rate of population increases (rm) (i.e., population growth rate per capita per day). The data for the demographic performance (R_0, rm) are means and standard errors obtained using the Jackknife method. Columns marked with and asterisk (*) indicate significant differences between Cry1Ac-Unexposed and Cry1Ac-Exposed (two-tailed *t*-tests, $P < 0.05$) and *ns* indicate not significant.

CHAPTER II – ABSTRACT

RABELO, Marcelo Mendes,D.Sc., Universidade Federal de Viçosa, April, 2020. **Demographic performance of *Helicoverpa zea* populations on dual and triple-gene Bt cotton** Adviser: Eliseu Jose Guedes Pereira. Co-adviser: Silvana Vieira de Paula Moraes.

Insecticidal toxins from *Bacillus thuringiensis* (Bt) provide valuable tools for pest management worldwide, helping combat insect vectors of human diseases and phytophagous insect pests of agriculture and forestry. Here, we report the effects of dual and triple Bt toxins expressed in transgenic cotton cultivars on the fitness and demographic performance of *Helicoverpa zea* (Boddie), a noctuid pest known as cotton bollworm and corn earworm. Life-history traits were determined for individuals of three field populations from a region where *H. zea* likely overwinter. Triple-gene Bt cotton cultivars expressing Cry and Vip3Aa toxins killed 100% of the larvae in all populations tested. In contrast, dual-gene Bt cotton expressing Cry1Ac+Cry1F and Cry1Ac+Cry2Ab2 allowed population growth with the intrinsic rate of population growth (r_m) 38% lower than on non-Bt cotton. The insects feeding on Bt cotton plants expressing Cry1Ac+Cry2Ab2, Cry1Ac+Cry1F, and Cry1Ab+Cry2Ae exhibited reduced larval weight, survival rate, and increased development time. Additionally, fitness parameters varied significantly among the insect populations even on non-Bt cotton plants, likely because of their different genetic background and/or previous Bt-toxin exposure. This is the first report of comparative fitness of field populations of *H. zea* on dual-gene Bt cotton after the recent development of field resistance to certain Bt toxins. These results document the population growth rates of *H. zea* from an agricultural landscape with 100% Bt cotton cultivars. Our results will help to refine models designed to predict resistance evolution and improve insect resistance management for Bt crops.

Keywords: Fitness. Life table. Cotton Bollworm. Corn Earworm.

1. INTRODUCTION

Transgenic crops expressing insecticidal toxins from the bacterium *Bacillus thuringiensis* (Berliner) (Bt) provide valuable pest management options in field crops worldwide (Bates et al. 2005, Naranjo 2011, Koch et al. 2015). Positive socio-economic and environmental impacts of the adoption of Bt crops have been reported since commercial release in 1996 (ISAAA 2017, Fleming et al. 2018). In the United States (U.S.), the Bt technology has provided control of major cotton pests, *Chloridea virescens* (Fabricius) (Lepidoptera: Noctuidae), *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelichiidae), and *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) (Luttrell and Jackson 2012, Braswell et al. 2019). Bt cotton has been shown to improve management of other lepidopteran pests such as *Spodoptera exigua* (Hübner), *Trichoplusia ni* (Hübner), *Spodoptera frugiperda* (J.E. Smith), *S. eridania* (Stoll), and *Chrysodeixis includens* (Walker) (Hardee et al. 2001, Stewart et al. 2009, Sorgatto et al. 2015, Rabelo et al. 2020). From 1996-2003, commercial Bt cotton in the U.S. was limited to events that expressed on the Cry1Ac toxin (Tabashnik et al. 2008). Since 2003, second generation dual-gene Bt cotton, expressing the toxins Cry1Ac+Cry2Ab2, Cry1Ac+Cry1F, and Cry1Ab+Cry2Ae became available and widely adopted (Luttrell and Jackson 2012, EPA 2019). The goal of these second generation cotton events is to reduce the risk of resistance evolution by targeting unique and independent target sites. Recently, the third generation of cotton events with triple Bt traits has been commercially available, including those expressing Cry1Ac+Cry1F+Vip3Aa20, Cry1Ac+Cry2Ab2+Vip3Aa20, and Cry1Ab+Cry2Ae+Vip3Aa20. The Cry and Vip families (vegetative insecticidal protein) are produced during different stages of the *B. thuringiensis* life cycle. Although they are thought to have a similar mode of action against the target insects, the two toxin classes exhibit different receptors in the insect midgut(Pardo-López et al. 2013).

The cotton budworm, *C. virescens,* is highly susceptible to most Cry Bt toxins, and Bt cotton usually reaches the high-dose condition, killing almost all heterozygotes for Bt resistance (Hardee et al. 2001, Blanco 2012) even for single toxin events. However, *H. zea* is less susceptible to Cry toxins expressed in cotton and corn, which do not satisfy high-dose criteria (Stone and Sims 1993). Toxicological bioassays performed with populations of *H. zea* from south U.S., including populations from Florida Panhandle have indicated a decrease in susceptibility of H. zea populations to Cry1Ab, Cry1Ac, Cry1A.105, and Cry2Ab2, but not to Vip3Aa (Fleming et al. 2018, Leite et al. 2018, Reisig et al. 2018, Kaur et al. 2019).

Field-evolved resistance in target pests is a threat to the success of the Bt technology, which may lead to control failures (Reisig and Kurtz 2018) and the need for traditional insecticides for supplemental control (Reisig et al. 2019). *Helicoverpa zea* exhibits a sequence of host crop utilization based on the temporal dynamics of the southeastern U.S. agricultural landscape. In general, the first generation of this pest feeds on corn, cultivated during the spring to beginning of summer. Corn is a major host plant of *H. zea.* However, this pest has a low impact on the yield of field corn when it is planted early in the season (Bibb et al. 2018). *L*ater in the summer, the subsequent generations of *H. zea* disperse from corn to cotton, which becomes the prevalent crop in the agricultural landscape of the southeastern U. S. until the end of the crop season. Thus, corn serves as a source of *H. zea* populations and if these source populations develop on Bt corn, a transition of survivors from corn to cotton is likely. Larval feeding in both crops producing the Vip3A toxin exerts continuous selection pressure and raises concerns about the selection of resistant populations(EPA 2019).

The United States Environmental Protection Agency (EPA) has defined the knowledge of pest biology and ecology as key elements in formulating a Bt insect resistance management (IRM) (EPA 2001). Some studies indicate that *H. zea* has high growth potential when feeding on non-Bt field corn and sweet corn (Storer et al. 2001, Olmstead et al. 2016). However, Cry1Ab Bt corn reduced the population growth of *H. zea* (Storer et al. 2001). The growth potential of *H. zea* populations feeding on dual- or triple-toxin Bt cotton remains undocumented. Developing life tables for *H. zea* on dual and triple Bt cotton events would allow the documentation of key fitness, such as survival, development time, fertility, and population growth (Birch 1948). Fitness components and rates of population growth (i.e., demographic performance), if available, can be used to inform models to predict the rate of resistance evolution in target pests or to comparatively assess different resistance management practices, such as the use of structured and/or natural refuges (Pittendrigh et al. 2004, Gassmann et al. 2009, Hackett and Bonsall 2016, Reisig and Kurtz 2018).

Helicoverpa zea populations from the Florida Panhandle represent valuable resources for documenting the fitness components and demographic performance. The region has a distinctive regional landscape consisting of natural vegetation, forests, and field crops. Cotton is cultivated in large areas, with an annually production value of over $45 million and with 100% adoption of Bt cultivars (Cook 2018, USDA/NASS 2019). Furthermore, the region is located in the Gulf Coastal Plain of the southeastern U.S., in an ecological transition zone between temperate and subtropical climates. In the region, *H. zea* populations can overwinter and disperse throughout the growing season (Raulston et al. 1986, Morey et al. 2012). The overwintering survival of *H. zea* could be a carry-over source of Bt resistance alleles for other regions and between seasons (EPA 2019). In this study, we report individual and population fitness of representative *H. zea* larvae challenged with dual- and triple-toxin Bt cotton technologies, which is important to inform resistance management recommendations.

2. MATERIALS AND METHODS

2.1.Cotton plants

This study was conducted during 2018 at the West Florida Research and Education Center (WFREC), University of Florida at Jay, FL. The cotton cultivars utilized are described in Table 1. The cultivars were planted in a Randomized Complete Block Design with four replications. The agronomic practices adopted were based on standard recommendations for the region (Wright et al. 2003), and no application of pesticides were performed in the experimental plots. Cotton leaves were collected from the upper part of the plant canopy in each plot, placed in a ziplock bag, and held in Styrofoam boxes with an ice pack. In the laboratory, the cotton leaves were tested using Envirologix GMO quick Stix to confirm Cry1Ac, Cry2Ab, Cry1F, and Vip3Aa expression (EnviroLogix Kit, Portland, ME) among the different events. Similar procedures were used to collect blooms, squares, and bolls during the first bloom to open boll plant stages.

2.2.Insect populations

Three *H. zea* populations were collected during the 2018 crop season from commercial fields located in the main cotton-producing counties in the Florida Panhandle: Santa Rosa, Escambia, and Jackson. Cotton fields were located in areas where a peanut/cotton rotation is adopted with corn planted in small scale. The populations from Santa Rosa and Jackson were collected from ears of Bt corn (Cry1A.105+Cry2Ab2). The population from Escambia was collected from blooms and bolls of Bt cotton (Cry1Ac+Cry2Ab2). Information on the collections, including location, and the number of generations in the laboratory are shown in Table 2.

Collected larvae were identified based on the morphology and validated after adult emergence (Hardwick 1965). The larvae were removed from the plant individually into plastic cups containing a multispecies lepidopteran diet (Southland Products, Lake Village, AR). The cups were held in Styrofoam boxes with an ice pack during transport to the laboratory where they were maintained at 25 ± 2 ºC, 70 ± 10% relative humidity and 14L: 10D photoperiod. Pupae were transferred to Petri dishes and covered with vermiculite moistened with water and placed in rearing cages (22 x 30 cm) for adult emergence. The adults were fed with a solution of 10% honey replaced every two days. Paper towels (Great Value, Bentonville, AR) were used to cover the internal walls of the cages as an oviposition substrate. The eggs were collected and transferred to ziplock bags until hatching. Neonates were transferred to a multispecies lepidopteran diet (Southland Products, Lake Village, AR) in rearing containers (Southland Products, Lake Village, AR) and maintained individually until pupation.

2.3.*Helicoverpa zea* life-history traits and life table parameters

One hundred neonates of each population (Santa Rosa, Escambia, and Jackson) were transferred in groups of five to 473-ml polypropylene containers (Fabri-Kal Corp. Kalamazoo, MI) and fed with cotton leaves, blooms, squares, and bolls of the cultivars described in Table 1. After five days, the larvae were placed in individual rearing containers to avoid cannibalism, as previously described (Capinera 2000). The plant tissues were replaced every four days until pupation. When the larvae reached 4th instar, wet vermiculite was added to the bottom of the rearing containers as a substrate for pupation and to avoid desiccation. The pupae were left in the containers until adult emergence. Larvae weight was determined after seven days. Once larval development was completed, and within 24 h after pupation, each pupa was weighed, and the sex was determined. Other life history components were recorded, including survival rate (neonate to pupa) and development time of larvae, pre-pupae, pupae, and adults. The experiment was arranged in a completely randomized design with 100 larvae per cotton cultivar (1 larva per replication) for each population.

One male and female from each cultivar that emerged within two days of one another were confined in mating cages (30 cm high x 20 cm diameter polymerized vinyl chloride tube). The cages were covered with a waxed brown paper (Roberts Consolidated Industries Inc., Boca Raton, FL) as an oviposition substrate, and supplied with a 10% aqueous honey solution, replaced every day. Adult survival and the number of eggs were recorded daily. The brown paper containing the eggs was transferred to ziplock bags until hatching. An additional egg viability estimation was based on a daily evaluation of the presence of the neonates in each ziplock bag. The sex ratio, number of eggs, survival, and age of females at the onset of egg-laying were determined to estimate the life table parameters. These include the net reproductive rate (R_0), which represents the multiplication rate per generation, the intrinsic rate of population increase (r_m), which reflects the ability of one female to generate another female per unit of time, and generation time (T), the mean time between two successive generations. The life table experiment was conducted in a completely randomized design with 16 to 18 replications (couples) per cotton cultivar.

2.4.Statistical analyses

Differences in the survival rate, body weight, development time, and egg viability of the three populations reared on the seven cotton cultivars were compared using a two-way analysis of variance in R software (version 3.5.1). The fixed effects tested were the *H. zea* population, cotton cultivar, and their interaction. Pairwise comparisons were made using Tukey's HSD post hoc test using a level of significance of 0.05. The population growth parameters (R_0, r_m, T) were determined using the SAS programming developed by Maia (2000) (Maia et al. 2000), and the variances associated with the estimates were obtained by the Jackknife method. This procedure allows the construction of confidence intervals for the estimated parameters in addition to comparisons by the *t*-test.

3. RESULTS

3.1. *Helicoverpa zea* life-history traits

The interaction between cotton cultivar x insect population was significant (Table 3, $P <$ 0.05) for larval weight, larval development time, larval survival, and pupal development time.

Pupal weight, pre-pupa time varied only with the main effects of either cultivar or population or both (Table 3), and the pupal viability did not vary significantly ($P > 0.05$).

The dual-gene Bt cotton cultivars significantly ($P < 0.05$) reduced larval and pupal weights in all populations tested (Table 4). The population from Escambia County exhibited the lowest larval weight on Cry1Ab+Cry12Ae and Cry1Ac+Cry2Ab relative to non-Bt cotton while Cry1Ac+Cry1F had the lowest negative impact on larval and pupal weights relative to the other cultivars. All three dual-gene Bt cotton cultivars reduced larval weight equally in the Santa Rosa population compared to the non-Bt cotton. The population from Jackson had the lowest larval weight on Cry1Ac+Cry2Ab2 and both lowest larval and pupal weights on Cry1Ab+Cry12Ae, while Cry1Ac+Cry1F cotton did not impact larval or pupal weights compared to non-Bt cotton. Among populations, *H. zea* from Jackson had the lowest larval and pupal weights, even when feeding on non-Bt cotton.

Regarding larval survival rates, the triple-gene Bt cotton cultivars caused 100% mortality of all populations tested and were therefore not be included in further analysis of life-history traits (Table 5). *Helicoverpa zea* from Escambia and Jackson had similar larval survival on non-Bt and Cry1Ac+Cry1F cotton. However, larval survival was reduced on Cry1Ac+Cry2Ab2 and Cry1Ab+Cry12Ae. *Helicoverpa zea* from Santa Rosa had larval survival reduced by all dual-gene Bt cotton, with Cry1Ab+Cry12Ae being the most severe. *Helicoverpa zea* from Escambia had high survival rates on Bt and non-Bt cotton compared to Santa Rosa and Jackson populations. Among dual-gene Bt cotton cultivars, Cry1Ac+Cry1F and Cry1Ab+Cry12Ae allowed the highest and lowest larval survival, respectively. The survivorship of pupa (i.e., pupal viability) ranged from 91 to 100% and did not vary among cotton cultivars or insect populations (Table 5).

The larvae developed more slowly on dual-gene Bt cotton than on non-Bt cotton (Table 6). Likewise, the insects from Jackson county had longer larval development time when feeding on non-Bt and Cry1Ab+Cry2Ae than the other populations. The Santa Rosa insects had longer pre-pupa development time on Cry1Ac+Cry2Ab2, but this trait was not affected by the other cultivars, populations, or their interaction (Table 6). The duration of the pupal stage was shorter for insects feeding on Cry1Ac+Cry1F than on non-Bt or the other Bt cotton cultivars (Table 6).

3.2. *Helicoverpa zea* life table parameters

The demographic performance of *H. zea* feeding on non-Bt, Cry1Ac+Cry2Ab2, and Cry1Ac+Cry1F varied among the cotton cultivars and insect populations (Table 8, Figure 1). Reproductive capacity on the other cultivars was not determined due to low survivor. The net reproductive rate (R_0) of insects reared on non-Bt cotton was approximately 50% higher than those on Cry1Ac+Cry1F and Cry1Ac+Cry2Ab2. The intrinsic rate of population increase (r_m) of the insects reared on non-Bt cotton was 30% greater than on Cry1Ac+Cry1F and Cry1Ac+Cry2Ab2, except those from Jackson County, which exhibited the same r_m value on non-Bt and Cry1Ac+Cry1F. The generation time (T) was nearly ten days shorter for insects reared on non-Bt cotton compared to those on the other cultivars, except for Jackson insects, which exhibited the same generation time on non-Bt and Cry1Ac+Cry1F. In contrast, the Jackson population had a higher fitness (higher R_0, r_m, and lower T) on Cry1Ac+Cry1F compared to the others. The egg viability was similar in all-cotton cultivars but was higher for the Escambia population (Table 7).

4. DISCUSSION

In this study, the life-history traits and demographic performance of *H. zea* from the Florida Panhandle varied among cotton cultivars and field populations, indicating differences among the cultivars in the efficacy against *H. zea* and the current population susceptibility to the Bt toxins. Gassmann *et al.* (2009) suggest that survival, developmental time, and body weight are key individual fitness parameters. Here, the effects on immature fitness components associated with both population and cotton cultivar translated to negative effects on the population growth potential of *H. zea*. Cotton plants are rich in terpenoid compounds, which may function as a barrier against herbivores impairing growth/development and/or behavioral traits. Nevertheless, the cotton plant has been modified during domestication and breeding for high yield and quality, including low gossypol oil in cottonseeds, which may have lessened the content of anti-herbivory secondary metabolites (such as gossypol) (Ti et al. 2009, Chen et al. 2015, Bernal et al. 2018). Information on secondary compounds in the cotton cultivars are generally scarce. However, other studies comparing non-Bt cotton cultivars have shown low or no change in *Spodoptera* life history (Rabelo et al. 2020). Thus, the differences in the effects on *H. zea* life history documented here are likely associated with the presence and content of Bt toxins in cotton tissues.

While the life-history traits of the insects from Escambia appear to be generally less affected by the Bt cotton cultivars, those of the Santa Rosa and Jackson populations had large negative effects on the body weight, survival, and development time. *Helicoverpa zea* populations were collected in three locations separated by at least 78 km. The agricultural landscape of the three counties has a prevalence of cotton and peanut. The acreage of cotton (Cry1Ac+Cry2Ab2 is the prevalent trait) is variable in different counties over the years. However, a pattern in the last five years indicates a predominance of peanut in Jackson county (USDA/NASS 2019), which may have reduced the exposure of this population to Bt hosts. Therefore, a parental effect due to the exposure to Cry1Ac+Cry2Ab2 (Von Kanel et al. 2016, Rabelo et al. 2020) may partially explain the better fitness (weight, development time, and survival) detected during the development of the Escambia population. Likewise, these life-history traits were more affected by Cry1Ab+Cry2Ae and Cry1Ac+Cry2Ab2, while Cry1Ac+Cry1F caused fewer negative impacts, causing significant mortality only in Santa Rosa. This result is probably associated with the fact Cry1Ac and Cry1F on *H. zea* do not satisfy high-dose criteria larvae (Yang et al. 2019). Cry1Ac has been expressed in Bt cotton cultivars since its first commercial release, with resistance documented 15 years later in the U.S. (Tabashnik et al. 2008, Carrière et al. 2015). The less-than-high-dose expression of Cry2 toxins and the recent widespread resistance to Cry2Ab in *H. zea* populations (Sivasupramaniam et al. 2008, Braswell et al. 2019, Kaur et al. 2019) may be a contributing factor to the considerable rates of larval survival in Cry1Ac+Cry2Ab2 and Cry1Ab+Cry2Ae. The triple-gene Bt cotton expressing Vip3Aa (Cry1Ac+Cry1F+Vip3Aa20, Cry1Ac+Cry2Ab2+Vip3Aa20, and Cry1Ab+Cry2Ae+Vip3Aa20) caused 100% larval mortality in all *H. zea* populations, which reinforces the high efficacy of this toxin in *H. zea* control (Leite et al. 2018, Reisig and Kurtz 2018). Vip toxins show limited amino-acid sequence homology with Cry toxins and cause pore formation with unique properties, thus having a low risk for cross-resistance between them (Dourado et al. 2016, Jurat-Fuentes and Crickmore 2017). Although this scenario suggests a favorable condition for resistance management, studies showed high LC_{50} for Vip3Aa (40-2100 ng/cm^2) indicated that this toxin might not be very potent against *H. zea* (Leite et al. 2018).

Also, some Cry toxins co-expressed in some Bt cotton cultivars have reportedly low impact on the larvae or are no longer effective (Tabashnik et al. 2008, Dively et al. 2016), which compromises the pyramid of Bt genes. Therefore, the efficacy of Bt cotton expressing Cry1, Cry2, and Vip toxins should be monitored to avoid selecting Bt-resistant populations earlier than expected. Overall, our data on life-history traits (survival rates, body weight, developmental time) are consistent with previous reports that Cry1Ac+Cry1F affects *H. zea* larvae less than Cry1Ac+Cry2Ab2, Cry1Ab+Cry12Ae, and cultivars expressing Vip3Aa (Luttrell and Jackson 2012, Fleming et al. 2018, Reisig et al. 2018).

Sublethal effects on *H. zea*, as indicated by reduced body weight and the prolonged larval development, may have implications for pest management. Delayed larval development and low body weight are expected to increase the likelihood of exposure to other mortality factors. For example, early-instar larvae are unable to bore into the cotton bolls (Ellsworth and Bradley 1992). Consequently, they may be more exposed to insecticide applications and vulnerable to natural enemies (Liu et al. 2014). Also, slow larval growth tends to increase the intervals for insecticide applications, which target the smaller larvae (about 1 cm) at the most vulnerable stage(Pedigo and Rice 2009).

Cotton is the last crop to be planted in the Florida Panhandle region, remaining longer period then other crops in the agricultural landscape prior to the fallow season. The longer larval development time of *H. zea* when feeding on Cry1Ac+Cry2Ab2, associated with infestations during mid- and late season, could expose larvae to shorter days and dropping temperatures, factors that regulate insect diapause (Meola and Gray 1984, Clemmensen and Hahn 2015). Diapausing populations from the Florida Panhandle may contribute to infestations in the corn and cotton belts North to 40 N latitude, where *H. zea* cannot permanently survive (Morey et al. 2012). The Florida Panhandle is already considered a “hybrid zone” of populations of noctuids, such as *S. frugiperda*, which flies from South Florida and Texas to the North of the U.S. (Nagoshi et al. 2012, 2014).

The pupal viability of *H. zea* was similar in different cotton cultivars and populations. On the other hand, feeding on different cotton cultivars affected pupal weight. Pupal weight is often correlated with fecundity(Leuck and Perkins 1972), although this correlation might also be affected by several other factors(Bilbo et al. 2018). The heavier pupal weight was linked with higher fecundity, which agrees with reports for other noctuids, such as *H. armigera*(Reigada et al. 2016).

Cumulative effects on specific life-history traits of *H. zea* (i.e., larval survival and development time) impacted the population growth potential on Bt cotton cultivars. The life-table parameters indicated that all *H. zea* populations are expected to grow when the larvae feed on Cry1Ac+Cry1F or Cry1Ac+Cry2Ab2 Bt cotton, but with reduced growth rates (i.e., R_0, r_m). Overall, insects feeding on Cry1Ac+Cry1F and Cry1Ac+Cry2Ab2 are expected to generate 30% and 42% fewer individuals per day compared to non-Bt cotton, respectively. The growth potential of *H. zea* from Jackson county was similar when feeding on non-Bt or on Cry1Ac+Cry1F, which could result in a higher number of exposed offspring (Tabashnik et al. 2008). However, the insects of the Jackson population had lower growth rates on non-Bt cotton when compared with Escambia and Santa Rosa populations, indicating the presence of fitness costs (Jakka et al. 2014) when they do not feed on Cry1Ac+Cry1F cotton. In a theoretical scenario in which only Cry1Ac+Cry1F cotton is cultivated in the Florida Panhandle, the *H. zea* population from Jackson county is expected to produce in one generation 10-27 % more females per female than the populations from Escambia and Santa Rosa. These differences between *H. zea* populations reinforce that resistance may develop because of local selection(Andow 2008).

During over 23 years of commercialized Bt crops in the U.S., IRM programs have relied on models to predict how quickly resistance to Bt may occur in different scenarios (Caprio 1998, Kennedy and Storer 2000). The information provided in this study can contribute to refine predictive models aiming to delay resistance to important Bt toxins, such as Vip3Aa (Reisig and Kurtz 2018). Our results reinforce the need for region-specific knowledge of target pests of Bt technology when designing IRM strategies (Kennedy and Storer 2000). Fitness components and life-table parameters of target pests in addition to their variability across environments should be taken into consideration in the simulation of predictive models (Kennedy and Storer 2000).

In conclusion, this study has quantified the dual and triple-gene Bt cotton effect on the life history and demographic performance of three populations of *H. zea* from the Florida Panhandle. Triple-gene Bt cotton caused 100% larval mortality in all populations tested. Dual-gene Bt cotton affected fitness and demographic growth. In addition, an interaction between *H. zea* populations and cotton cultivars (Bt and non-Bt) was documented, influencing the magnitude of the effect in the life history and population growth. These findings improve our understanding of demographic growth rates of *H. zea* in a landscape containing 100% Bt cotton. The results of this study are also useful to inform models to better predict the risk of resistance evolution and validate resistance management strategies, including refuge recommendations.

5. ACKNOWLEDGMENTS

We thank the extension agent Ethan Carter and all Entomology team at WFREC for assisting with the insect collections. We also acknowledge the farmers from the Florida Panhandle for providing access to their commercial fields and the companies Bayer, BASF, and Corteva for providing the cotton seeds. Funding and resources for this research were supported by the USDA National Institute of Food and Agriculture, Hatch project 005643 and CPPM EIP FLA-ENY-005649.

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7. TABLES AND FIGURE

Table 1. Non-Bt and Bt cotton cultivars expressing *B. thuringiensis* toxins used in the study.

Trade name	Cultivar	Family	Bt event name	Year launched	Bt toxin
Non-Bt	DP 1822XF	Deltapine	-	-	-
Bollgard II	DP 1646B2XF	Deltapine	MON15985	2003	Cry1Ac, Cry2Ab
WideStrike	PHY 444WRF	Phytogen	3006-210-23, 281-24-236	2005	Cry1Ac, Cry1F
TwinLink	ST 5122GLT	Stoneville	T304-40, GHB119	2014	Cry1Ab, Cry2Ae
Bollgard III	DP 1851B3XF	Deltapine	MON15985, COT102	2014	Cry1Ac, Cry2Ab2, Vip3Aa20
WideStrike III	PHY4803FE	Stoneville	3006-210-23, 281-24-236, COT102	2015	Cry1Ac, Cry1F, Vip3Aa20
TwinLink Plus	ST 5471GLTP	Stoneville	T304-40, GHB119, COT102	2017	Cry1Ab, Cry2Ae, Vip3Aa20

Table 2. *Helicoverpa zea* populations from the Florida Panhandle, 2018 crop season.

County	Geospatial coordinate		Number of insects collected	Generation tested
	Latitude	Longitude		
Santa Rosa	30.8695	-85.1454	100	F3
Escambia	30.8041	-85.0805	40	F2
Jackson	30.7757	-87.1432	130	F3

Table 3. Two-way ANOVA for life-history traits of *H. zea* populations feeding on cotton cultivars.

Variable	Source of variation	*F*	*P*
Larval weight	Population	8.867	0.0003
	Cultivar	75.853	<0.0001
	Population × Cultivar	8.471	<0.0001
Larval development time	Population	12.959	<0.0001
	Cultivar	174.10	<0.0001
	Population × Cultivar	4.927	0.0002
Larval survival	Population	7.136	0.0010
	Cultivar	182.51	<0.0001
	Population × Cultivar	4.529	<0.0001
Pre-pupal development time	Population	0.99	0.3717
	Cultivar	5.18	0.0016
	Population × Cultivar	1.715	0.1302
Pupal weight	Population	9.43	0.0001
	Cultivar	15.70	<0.0001
	Population × Cultivar	1.58	0.1647
Pupal development time	Population	0.933	0.3943
	Cultivar	31.435	<0.0001
	Population × Cultivar	4.846	<0.0001
Pupal survival	Population	0.271	0.764
	Cultivar	0.691	0.561
	Population × Cultivar	0.520	0.760
Egg viability	Population	3.712	0.0275
	Cultivar	0.134	0.8751
	Population × Cultivar	0.129	0.9427

P values of 0.05 or lower were considered significant as calculated using two-way ANOVA in R software (version 3.5.1).

Table 4. Larval and pupal weight (mg) of *H. zea* reared on Bt and non-Bt cotton cultivars.

Stage	Population	Cotton cultivar						
		Non-Bt	Cry1Ac+ Cry1F	Cry1Ac+ Cry2Ab	Cry1Ab+ Cry2Ae	Cry1Ac+ Cry1F+ Vip3Aa	Cry1Ac+ Cry2Ab+ Vip3Aa	Cry1Ab+ Cry2Ae+ Vip3Aa
Larva	Escambia	59.6 ± 24.4 Aa	25.5 ± 6.18 Ba	2.02 ± 2.14 Ca	0.29 ± 0.14 Ca	*	*	*
	Santa Rosa	56.8 ± 16.0 Aa	6.79 ± 1.96 Bb	2.31 ± 1.91 Ba	0.34 ± 0.17 Ba	*	*	*
	Jackson	20.1 ± 7.98 Ab	22.6 ± 6.03 Aa	0.26 ± 0.02 Bb	1.64 ± 1.66 Ba	*	*	*
Pupa	Escambia	429.0 ± 69.3 Aa	363.0 ± 49.7 Ca	393.0 ± 49.0 Ba	401.0 ± 00.0 Ba	*	*	*
	Santa Rosa	427.0 ± 67.7 Aa	386.0 ± 67.9 Ba	409.0 ± 40.4 Aa	324.0 ± 00.0 Ba	*	*	*
	Jackson	391.0 ± 70.7 Ab	373.0 ± 53.2 Aa	*	302.0 ± 80.8 Ba	*	*	*

Means (± SE) followed by the same capital letter within lines or the same lowercase latter within columns for each parameter do not significantly differ ($P > 0.05$; Tukey HSD). *not determined due to the high larval mortality.

Table 5. Larva and pupal survival rates (%) of *H. zea* reared on Bt and non-Bt cotton cultivars.

Stage	Population	Cotton cultivar						
		non-Bt	Cry1Ac+ Cry1F	Cry1Ac+ Cry2Ab	Cry1Ab+ Cry2Ae	Cry1Ac+ Cry1F+ Vip3Aa	Cry1Ac+ Cry2Ab+ Vip3Aa	Cry1Ab+ Cry2Ae+ Vip3Aa
Larva	Escambia	93 ± 4.83 Aa	80 ± 0.7 Aa	30.0 ± 28.3 Ba	2.0 ± 0.4 Ca	0.0 ± 0.0 Ca	0.0 ± 0.0 Ca	0.0 ± 0.0 Ca
	Santa Rosa	83 ± 10.6 Aab	40 ± 1.0 Bb	18.0 ± 16.2 Bab	8.0 ± 0.8 Ca	0.0 ± 0.0 Ca	0.0 ± 0.0 Ca	0.0 ± 0.0 Ca
	Jackson	77 ± 16.4 Ab	80 ± 1.5 Aa	4.22± 2.00 Bb	13 ± 16.4 Ba	0.0 ± 0.0 Ca	0.0 ± 0.0 Ca	0.0 ± 0.0 Ca
Pupa	Escambia	96.9 ± 6.5 Aa	93.0 ± 11.4 Aa	95.2 ± 12.6 Aa	100 ± 0.0 Aa	*	*	*
	Santa Rosa	100 ± 0.0 Aa	96.3 ± 11.1 Aa	91.7 ± 20.0 Aa	100 ± 0.0 Aa	*	*	*
	Jackson	100 ± 0.0 Aa	95.9 ± 10.8 Aa	*	100 ± 0.0 Aa	*	*	*

Means (± SE) followed by the same capital letter within lines or the same lowercase latter within columns for each parameter do not significantly differ (P > 0.05; Tukey HSD). *not determined due to the high larval mortality.

Table 6. Development time (days) of *H. zea* reared on Bt and non-Bt cotton cultivars.

Stage	Population	Cotton cultivar						
		non-Bt	Cry1Ac+ Cry1F	Cry1Ac+ Cry2Ab	Cry1Ab+ Cry2Ae	Cry1Ac+ Cry1F+ Vip3Aa	Cry1Ac+ Cry2Ab+ Vip3Aa	Cry1Ab+ Cry2Ae+ Vip3Aa
Larva	Escambia	21.7 ± 1.43 Cb	28.3 ± 3.22 Ba	32.5 ± 4.79 Aa	21.00 ± 0.00 Cb	*	*	*
	Santa Rosa	21.9 ± 2.02 Cb	28.7 ± 2.89 Ba	32.9 ± 3.51 Aa	20.00 ± 3.50 Cb	*	*	*
	Jackson	24.3 ± 3.63 Ca	28.0 ± 3.18 Ba	*	37.2 ± 3.50 Aa	*	*	*
Pre-Pupa	Escambia	3.38 ± 0.71 Aa	3.45 ± 1.03 Aa	3.75 ± 1.08 Aa	3.00 ± 0.00 Aa	*	*	*
	Santa Rosa	3.52 ± 0.89 Ba	3.27 ± 0.84 Ba	4.36 ± 0.80 Aa	3.00 ± 0.00 Ba	*	*	*
	Jackson	3.48 ± 1.02 Ba	3.75 ± 0.91 Ba	*	2.75 ± 0.50 Aa	*	*	*
Pupa	Escambia	19.0 ± 1.05 Aa	17.4 ± 2.00 Ba	19.2 ± 1.18 Aa	21.00 ± 0.00 Aa	*	*	*
	Santa Rosa	18.4 ± 1.44 Ba	17.2 ± 2.78 Ca	20.9 ± 1.14 Aa	20.00 ± 1.81 Aa	*	*	*
	Jackson	19.3 ± 2.04 Aa	17.4 ± 2.00 Ba	*	17.00 ± 0.00 Ab	*	*	*

Means (± SE) followed by the same capital letter within lines or the same lowercase latter within columns for each parameter do not significantly differ (P > 0.05; Tukey HSD). *not determined due to the high larval mortality.

Table 7. Egg viability (%) of *H. zea* reared on Bt and non-Bt cotton cultivars.

Population	Cotton cultivar						
	non-Bt	Cry1Ac+ Cry1F	Cry1Ac+ Cry2Ab	Cry1Ab+ Cry2Ae	Cry1Ac+ Cry1F+ Vip3Aa	Cry1Ac+ Cry2Ab+ Vip3Aa	Cry1Ab+ Cry2Ae+ Vip3Aa
Escambia	65.2 ± 37.6 Aa	59.0 ± 44.3 Aa	69.2 ± 34.2 Aa	*	*	*	*
Santa Rosa	44.3 ± 39.5 Ab	44.5 ± 33.9 Ab	43.8 ± 39.8 Ab	*	*	*	*
Jackson	43.3 ± 32.6 Ab	46.7 ± 36.6 Ab	43.3 ± 32.6 Ab	*	*	*	*

Means (± SE) followed by the same lowercase latter within columns do not significantly differ (P > 0.05; Tukey HSD). * not determined due to the high larval mortality.

Table 8. Life table of *H. zea* populations from different counties in the Florida Panhandle reared on Bt and non-Bt cotton cultivars.

Parameter	Population	Cotton cultivar						
		non-Bt	Cry1Ac+ Cry1F	Cry1Ac+ Cry2Ab	Cry1Ab+ Cry2Ae	Cry1Ac+ Cry1F+ Vip3Aa	Cry1Ac+ Cry2Ab+ Vip3Aa	Cry1Ab+ Cry2Ae+ Vip3Aa
R_0	Escambia	320.62 ± 61.74 Aa	139.32 ± 18.99 Bb	133.54 ± 16.94 Ba	*	*	*	*
	Santa Rosa	406.80 ± 67.24 Aa	87.26 ± 12.71 Bc	80.57 ± 20.34 Ba	*	*	*	*
	Jackson	289.59 ± 53.32 Aa	270.09 ± 35.50 Aa	*	*	*	*	*
r_m	Escambia	0.13 ± 0.006 Aa	0.10 ± 0.003 Bb	0.08 ± 0.003 Ca	*	*	*	*
	Santa Rosa	0.13 ± 0.006 Aa	0.08 ± 0.003 Bc	0.07 ± 0.005 Bb	*	*	*	*
	Jackson	0.11 ± 0.05 Ab	0.11 ± 0.03 Aa	*	*	*	*	*
T	Escambia	41.94 ± 0.67 Ab	48.88 ± 0.65 Bb	55.37 ± 0.65 Cb	*	*	*	*
	Santa Rosa	45.08 ± 0.95 Aa	52.99 ± 0.87 Ba	60.34 ± 1.73 Ca	*	*	*	*
	Jackson	47.62 ± 1.20 Aa	49.57 ± 0.75 Ab	*	*	*	*	*

Means (± SE) followed by the same capital letter within lines or the same lowercase latter within columns for each parameter do not significantly differ ($P > 0.05$) through pairwise comparisons using two-tailed t-tests after the jackknife method to estimate variance. R_0 - Intrinsic rate of population increase (females per female per generation); r_m - Net reproductive rate (females per female per day); T - Generation time (days). * not determined due to the high larval mortality.

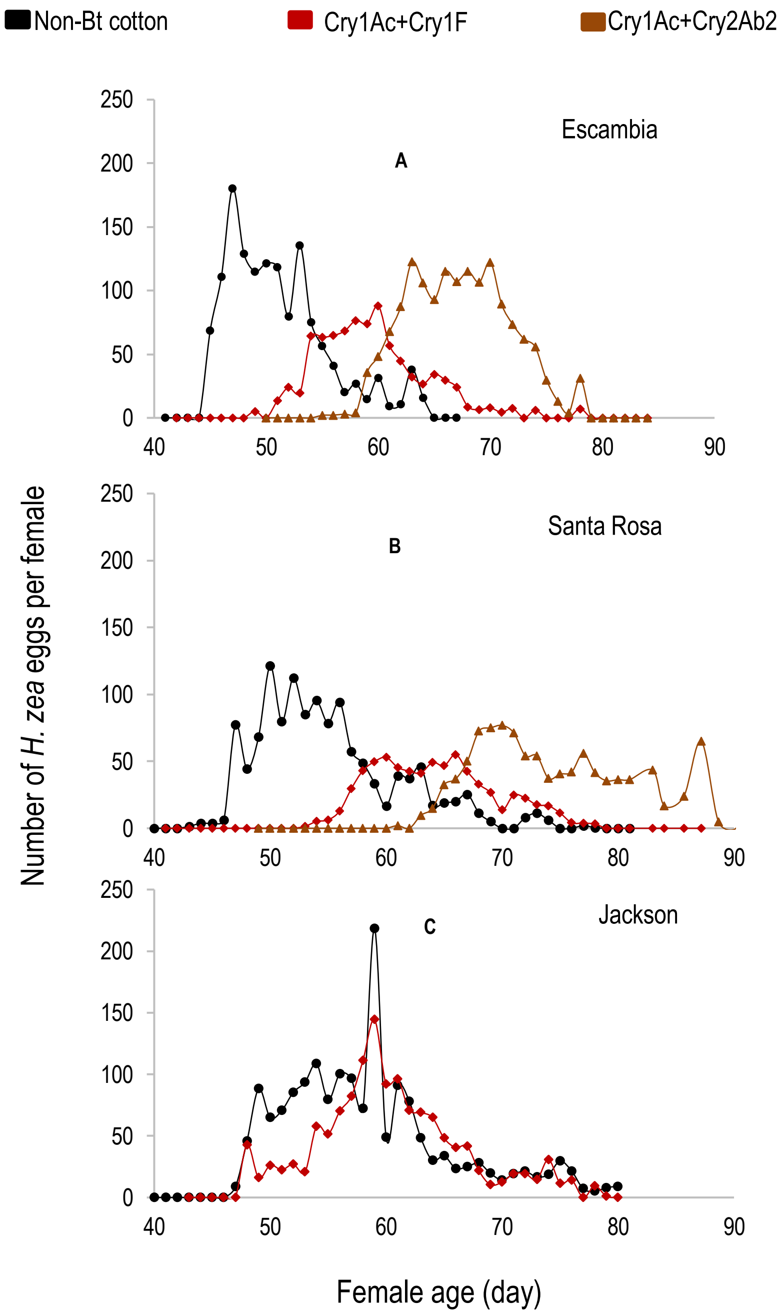

Figure 1. Reproductive schedule of *H. zea* feeding on non-Bt and Bt cotton cultivars as represented by fecundity (number of eggs per day) and female longevity. Each line represents an average of 16 *H. zea* females mated in pairs in mating cages. Panels A, B, and C represent the populations from Escambia, Santa Rosa, and Jackson county, respectively. The black line refers to insects feeding on non-Bt cotton, while the red and green are for insects feeding on Bt cotton Cry1Ac+Cry1F and Cry1Ac+Cry2Ab2, respectively.

CHAPTER III – ABSTRACT

RABELO, Marcelo Mendes,D.Sc., Universidade Federal de Viçosa, April, 2020. **Contrasting susceptibility of lepidopteran pests to diamide and pyrethroid insecticides in a region of overwintering and migratory intersection** Adviser: Eliseu Jose Guedes Pereira. Co-adviser: Silvana Vieira de Paula Moraes.

Pesticide resistance is a growing issue worldwide, and susceptibility of pest populations should be monitored in migratory intersection regions for successful resistance management. We determined the susceptibility of eight noctuid species from the Florida Panhandle to bifenthrin (pyrethroid) and chlorantraniliprole (diamide). Larvae from field and laboratory populations were exposed to commercial insecticide formulations using the leaf-dip method in concentration-mortality bioassays. The field populations of *Helicoverpa zea* (Boddie), *Spodoptera frugiperda* (Smith), *S. eridania* (Stoll), *S. exigua* (Hubner), and *Chloridea virescens* (Fabricius) had reduced susceptibility to bifenthrin compared with the laboratory populations. Resistance ratios to bifenthrin were as high as 10,071-fold in *S. exigua* and 436-fold in *S. frugiperda,* while there was no reduced susceptibility in *Agrotis ipsilon* (Hufnagel). The susceptibility to chlorantraniliprole was similar between the field and laboratory populations studied, except for *S. exigua* that exhibited 630-fold resistance to the diamide. The probit regression equations indicated that the larval mortality of *S. exigua* and *S. frugiperda* populations was less than 80% with bifenthrin at the concentration equivalent to the label rate. Likewise, the estimated mortality of *S. exigua* larvae with chlorantraniliprole at the label rate concentration was less than 80%. The lepidopteran pest populations tested were variable in susceptibility to bifenthrin in contrast to more consistent susceptibility to chlorantraniliprole. These results help in the choice of effective insecticides for integrated pest management and resistance management in cropping systems colonized by migratory lepidopteran pests from the United States Gulf Coast region.

Keywords: Diamide. Pyrethroid. Resistance. Bioassay. Armyworm. Risk of Control Failure.

1. INTRODUCTION

Lepidoptera has about 120 thousand species described, and virtually all of them are phytophagous (Scoble 1992). As a result, there are many lepidopteran pests in agroecosystems worldwide, mostly in the family Noctuidae (Bradshaw et al. 2016, Suckling et al. 2017). The United States (U.S.) Gulf Coast can function as a region of overwintering and migratory intersection for a number of polyphagous noctuids, including armyworms, cutworm and heliothines (Showers 1997, Nagoshi et al. 2009, 2012, Westbrook and López 2010). Often times they cause economic losses in row and vegetable crops (King 1981, Pogue 2002, Blanco 2012, Luttrell and Jackson 2012). These noctuids include the cotton bollworm *Helicoverpa zea* (Boddie) (Luttrell and Jackson 2012), tobacco budworm *Chloridea virescens* (Fabricius) (Blanco 2012), fall armyworm *Spodoptera frugiperda* (Smith), southern armyworm *Spodoptera eridania* (Stoll), beet armyworm *Spodoptera exigua* (Hubner), yellowstriped armyworm *Spodoptera ornithogalli* (Guenee), velvet armyworm *Spodoptera latifascia* (Walker) (Pogue 2002), and black cutworm *Agrotis ipsilon* (Hufnagel) (Rings et al. 1975).

The Gulf Coastal Plain of the southeastern U.S., known as the Florida Panhandle, has a unique landscape consisting of a mosaic of natural vegetation, forests, and row crops. These include corn (*Zea mays* L.), cotton (*Gossypium hirsutum* L.), peanut (*Arachis hypogaea* L.), soybean (*Glycine max* L.), and wheat (*Triticum aestivum* L.), which had an overall production value of $354 million in 2017 (FDACS 2018, USDA/NASS 2019). Nevertheless, growers face a challenge with noctuid pests in their crops. Corn yield losses from fall armyworm and black cutworm can reach up to 45% and 20%, respectively (Hruska and Gladstone 1988, Oloumi-Sadeghi et al. 1992a). Two other major North American *Spodoptera* pests are the beet armyworm and southern armyworm (Pogue 2002). Prior to the introduction of *Bacillus thuringiensis* (Bt) cotton defoliation by the beet armyworm caused severe production losses, which exceeded $19 million annually (Mascarenhas et al. 1996, Luttrell et al. 2015). The heliothines cotton bollworm and tobacco budworm also caused heavy losses ($1 billion in cotton alone) before the introduction of Bt cotton (Stadelbacher et al. 1986, Blanco 2012, Luttrell and Jackson 2012). Moreover, cotton bollworm (also referred to as the corn earworm) feed on other cultivated crops such as peanut and corn (Abbott et al. 2019, Yang et al. 2019).

Historically, lepidopteran infestations have been managed by using synthetic insecticides, including carbamates, organophosphates, pyrethroids, and diamides (Lai and Su

2011, Nauen and Steinbach 2016). Since 1996, Bt crop cultivars became available to management lepidopteran pests. However, this technology is available only in cotton and corn in the U.S. In addition, some lepidopteran pests may not be susceptible to the Bt toxins because of tolerance and/or resistance, and the use of insecticides remains a component required in many pest management programs (Dively et al. 2016, Reisig and Kurtz 2018, Rabelo et al. 2020a, Rabelo et al. 2020b). Pyrethroids have been used for control of lepidopteran pests for more than 25 years in the U.S., (Soderlund and Bloomquist 1989) and there are cases of pyrethroid resistance in populations of heliothines, plusines and *Spodoptera* spp.(Portillo et al. 1993, Carvalho et al. 2013, Reisig et al. 2019). Diamide insecticides were introduced in the 2000's and are generally potent against multiple lepidopteran pests (Teixeira and Andaloro 2013). Diamides are modulators of ryanodine receptors, a unique mode of action, having low toxicity to mammals, fish, birds, and many beneficial insects (Teixeira and Andaloro 2013). These properties make them an alternative for pest management, particularly in regions where resistance to other insecticides has evolved (Sparks and Nauen 2015). Reliance on diamides may increase the selection pressure on target pests and lead to high levels of field resistance as reported for beet armyworm, diamondback moth *Plutella xylostella* (Linnaeus), and the tomato leafminer *Tuta absoluta* (Meyrick) in populations from China, Thailand, and Italy (Troczka et al. 2012, Che et al. 2013, Roditakis et al. 2015, Nauen and Steinbach 2016).

Insecticide resistance management (IRM) programs need to be regional due to the unique features of cropping systems (WHO 2010). Many species of lepidopteran have long- and short-range migrations and can introduce the insecticide resistance alleles in regions where they were at low frequency. For example, many of the annual lepidopteran pest infestations in North America are migratory populations from subtropical and tropical regions such as Florida and Texas where the winter temperatures are not extreme (Nagoshi et al. 2009, 2012, Westbrook and López 2010). The same concern holds for invasive lepidopteran pest (Goergen et al. 2016, Georgen 2018, Rabelo et al. 2020a).

The insecticide susceptibility of noctuid pest populations collected in the Florida Panhandle has not been documented. This assessment is critical for IPM programs and has wide implications for local and wide-range IRM. Such susceptibility data can help predict the efficacy of insecticides and the risk of pest resistance evolution (Robertson et al. 2017). Because of the historical use of pyrethroids and the recent increase in the use of diamides, we documented the susceptibility to bifenthrin (pyrethroid) and chlorantraniliprole (diamide) in noctuid populations of eight species from a region of overwintering and migratory intersection.

2. MATERIALS AND METHODS

2.1. Insect collection and colonies

Larvae and moths were collected during the 2018 crop season in commercial and experimental fields located in the three counties in the Florida Panhandle with the highest cotton and peanut production: Santa Rosa, Escambia, and Jackson (Table 1). Fields of cotton, corn, peanut (each 60 to 80 ha), and tomato (0.2 ha) were scouted, sampling at least 100 plants/field. Peanut fields were sampled using a 1-m long, white beat cloth while the others were sampled by visual plant inspection.

The larvae collected were placed in 2.5-ml plastic tubes containing a multispecies lepidopteran diet (Southland Products, Lake Village, AR). In the same fields used larvae were collected, moths were captured in UV light traps and transferred to 2.5 ml-plastic containers. The larvae and moths captured were held in styrofoam boxes with an ice pack and transported to the entomology laboratory at the West Florida Research and Education Center, Jay, FL. The insects were maintained at 25 ± 2 ºC, 70 ± 10% relative humidity, and 14L:10D photoperiod. The pupae were transferred to Petri dishes containing moistened vermiculite. The pupae and moths were placed in mating cages (23 cm diameter, 30 cm height) internally lined with paper toweling as an oviposition substrate. The moths were fed with a solution of 10% honey, changed every two days. The eggs were collected and transferred to zip lock bags until hatching. A large group of neonates (over a 100) were transferred to 250-ml cups containing the multispecies lepidopteran diet and reared until the appropriate instar for the bioassays. One colony for each field-derived population was established in the laboratory. The species, location, host, collection method, number of insects collected, and the number of generations in the laboratory until the bioassays are listed in Table 1. The field-derived insects were identified based on morphological characteristics of the larvae and adults and confirmed by sending specimens to the Florida Department of Agriculture and Consumer Services. Eight species were collected (Table 1). In addition, Benzon Research Inc. (Carlisle, PA) provided insect colonies of the species studied, except yellowstriped and velvet armyworms, which were unavailable at the time. These colonies were used as susceptible populations in the bioassays. The insects were maintained using the same procedures previously described.

2.2. Insecticide susceptibility

Concentration-mortality bioassays were conducted using late second/early third instar larvae. Dilutions of the commercial insecticides were prepared in distilled water without any adjuvants. Bifenthrin (Brigade 2EC; FMC Corporation, Philadelphia, PA) and chlorantraniliprole (Prevathon; FMC Corporation, Newark, DE) were tested at seven concentrations, including a control (water only). The concentrations varied according to the population susceptibility to the insecticide. In brief, the dilutions were prepared by taking the label rate to control larvae in cotton (see below) as a starting point and then making serial dilutions in a logarithmic (multiplicative inverse) scale. The bioassays were conducted using 4-cm leaf disks of a non-Bt cotton cultivar, DP1822XF (Monsanto, St. Louis, MO). The leaf-dip technique recommended by the Insecticide Resistance Action Committee (Method 007) was used. The cotton leaves were removed from the middle and upper part of the plants during the growth stages of canopy development and first bloom. Leaf disks (4 cm diameter) were cut and dipped individually into the insecticide dilution for five seconds with gentle agitation. The disks were placed on paper toweling, allowed to dry for approximately five minutes, and carefully transferred to Petri dishes (10 cm diameter × 15 cm height) using tweezers, not interfering with the surface treated. A single larva was transferred to the leaf disk in the case of black cutworm, cotton bollworm, fall armyworm to avoid cannibalism; otherwise, four or five larvae (depending on availability) were transferred to the leaf disk. The dishes were covered and placed in a growth chamber at 25 ± 2 °C, 70 ± 10% relative humidity, and 14L:10D photoperiod. The bioassays were replicated at least five times using 30-40 larvae per concentration. Larval mortality was recorded after exposure of 48 h to bifenthrin and 72 h to chlorantraniliprole. The larvae were considered dead if they did not move when prodded with a fine camelhair paintbrush.

2.3. Data analysis

Larval mortality data were analyzed using probit regression[42] in Polo Plus 1.0 version,[43] adjusting for natural mortality when needed. The susceptibility parameters estimated were the median lethal concentration (LC_{50}) and their respective 95% confidence limits (95% CL) as well as the slope and standard error for the response curves. Resistance ratios and their respective 95% confidence intervals were determined using the laboratory populations as references for all comparisons (Robertson et al. 2017). Relative tolerance ratios and their respective 95% confidence intervals were determined using the most susceptible species as reference for all comparisons within each insecticide. A single response curve was generated for the field populations of cotton bollworm by pooling the mortality data and running the probit analysis as described above.

In addition, the concentration-mortality regression equation of the insecticide for each population was used to predict larval mortality at the concentration corresponding to the recommended field rate in cotton. This was done to assess the risk of control failure in a hypothetical best-case scenario. We used the relationship between the insecticide concentration and mortality, given by the concentration-mortality (probit) regression equation fitted to the bioassay data. To translate the field rate (l oz./acre) into the unit of concentration of the leaf-dip bioassay (ug/mL or mg/L), we used the highest value of the recommended field rate (i.e., the greatest dosage indicated by the manufacturer on the product label). We assumed a carrier volume of 10 gallons per acre (93.5 L/ha) as typically applied using state-of-art technology (such as a modern ground rig used to apply pesticides in field crops). More specifically, the highest label concentration recommended for controlling lepidopteran pests in cotton was used in the probit equation. The following values were used: bifenthrin: 1.198 g/L, equivalent to the field rate of 0.112 kg/ha (0.10 lb/acre); chlorantraniliprole: 1.078 g/L, equivalent to 0.101 kg/ha (0.09 lb/acre); spray volume: 93.5 L/ha (10 gal/acre).

3. RESULTS

3.1. Susceptibility to bifenthrin

The probit model fit the bioassay data properly, as indicated by *P*-values higher than 0.05 in the chi-squared tests for lack of fit (Table 2, 3), thus generating reliable estimates of the susceptibility parameters. For cotton bollworm, significant differences in LC_{50} values ($P < 0.05$) were observed when field and laboratory populations of cotton bollworm were exposed to bifenthrin (Table 2). The LC_{50} value of bifenthrin did not vary among field populations of cotton bollworm from Santa Rosa, Escambia, or Jackson counties (Table 2). However, the resistance ratio indicated these three populations were 8- to 14-fold less susceptible than the laboratory population, but all LC_{50} values were lower than the concentration equivalent to the field rate (1,198 mg/L) (Table 2). When combining the populations from the three counties, the overall resistance ratio for bifenthrin compared to the susceptible reference population was approximately 10 (Figure 1).

Among the other seven noctuid species, the larvae of field populations of cotton bollworm, fall armyworm, southern armyworm, and beet armyworm were less susceptible than those of the respective laboratory population (Table 3). Beet armyworm and fall armyworm exhibited the highest resistance ratio between field and laboratory populations, 3,310, and at least 2,228, respectively (Table 3). The field-collected larvae of these two species had LC_{50} values lower than the concentration equivalent to the recommended field rate (1,198 mg/L). There was no difference in susceptibility to bifenthrin between laboratory and field black cutworm populations. The LC_{50} value for yellowstriped armyworm and velvet armyworm were 1.49 mg/L and 11.37 mg/L, respectively (Table 3).

3.2. Susceptibility to chlorantraniliprole

Again, the probit model fit properly to the bioassay data ($P > 0.05$), thus allowing valid estimates of the susceptibility parameters (Table 4). No significant differences in the LC_{50} values ($P > 0.05$) were observed when field and laboratory populations were exposed to chlorantraniliprole, except for beet armyworm (Table 4). The LC_{50} values for cotton bollworm from Santa Rosa and Jackson counties were 1.20 and 1.31 mg/L, respectively, and 0.56 mg/L for the laboratory population (Table 2). The resistance ratio values ranged from 0.8 to 5.6 (95 % CL), with a mean of 2.23 (Figure 1). Fall armyworm and southern armyworm showed resistance ratios (95 % CL) of 0.11 (0.03 – 0.34) and 1.72 (0.77 – 3.84), respectively (Table 4). Beet armyworm was the only species among the armyworms where the field population had significantly lower susceptibility to chlorantraniliprole compared to the laboratory population. There was no mortality in beet armyworm larvae from the field population, even with the highest concentration tested (139.92 mg/L) (Table 4). The yellowstriped armyworm and velvet armyworm presented LC_{50} values of 2.94 and 0.19 mg/L, respectively (Table 4). None of the populations tested had LC_{50} values higher than the concentration equivalent to the recommended field rate (1,078 mg/L).

3.3. Estimated mortality at the field-rate concentration of the insecticide

The estimated mortality is based on the relationship between the insecticide concentration and the probit regression equation from the bioassay. Estimates of larval mortality for the field populations of beet armyworm and fall armyworm, at the bifenthrin concentration equivalent to the field rate were lower than 80% in a best-case scenario (Figure 2). In contrast, the expected larval mortality was higher than 80% for all the lepidopteran pest species at the field rate of chlorantraniliprole, except for a field population of beet armyworm (Figure 2).

3.4. Tolerance of the noctuid species

The tolerance to bifenthrin and chlorantraniliprole varied among the field-collected larvae of the eight species studied (Figure 3). The black cutworm and tobacco budworm larvae were the least tolerant and used as references to compare the tolerance ratio for bifenthrin (Figure 3a) and chlorantraniliprole (Figure 3b), respectively. The beet armyworm larvae exhibited the highest tolerance to both insecticides, with tolerance ratio values over 250 times higher than the least tolerant species. Whereas fall armyworm and cotton bollworm larvae were among the three most tolerant larvae to bifenthrin, they were two of the least tolerant of chlorantraniliprole.

4. DISCUSSION

The results of this study indicate a significant reduction in the susceptibility of field populations of cotton bollworm, tobacco budworm, fall armyworm, southern armyworm, and beet armyworm to bifenthrin in the Florida Panhandle. The selection for resistance by the extensive adoption of pyrethroids for pest management in the last 25 years in several crops is likely a contributing factor to this outcome. The three populations of cotton bollworm from different locations in the Florida Panhandle had similar susceptibility to bifenthrin but were in on average 10-fold less susceptible than a laboratory population (Figure 1). These low resistance ratio values for bifenthrin are similar to those for other U.S. cotton bollworm populations tested recently (Jacobson et al. 2009, Margus et al. 2019, Reisig et al. 2019) or over 20 years ago, (Stadelbacher et al. 1990, Abd-Elghafar et al. 1993, Kanga et al. 1996, Brown et al. 1998, McCaffery 1998) likely caused by increased detoxification. This similarity among populations may be associated with the high dispersal capacity and reproductive biology of cotton bollworm, leading to extensive gene flow (Perera and Blanco 2011, Seymour et al. 2016). Host plants and their secondary metabolites could also influence insect susceptibility to bifenthrin. In addition to gossypol, cotton plants have several other related sesquiterpene aldehydes, which can induce P450 enzyme activity and increase tolerance to pyrethroid (Tao et al. 2012).

The field population of tobacco budworm also had reduced susceptibility to bifenthrin. Cotton is the primary host plant of this species, and there are reports of its suppression due to high-dose events of Bt cotton (Ali et al. 2006, Blanco 2012, Fleming et al. 2018). This may have reduced the infestations in other non-Bt host crops, including peanut and soybean. Still, multiple insecticide sprays against other incidental pests on these crops, such as the velvetbean caterpillar *Anticarsia gemmatalis* (Hubner), may exert selection pressure on eventual infestations by tobacco budworm.

Five armyworm species collected in the Florida Panhandle had their insecticide susceptibility documented. Field populations of the beet armyworm, fall armyworm, and southern armyworm had lower susceptibility to bifenthrin compared with laboratory populations. Fall armyworm populations have developed high levels of resistance to diverse insecticide classes, including organophosphates, carbamates, benzoylureas, and pyrethroids (Carvalho et al. 2013). A high infestation of fall armyworms often occurs in all American continents, causing economic losses in a wide range of host crops, especially corn (Santos-Amaya et al. 2015, Burtet et al. 2017). The moths are known to migrate from Florida and Mexico to the central and eastern of the U.S., extending as far north as Canada (Nagoshi et al. 2012). The high level of pyrethroid resistance documented in the present study may occur in other regions across the U.S. and should be better documented. In addition, fall armyworm and southern armyworm were recently reported in Africa and Asia (Goergen et al. 2016, Georg Goergen 2018, Sharanabasappa et al. 2018). and the low efficacy of some insecticides has already been reported in some populations of these invasive species (Kumela et al. 2018).

Overall, the field populations of lepidopteran pests from the Florida Panhandle were similarly susceptible to chlorantraniliprole compared to laboratory populations. No change in the susceptibility to the diamide was observed in cotton bollworm populations. This result agrees with those obtained for populations from seven states across the Midsouthern and Southeastern U. S. (Adams et al. 2016). Our study extended coverage to some populations in Florida, which were not included in that baseline assessment. In a scenario of increased cotton bollworm population levels in the Florida Panhandle associated with reduced efficacy of some Bt technologies (Luttrell and Jackson 2012), chlorantraniliprole can be one valuable tool in a resistance mitigation program.

Among the armyworm species tested, only beet armyworm presented a high resistance level to both insecticides compared to the laboratory population. This is the first report of low susceptibility of beet armyworm to chlorantraniliprole (630-fold) in the U. S. Some populations of this pest rapidly evolve insecticide resistance, as reported in China (Lai et al. 2011, Che et al. 2013). Beet armyworm has around 90 hosts in North America, including crop plants such as cotton, cabbage, peanut, and sunflower (Greenberg et al. 2001). The low susceptibility to bifenthrin and chlorantraniliprole poses a challenge to manage this pest because of the limited number of effective insecticides available.

On the other hand, black cutworm had no significant difference in the susceptibility between field and laboratory populations to both insecticides. Black cutworm can be difficult to control with insecticides because of the subterranean larval habit (Oloumi-Sadeghi et al. 1992b). The limited larval exposure to insecticidal sprays would pose weak selection pressure. While black cutworm and yellowstriped armyworm were the most susceptible species to bifenthrin (i.e., having the lowest relative tolerance, Figure 3), yellowstriped armyworm and velvet armyworm were among the most tolerant species to chlorantraniliprole. With the use of diamides and pyrethroids, to which yellowstriped and velvet armyworms are relatively tolerant, their prevalence in the agroecosystem may increase as has occurred with some armyworms in other scenarios (Rabelo et al. 2020a, Rabelo et al. 2020b).

The contrasting variation between the susceptibility to bifenthrin and chlorantraniliprole is likely associated with the more recent adoption of the latter insecticide to manage lepidopteran pests. Diamides have become an important tool for IPM programs in regions where pyrethroids and other insecticides are no longer effective (Yu 1991, Portillo et al. 1993, McCaffery 1998, Reisig et al. 2019). The mode of action of chlorantraniliprole involves activation of ryanodine receptors resulting in abnormal calcium release in muscle cells, and therefore unlikely to have target-site cross-resistance with neurotoxic insecticides (Lahm et al. 2007). Despite the development of resistance in some pest populations, pyrethroids are still used to manage lepidopteran pests due to their relatively low price, high control efficacy when initially introduced, and grower convenience and tradition (Wirtz et al. 2009, Dewar 2016).

The bioassays using the leaf-dip method allowed assessing the risk of control failure in a hypothetical best-case scenario of insecticide coverage and larval exposure. In this situation, the estimates of expected mortality obtained were intentionally overestimated and should not be taken as actual rates of larval mortality under field conditions. There, issues of weathering, application speed, coverage, etc., all affect insecticide efficacy, and studies show that proper coverage may not be achieved even using state-of-the-art application technology (Montezano et al. 2019, Souza et al. 2019). Our results indicate that the risk of control failure is higher for bifenthrin than chlorantraniliprole. It is unlikely that bifenthrin at the recommended field rate will provide proper control efficacy (> 80%) of beet armyworm and fall armyworm in the Florida Panhandle (Figure 2). This inference is possible because the leaf-dip bioassay used represents the best-case scenario of exposure of a noctuid larva to the insecticide, and the field rate indicated in the product label can be converted to that in the bioassay. In addition, the current populations of lepidopteran species had variable tolerance to both insecticides, and, overall, *Spodoptera* species (i.e., beet armyworm, fall armyworm) were relatively more tolerant to the insecticides than were the other species (Figure 3). The relative toxicity data provide useful information for multiple-species management decisions in several crops, including peanut in the southern U.S.

The rotation of insecticides with different modes of action is one of the strategies recommended to delay resistance in lepidopteran pest populations (Sparks and Nauen 2015). Though managing pest susceptibility is central, other factors can reduce the efficacy of chemical control, including inadequate spray coverage, insufficient field rates, poor application timing, or spraying in late insect developmental stages (Gontijo et al. 2012, Montezano et al. 2019, Reisig et al. 2019). These operational factors should also be considered when designing a locally adapted IRM program.

Our efforts generated region-specific information on the susceptibility of populations of eight lepidopteran pest species, which helps to assess the resistance risk to pyrethroid and diamide insecticides. This assessment is of broad interest because insecticide resistance can spread to other regions by migrant individuals from species such as cotton bollworm and fall armyworm.

5. ACKNOWLEDGMENTS

We thank the extension agent Ethan Carter and all Entomology team at WFREC for the help in the insect collections. We also acknowledge the farmers from the Florida Panhandle for providing access to their commercial fields and FMC Corporation for the insecticide samples used in the bioassays. This study was supported by the National Institute of Food and Agriculture, USDA, NIFA CPPM EIP project # 005649, NIFA hatch project # 005643, and Florida Peanut Checkoff funds.

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7. TABLES AND FIGURES

Table 1. Lepidopteran pest species from the Florida Panhandle, 2018 crop season.

Insect species		County	Geospatial coordinate		Host or collection method	Number of insects collected	Generation tested
Latin name	Common name		Latitude	Longitude			
H. zea	Cotton bollworm	Santa Rosa	30.8695	-85.1454	Corn	100	F_1
	(corn earworm)	Escambia	30.8041	-85.0805	Cotton	39	F_1
		Jackson	30.7757	-87.1432	Corn	130	F_3
C. virescens	Tobacco budworm	Jackson/ Santa Rosa[⁋]	30.9842	-87.4696	Light trap	20	F_3
S. frugiperda	Fall armyworm	Santa Rosa	30.8695	-85.1454	Corn	70	F_1
S. eridania	Southern armyworm	Jackson	30.9842	-87.4696	Peanut	83	F_1
S. exigua	Beet armyworm	Jackson	30.9842	-87.4696	Peanut	25	F_2
S. ornithogalli	Yellowstriped armyworm	Santa Rosa	30.8695	-85.1454	Light trap	5	F_2
S. latifascia	Velvet armyworm	Santa Rosa	30.8695	-85.1454	Tomato	120	F_1
A. ipsilon	Black cutworm	Santa Rosa	30.8695	-85.1454	Light trap	8	F_3

[⁋] The tobacco budworm population was derived from moths collected in both Jackson (17) and Santa Rosa (3) counties.

Table 2. Susceptibility of populations of cotton bollworm (*Helicoverpa zea*) (late second/early third-instar larvae) to bifenthrin and chlorantraniliprole.

Insecticide	Population	N[a]	LC_{50}[b] (95% CL) ------ mg/L------	Slope ± SE	χ^2	*P* [c]	RR [d] (95% CL)
Bifenthrin	Laboratory	250	3.50 (2.21 – 5.36)	1.41 ± 0.19	1.95	0.58	1
	Santa Rosa	250	48.99 (31.29 – 77.41)	1.47 ± 0.17	2.14	0.54	14.00 (7.48 – 26.18)
	Jackson	200	30.41 (11.01 – 77.39)	1.60 ± 0.22	4.42	0.21	8.68 (4.71 –16.02)
	Escambia	240	27.00 (8.87 – 76.27)	1.42 ± 0.18	4.82	0.18	7.70 (4.10 – 14.49)
Chlorantraniliprole	Laboratory	177	0.56 (0.14 – 1.14)	1.73 ± 0.49	0.36	0.94	1
	Santa Rosa	240	1.31 (0.74 – 2.32)	0.94 ± 0.10	2.27	0.51	2.33 (0.84 – 6.40)
	Jackson	278	1.20 (0.67 – 2.14)	0.91 ± 0.09	3.81	0.43	2.14 (0.77 – 5.91)

[a] N, Number of individuals tested.

[b] LC_{50}, Lethal concentration to cause mortality in 50% of individuals.

[c] *P*-value associated with the chi-squared, goodness-of-fit test.

[d] RR, Resistance ratio and 95% confidence limits (95% CL). RR values are considered significant (concerning the respective laboratory population) if the 95% CL does not include 1.

Table 3. Susceptibility of populations of seven species of noctuids (late second/early third-instar larvae) to bifenthrin.

Insect species	Population	N[a]	LC_{50}[b] (95% CL) ------ mg/L------	Slope ± SE	χ^2	P[c]	RR[d] (95% CL)
C. virescens	Laboratory	200	2.88 (1.76 – 4.61)	1.34 ± 0.18	1.40	0.70	1
	Jackson/ Santa Rosa	240	24.05 (8.33 – 72.25)	0.95 ± 0.10	3.52	0.31	8.35 (3.96 – 17.60)
S. frugiperda	Laboratory	240	5.10 (2.43 – 10.87)	1.05 ± 0.14	0.84	0.83	1
	Santa Rosa	240	> 2,227.99	nc[e]	nc	nc	> 436.00
S. eridania	Laboratory	300	0.55 (0.35 – 0.70)	2.10 ± 0.44	0.84	0.83	1
	Jackson	300	9.54 (6.03 – 14.90)	1.25 ± 0.13	3.03	0.38	17.29 (10.02 – 29.84)
S. exigua	Laboratory	300	0.32 (0.16 – 0.53)	1.76 ± 0.35	0.02	0.99	1
	Jackson	300	3,310 (2,085 – 15,520)	1.76 ± 0.63	2.04	0.56	10,07 (4,426 – 22,916)
S. ornithogalli	Escambia	600	1.49 (0.40 – 5.75)	1.55 ± 0.18	8.41	0.20	nc
S. latifascia	Santa Rosa	300	11.37 (8.06 – 16.03)	2.06 ± 0.28	0.24	0.99	nc
A. ipsilon	Laboratory	200	0.27 (0.79 – 0.92)	1.22 ± 0.15	5.09	0.53	1
	Santa Rosa	200	0.42 (0.15 – 1.06)	1.06 ± 0.13	3.13	0.79	1.53 (0.73 – 3.20)

[a] N, Number of individuals tested.

[b] LC_{50}, Lethal concentration to cause mortality in 50% of individuals in 48 h exposure.

[c] *P*-value associated with the chi-squared, goodness-of-fit test.

[d] RR, Resistance ratio and 95% confidence limits (95% CL). RR values are considered significant (concerning the respective laboratory population) if the 95% CL does not include 1.

[e] nc, Not calculated due to lack of mortality even at the highest concentration tested.

Table 4. Susceptibility of populations of seven species of noctuids (late second/early third-instar larvae) to chlorantraniliprole.

Insect species	Location	N[a]	LC_{50}[b] (95% CL) ------ mg/L------	Slope ± SE	χ^2	P[c]	RR[d] (95% CL)
C. virescens	Laboratory	240	0.66 (0.09 – 2.94)	0.55 ± 0.75	5.39	0.24	1
	Jackson/ Santa Rosa	200	0.11 (0.03 – 0.62	0.43 ± 0.09	1.82	0.61	0.18 (0.01 – 2.14)
S. frugiperda	Laboratory	240	6.35 (2.53 – 11.41)	1.55 ± 0.34	1.49	0.47	1
	Santa Rosa	196	0.71 (0.27 – 1.65)	0.65 ± 0.90	1.46	0.69	0.11 (0.03 – 0.34)
S. eridania	Laboratory	300	6.28 (1.98 – 31.38)	0.81 ± 0.09	3.78	0.28	1
	Jackson	300	10.80 (3.60 – 34.69)	1.44 ± 0.16	6.37	0.09	1.72 (0.77 – 3.84)
S. exigua	Laboratory	300	2.12 (1.31 – 3.46)	1.19 ± 0.13	2.29	0.51	1
	Jackson	144	>139.92	nc[e]	nc	nc	630.00
S. ornithogalli	Escambia	293	2.94 (0.82 – 11.13)	1.55 ± 0.18	8.18	0.08	nc
S. latifascia	Santa Rosa	300	0.19 (0.014 – 1.07)	0.72 ± 0.92	5.62	0.08	nc
A. ipsilon	Laboratory	252	0.57 (0.23 – 1.56)	1.27 ± 0.15	3.90	0.27	1
	Santa Rosa	233	0.80 (0.26 – 2.12)	1.03 ± 0.13	3.35	0.34	1.38 (0.66 – 2.86)

[a] N, Number of individuals tested.

[b] LC_{50}, Lethal concentration to cause mortality in 50% of the individuals in 72 h exposure.

[c] *P*-value associated with the chi-squared, goodness-of-fit test.

[d] RR, Resistance ratio and 95% confidence limits (95% CL). RR values are considered significant (relative to the respective laboratory population) if the 95% CL does not include 1.

[e] nc, Not calculated due to lack of mortality even at the highest concentration teste

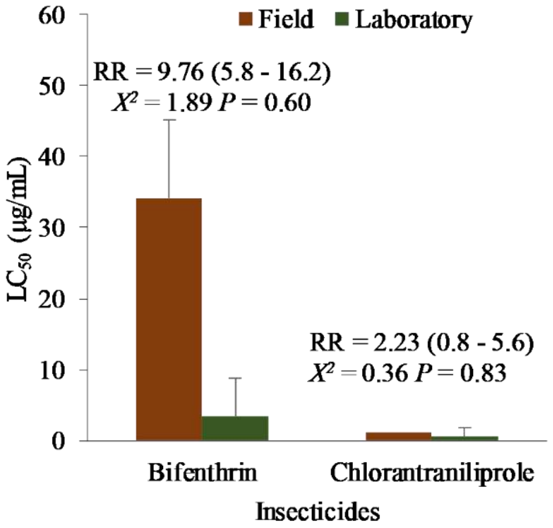


Figure 1. Overall median lethal concentration (LC_{50}) for and resistance ratio (RR) of populations of cotton bollworm (*Helicoverpa zea)* to bifenthrin and chlorantraniliprole. The populations were collected in 2018 in the Florida Panhandle, FL, U.S. The error bars and numbers in parenthesis represent the 95% confidence limits. RR = LC_{50} for field insects/LC_{50} for lab insects. RR values are considered significant (relative to the respective laboratory population) if the 95% CL does not include 1.

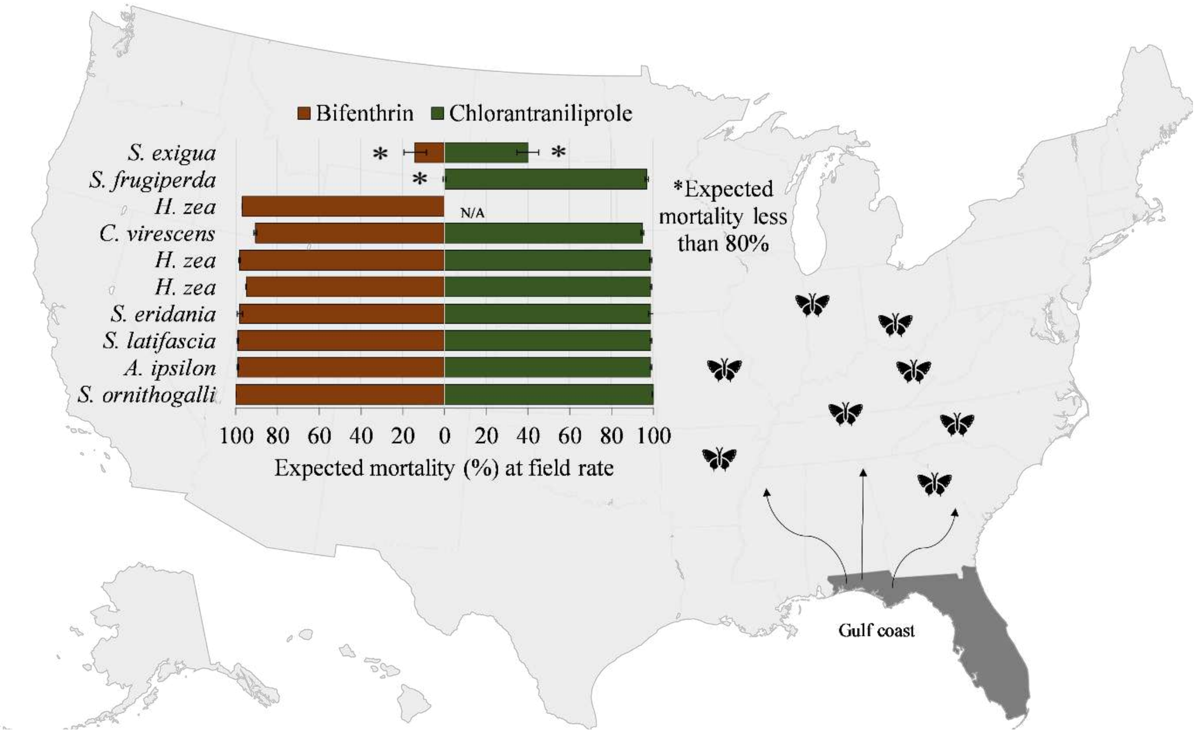
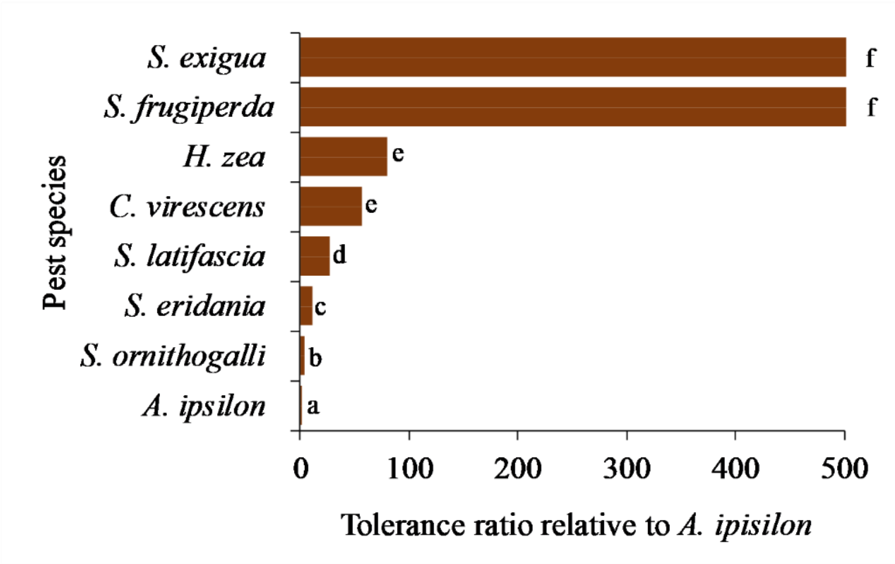


Figure 2. Expected mortality by bifenthrin and chlorantraniliprole at the concentration corresponding to the field rate (in a best-case scenario of exposure). The mortality was estimated by using the concentration-mortality regression equations obtained from the bioassay data for each lepidopteran species and population (Table 2, 3, 4). The highest label concentration recommended to control lepidopteran pests in cotton was plugged in the probit equation to obtain predicted mortality value using appropriate calculations (see Materials and Methods). The map shows the Florida Panhandle area, where the populations were collected, and the potential moth migration paths. Data are means and standard errors. Asterisk (*) indicates that in a best-case scenario, the field rate of the insecticide is likely to cause mortality lower than 80%, the minimum usually expected for suitable pest control. N/A means not available.

A) Bifenthrin



B) Chlorantraniliprole

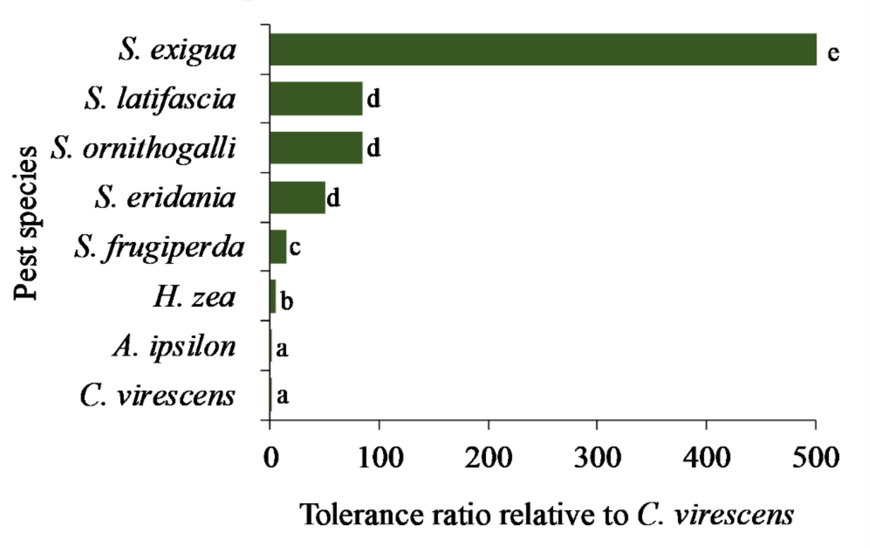


Figure 3. Relative tolerance of eight lepidopteran species to pyrethroid and diamide insecticides. Black cutworm (*A. ipsilon*) and tobacco budworm (*C. virescens*) showed the lowest tolerance to the insecticides and were used as references for the pairwise comparisons between species. Distinct letters indicate a significant difference ($P < 0.05$) in the relative tolerance ratio.

CONCLUSIONS AND IMPLICATIONS

This work advances our understanding of potential responses of target and nontarget pest species exposed to insecticides and Bt toxins, which help to design pest management programs for lepidopteran species in the Americas. In summary, the low susceptibility of *S. cosmioides* larvae to Cry toxins and some Bt cotton cultivars emphasizes that the Bt technology should be used within an integrated pest management framework to properly manage populations of *S. cosmioides*. The Cry1Ac-induced parental effect in the *S. cosmioides* larva advances our understanding of the consequences of the exposure of insect species of low-inherent susceptibility to Bt toxins. The parental effect described here does not seem troublesome for pest management of *S. cosmioides* as the offspring of the larvae developing on Cry1Ac cotton did not have increased performance.

Dual-gene Bt cotton affects the fitness and demographic growth of *H. zea* from the Florida Panhandle. In addition, an interaction between *H. zea* populations and cotton cultivars (Bt and non-Bt) was documented, which drove the magnitude of the effect in the life history, and consequently, in the life table parameters. These outcomes improve our understanding of demographic growth rates of *H. zea,* and our finds can also inform models better to predict the risk of resistance evolution to Bt technology and validate resistance management strategies, such as refuge recommendations.

Our efforts also generated region-specific information on the susceptibility of populations of eight lepidopteran pest species from the Florida Panhandle, which helps to assess the resistance risk to pyrethroid and diamide insecticides. This assessment is of broad interest because insecticide resistance can spread to other regions by migrant individuals from species such as cotton bollworm and fall armyworm.