

**University of São Paulo  
Luiz de Queiroz College of Agriculture**

**Risk of resistance evolution of *Spodoptera frugiperda* (Lepidoptera:  
Noctuidae) to emamectin benzoate in Brazil**

**Dionei Schmidt Muraro**

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

**Piracicaba  
2023**

**Dionei Schmidt Muraro**  
**Agronomist**

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versão revisada de acordo com a Resolução CoPGr 6018 de 2011

Advisor:  
Prof. Dr. **CELSO OMOTO**

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## RESUMO

### **Risco de evolução da resistência de *Spodoptera frugiperda* (Lepidoptera: Noctuidae) a emamectin benzoate no Brasil**

O uso de inseticidas químicos tem sido uma das principais estratégias no controle de *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae) no Brasil. No entanto, a redução da eficácia de alguns inseticidas em razão da evolução da resistência tem sido um dos grandes desafios na estruturação de programas de manejo integrado de pragas (MIP). Dentre os inseticidas, emamectin benzoate do grupo das avermectinas representa uma importante opção no manejo de *S. frugiperda*. Esse inseto-praga pode causar severos danos em diversas culturas de importância econômica, caso estratégias de controle não forem adotadas corretamente. Nesse sentido, estudos que fomentem a implementação de estratégias no manejo da resistência de insetos (MRI) são fundamentais para manter a eficácia de emamectin benzoate no controle de *S. frugiperda*. Os objetivos deste estudo foram: (i) Monitoramento da suscetibilidade a emamectin benzoate em populações de *S. frugiperda* no Brasil (ii) Seleção e caracterização do padrão de herança da resistência, resistência cruzada e uso de sinergistas em *S. frugiperda* resistente a emamectin benzoate; e (iii) avaliação do custo adaptativo em *S. frugiperda* resistente a emamectin benzoate. O monitoramento da suscetibilidade foi realizado em populações de *S. frugiperda* coletadas no campo de 2019 a 2021 em comparação com os dados de linha-básica suscetibilidade obtidas a partir de populações coletadas em 2003 e 2004. De 2003 a 2004, a sobrevivência larval na concentração diagnóstica de emamectin benzoate (3,2 µg i.a./mL) foi < 1,79%. De 2019 a 2021, houve redução significativa na suscetibilidade a emamectin benzoate em *S. frugiperda*, com valores de CL<sub>50</sub> variando de 0,78 a 158,0 µg i.a./mL (razão de resistência de até 632 vezes). A sobrevivência larval na concentração diagnóstica de emamectin benzoate foi alta em todas as populações avaliadas de 2019 a 2021, com valores de até 81,4%. A sobrevivência larval em folhas de milho pulverizadas com emamectin benzoate na dose recomendada para controle de *S. frugiperda* variou de 16,6 a 88,5% em populações de *S. frugiperda* coletadas em 2021. A linhagem resistente a emamectin benzoate foi selecionada por meio da técnica de F<sub>2</sub> “screen” e após 10 gerações de pressão de seleção essa linhagem apresentou uma razão de resistência de 2339 vezes. O padrão de herança da resistência de *S. frugiperda* a emamectin benzoate foi caracterizada como autossômica, incompletamente dominante e poligênia. Foi observada uma baixa resistência cruzada entre emamectin benzoate e outros inseticidas (methomyl, chlorpyrifos, lambda-cyhalothrin, spinetoram, indoxacarb e chlorantraniliprole), variando de 1,1 a 5,7 vezes. Além disso, a exposição aos sinergistas inibidores de enzimas de detoxificação não aumentou significativamente a suscetibilidade larval das linhagens, sugerindo que a resistência metabólica não é o principal mecanismo de resistência de *S. frugiperda* a emamectin benzoate. A presença de custo adaptativo associado à resistência de *S. frugiperda* a emamectin benzoate foi verificada com uma redução de ~35% no potencial de crescimento populacional. Em contraste, a ausência de custo adaptativo foi observada nos parâmetros comportamentais, incluindo tempo de mobilidade, distância percorrida, velocidade e taxa de predação do *Podisus nigrispinus* (Heteroptera: Pentatomidae). Os resultados deste estudo evidenciam o alto risco de evolução da resistência de *S. frugiperda* a emamectin benzoate no Brasil. Portanto, as informações obtidas no presente estudo demonstraram o alto risco de evolução da resistência de *S. frugiperda* a emamectin benzoate e estratégias de MRI devem ser implementadas com o intuito de prolongar a vida útil deste inseticida no manejo de *S. frugiperda* no Brasil.

Palavras-chave: Emamectin benzoate, Lagarta-do-cartucho do milho, Herança da resistência, Manejo da resistência de insetos

## ABSTRACT

### **Risk of resistance evolution of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) to emamectin benzoate insecticide in Brazil**

The use of chemical insecticides is one of the main strategies to control *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae) in Brazil. However, the reduction in the efficiency of insecticides due to the evolution of resistance has been one of the major challenges in the implementation of integrated pest management programs. Among the insecticides, emamectin benzoate from the avermectin group represents an important option for the management of *S. frugiperda*. This insect pest can cause severe damage to several crops of economic importance, if control strategies are neglected. In this sense, studies that promote the implementation of insect resistance management (IRM) strategies are essential to maintain the efficacy of emamectin benzoate against *S. frugiperda*. The goals of this study were: (i) monitoring of susceptibility to emamectin benzoate in populations of *S. frugiperda* in Brazil (ii) selection and characterization of the inheritance pattern, cross-resistance and synergism in *S. frugiperda* resistant to emamectin benzoate; and (iii) Assessment of fitness cost in *S. frugiperda* resistant to emamectin benzoate. Monitoring was carried out on *S. frugiperda* populations collected in the field from 2019 to 2021 in compare to the baseline susceptibility studies conducted with populations collected in 2003 and 2004. From 2003 to 2004, larval survival at the diagnostic concentration of emamectin benzoate (3.2  $\mu\text{g a.i./mL}$ ) was < 1.79%. From 2019 to 2021, susceptibility to emamectin benzoate in *S. frugiperda* decreased, with  $\text{LC}_{50}$  values ranging from 0.78 to 158.0  $\mu\text{g a.i. mL}^{-1}$  (resistance ratios up to 632-fold). Using the diagnostic concentration, we also observed an increase in larval survival of up to 81.4%. Larval survival on maize leaves sprayed with the field rate of emamectin benzoate ranged from 16.6 to 88.5% in populations of *S. frugiperda* collected in 2021. The emamectin benzoate resistant strain was selected using the  $\text{F}_2$  screen method and after 10 generations of selection pressure this strain showed a resistance ratio of 2,339-fold. The inheritance of emamectin benzoate resistance in *S. frugiperda* was characterized as autosomal, incompletely dominant, and polygenic. Low cross-resistance was observed among emamectin benzoate and other insecticides (methomyl, chlorpyrifos, lambda-cyhalothrin, spinetoram, indoxacarb and chlorantraniliprole), ranging from 1.1 to 5.7-fold. In addition, the exposure of the strains to different synergists known to inhibit detoxification enzymes did not result in significantly increased larval toxicity, suggesting a minor role for metabolic resistance. Also, we observed fitness cost associated with the resistance of *S. frugiperda* to emamectin benzoate, reducing  $\sim 35\%$  its potential for population growth on non-Bt maize and artificial diet. In contrast, a lack of fitness cost was observed in the behavioral parameters, including mobility time, distance traveled, speed, and predation rate by *Podisus nigrispinus* (Heteroptera: Pentatomidae). The results of this study demonstrate the risk of resistance evolution of *S. frugiperda* to emamectin benzoate in Brazil. Therefore, the information obtained in this study showed high risk of resistance evolution of *S. frugiperda* to emamectin benzoate and IRM strategies should be implemented to prolong the lifetime of this insecticide in the management of *S. frugiperda* in Brazil.

**Keywords:** Emamectin benzoate, Fall armyworm, Inheritance of resistance, Insecticide resistance management



## 1. INTRODUCTION

The evolution of insect pest resistance to insecticides is one of the greatest challenges in implementing integrated pest management (IPM) programs (Bass et al., 2015; Hawkins et al., 2019). Despite the increase in the adoption of IPM, the use of chemical insecticides is still the main method of controlling insect pests in several crops (Sparks and Nauen, 2015). The indiscriminate and often inappropriate use of insecticides increase selection pressure and, consequently, the selection of insecticide-resistant individuals in the field. Cases of resistance to insect species have gradually increased globally, and this situation has a large economic, environmental, and social impact on agricultural production (Sparks and Nauen, 2015; Nauen et al., 2019). Therefore, studies that provide bases for implementing insect resistance management (IRM) strategies are fundamental in maintaining the effectiveness of insecticides in controlling insect pests.

Emamectin benzoate belongs to the chemical group of avermectins (glutamate-gated chloride channel allosteric modulator) and has high activity against lepidopterans (Dybas et al., 1989; Lasota and Dybas, 1991; Lopez et al., 2011). This insecticide derives from macrocyclic lactones produced by the fermentation process of a soil microorganism, *Streptomyces avermitilis* (Campbell, 1989). Emamectin benzoate acts as a glutamate-gated chloride channel allosteric modulator by binding to a secondary site of the chlorine channel and continuously activating them. Glutamate-mediated chloride channels present in muscles and nerve cells, when continuously activated, produce an inhibitory effect causing the death of the insect by generalized paralysis (Jansson and Dybas, 1996). In addition, emamectin benzoate is sensitive to photodegradation, being degraded in less than 10 h (MacConnell et al., 1989). Despite being sensitive to photodegradation, this insecticide is quickly absorbed by plants due to the translaminar effect (Wright et al., 1985; Dybas, 1989).

Cases of resistance to emamectin benzoate have been reported in *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) and *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) in China (Che et al., 2013; Tong et al., 2013; Su et al., 2014; Xuegui et al., 2018) and Pakistan (Ishtiaq et al., 2014; Saleem et al., 2016; Ahmad et al., 2018); *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) in Pakistan (Qayyum et al., 2015); and *Plutella xylostella* L. (Lepidoptera: Plutellidae) in the United States (Shelton et al., 2000; Zhao et al., 2006) and Pakistan (Sayed et al., 2005). Insect resistance to the group of avermectins can occur due to metabolic detoxification or changes in the site of action (Wang and Wu, 2007; Liu et al., 2014; Riga et al., 2014; Xu et al., 2016; Wang et al., 2018). Resistance to

emamectin benzoate has been associated with metabolic detoxification in *S. exigua* (Zuo et al., 2018).

Despite cases of resistance evolution, emamectin benzoate represents an important tool in managing lepidopteran pests. One of the target pests is the fall armyworm *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), which is a polyphagous species with high dispersal and reproductive potential (Pogue, 2002). *Spodoptera frugiperda* is native to the Americas but has recently invaded Africa (Goergen et al., 2016; Day et al., 2017), Asia (Sharanabasappa et al., 2018; Sidana et al., 2018; Gilal et al., 2020; Jing et al., 2020), and Oceania (Piggott et al., 2021), posing a huge threat to the global production systems, if strategies to control this pest are not adopted (Abrahams et al., 2017). This species causes economic damage to many crops, especially corn (*Zea mays* L.), cotton (*Gossypium hirsutum* L.), sorghum (*Sorghum bicolor* L.), millet (*Pennisetum americanum* L.), wheat (*Triticum* spp.), rice (*Oryza sativa* L.), and soybean (*Glicine max* L. Merrill) (Barros et al., 2010; Montezano et al., 2018; Oliveira et al., 2019).

In Brazil, emamectin benzoate was released in 2017 to control *S. frugiperda*, but it has been used in the country since 2014. It was released on an emergency permit after the detection of *H. armigera* in the country. *Spodoptera frugiperda* and *Helicoverpa armigera* share the same cultivation systems (cotton, maize, and soybean) and populations of *S. frugiperda* have been exposed to the insecticide emamectin benzoate since its emergency registration to control *H. armigera* (Bentivenha et al., 2017; Malaquias et al., 2021). In addition, the intensive cropping systems and favorable climatic conditions in Brazil favor the occurrence of severe infestations of *S. frugiperda* throughout the year.

Insecticides and genetically modified plants that express proteins from *Bacillus thuringiensis* Berliner (Bt) are the main methods used to control *S. frugiperda*. However, due to the high selection pressure imposed on *S. frugiperda*, control failures were reported in the field for Cry1F and Cry1Ab proteins, increasing the use of chemical insecticides for *S. frugiperda* management in plants that express Bt proteins (Farias et al., 2014; Omoto et al., 2016; Blanco et al., 2016; Burtet et al., 2017; Muraro et al., 2019). Resistance evolution to several insecticides have been reported for *S. frugiperda*, such as lambda-cyhalothrin, lufenuron, spinosad, chlorantraniliprole, spinetoram, teflubenzuron, and chlorpyrifos (Diez-rodriguez et al., 2001; Carvalho et al., 2013; Nascimento et al., 2016; Okuma et al., 2017; Bolzan et al., 2019; Lira et al., 2020; Nascimento et al., 2021; Garlet et al., 2021). In addition, 144 cases of *S. frugiperda* resistance to insecticides have been reported worldwide (APRD, 2022).

To establish an IRM program, it is necessary to monitor the insect-pest susceptibility to insecticides and understand the genetic, bioecological, and operational factors affecting the evolution of resistance (Roush and Daly, 1990; French-Constant and Bass, 2017). Monitoring allows to assess changes in susceptibility in insect pest populations to a control agent can be performed using a diagnostic concentration such as the concentration capable of killing 99% of the insects. This concentration is estimated from the baseline susceptibility studies performed before the widespread use of the insecticide under field conditions (Roush and Miller, 1986; French-Constant and Roush, 1990). The detection of changes in susceptibility over time and space is important to support decisions focused on increasing assertiveness in proactive or curative strategies in IRM programs (Georghiou, 1972; Dennehy, 1987; Roush and McKenzie, 1987).

The inheritance pattern of resistance has a great impact on the evolution to resistance. When inheritance of resistance is dominant, heterozygotes that are the main carriers of the resistance alleles at early stages of resistance evolution survive the exposure to the control agent, thus favoring the evolution of resistance. Inheritance of resistance can be dominant or recessive, autosomal, sex-linked, or mitochondrial, and monogenic or polygenic (Georghiou and Taylor, 1977). In addition, in the field, rotation of insecticides with different modes of action are commonly used to control insect pests. For this strategy to be effective in IRM, it is assumed that there is no cross-resistance between the insecticides used in rotation and presence of fitness cost associated with resistant alleles (Georghiou and Taylor, 1977; Roush and McKenzie, 1987; Tabashnik, 1989, 1994; Tabashnik et al., 2005).

Fitness costs are related to the fact that the resistant individuals have a disadvantage in relation to the susceptible ones in the absence of selection pressure (Roush and McKenzie, 1987; Kliot and Ghanim, 2012). In addition to resistant and susceptible, it is also essential to assess heterozygous, as they are the main carriers of the resistance allele (Roush and Daly, 1990). The use of strains with similar genetic basis is important to more assertively assess the influence of the resistance allele on the biological and behavioral parameters to be observed (Bass, 2017; Riordan and Nadeau, 2017; Lenormand et al., 2018; Freeman et al., 2021). Although there are many studies with fitness cost associated with resistance (Gassmann, Carrière and Tabashnik, 2009; Kliot and Ghanim, 2012), there are few studies that evaluate the behavioral and defensive responses of resistant and susceptible insects to insecticides and the influence of behavioral responses in the predation rate of natural enemies (Malaquias et al., 2014; Muller et al., 2019; Jackson et al., 2020; Guillem-Amat et al., 2020). The presence of natural enemies in agroecosystems is one factor that needs to be considered in IRM.



Among the natural enemies, the predator *Podisus nigrispinus* (Dallas) (Heteroptera: Pentatomidae) contributes to the biological control of many lepidopteran species, including *S. frugiperda*, in several crops in Brazil (Zanuncio et al., 2008).

To support IRM strategies and preserve the effectiveness of the insecticide emamectin benzoate to control *S. frugiperda*, the objectives were:

- To monitor the susceptibility to emamectin benzoate in populations of *Spodoptera frugiperda* in Brazil (Chapter 2);
- To select a strain of *Spodoptera frugiperda* resistant to emamectin benzoate and characterize the inheritance pattern, cross-resistance and synergism (Chapter 3);
- To evaluate fitness costs associated with emamectin benzoate resistance in *S. frugiperda* (Chapter 4).

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## 2. EVIDENCE OF FIELD-EVOLVED RESISTANCE IN *Spodoptera frugiperda* (LEPIDOPTERA: NOCTUIDAE) TO EMAMECTIN BENZOATE IN BRAZIL

### Abstract

The fall armyworm, *Spodoptera frugiperda* (J. E. Smith, 1797), has evolved resistance to several insecticides and *Bacillus thuringiensis* Berliner (Bt) toxins expressed in transgenic plants worldwide. In this study, we presented laboratory studies that established the baseline susceptibility and defined a diagnostic concentration of emamectin benzoate for the *S. frugiperda* performed in 2002. Then, resistance to emamectin benzoate was monitored in field-collected populations of *S. frugiperda* in Brazil from 2003 to 2004 and 2019 to 2021 using different methods: (i) estimating concentration-mortality curves; (ii) determining larval survival at a diagnostic concentration of emamectin benzoate; and (iii) assessing its survival rate on maize leaves sprayed with the field rate of this insecticide. Populations of *S. frugiperda* presented similar susceptibility to emamectin benzoate in 2002 (LC<sub>50</sub> from 0.25 to 0.44 µg a.i. mL<sup>-1</sup>) and 2003/04 (LC<sub>50</sub> from 0.13 to 0.43 µg a.i. mL<sup>-1</sup>), not differing from the susceptible reference population (LC<sub>50</sub> of 0.22 µg a.i. mL<sup>-1</sup>). From 2003 to 2004, larval survival at the diagnostic concentration of emamectin benzoate of 3.2 µg a.i. mL<sup>-1</sup> was < 1.79%. From 2019 to 2021, the susceptibility to emamectin benzoate in field populations of *S. frugiperda* decreased, with LC<sub>50</sub> values ranging from 0.78 to 158.0 µg a.i. mL<sup>-1</sup>, indicating resistance ratios up to 632-fold. Larval survival at a diagnostic concentration of emamectin benzoate also increased, reaching up to 81.4%. Larval survival on maize leaves sprayed with the field rate of emamectin benzoate ranged from 16.6 to 88.5% in populations of *S. frugiperda* collected in 2021. Overall, our findings revealed significant shifts towards decrease in the susceptibility to emamectin benzoate in Brazilian populations of *S. frugiperda*, providing robust evidence of field-evolved resistance.

Keywords: Fall armyworm; Susceptibility; Glutamate-gated chloride channel allosteric modulator; Resistance management

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### 2.1. Introduction

The fall armyworm, *Spodoptera frugiperda* (J. E. Smith, 1797) (Lepidoptera: Noctuidae), has evolved resistance to several insecticides and *Bacillus thuringiensis* Berliner (Bt) toxins expressed in transgenic plants worldwide. According to the *Arthropod Pesticide Resistance Database*, the *S. frugiperda* has developed resistance to 40 active ingredients,

representing more than 140 resistance cases. This species is native to the Americas, but currently it has become a pest of global relevance being documented in Africa (Pogue, 2002; Goergen et al., 2016; Day et al., 2017), Asia (Sharanabasappa et al., 2018; Sidana et al., 2018; Gilal et al., 2020; Jing et al., 2020), and Oceania (Piggott et al., 2021). The *S. frugiperda* causes economic loss in various economically important crops, including maize (*Zea mays* L.), cotton (*Gossypium hirsutum* L.), sorghum (*Sorghum bicolor* L.), soybean (*Glycine max* L. Merrill), and millet (*Pennisetum americanum* (L.) (Barros et al., 2010; Montezano et al., 2018; Oliveira et al., 2019).

Control of the *S. frugiperda* has been performed mainly using insecticides and transgenic plants expressing Bt toxins (Malaquias et al., 2021a). Among the insecticides, emamectin benzoate, a glutamate-gated chloride channel allosteric modulator, has been registered since 2017 to control *S. frugiperda* in Brazil, although this active ingredient has been commonly used against *Helicoverpa armigera* (Hübner, 1809) (Lepidoptera: Noctuidae) since 2014. In the current Brazilian agricultural system, both species share the same ecological niche in cotton, maize, and soybean (Bentivenha et al., 2017; Malaquias et al., 2021b). Therefore, the exposure of *S. frugiperda* populations to emamectin benzoate has occurred since its registration in 2014. Field-evolved resistance of the *S. frugiperda* to Bt toxins (Farias et al., 2014; Omoto et al., 2016) has increased the number of insecticide applications against *S. frugiperda* in maize, cotton, and soybean crops (Blanco et al., 2016; Burtet et al., 2017; Muraro et al., 2019). The frequent use of insecticides against *S. frugiperda* favors the evolution of resistance, as reported in laboratory studies for chlorpyrifos, lufenuron, spinosad, chlorantraniliprole, spinetoram, and teflubenzuron (Diez-Rodríguez and Omoto, 2001; Carvalho et al., 2013; Nascimento et al., 2016; Okuma et al., 2018; Bolzan et al., 2019; Lira et al., 2020; Nascimento et al., 2021). Recently, was selected a laboratory-resistant strain of *S. frugiperda* to emamectin benzoate and showed high risk of resistance evolution based on incomplete dominant inheritance (Muraro et al., 2021).

Understanding the geographic variation in susceptibility of the *S. frugiperda* to emamectin benzoate is essential to support insect resistance management (IRM) programs. Thus, the baseline susceptibility of the *S. frugiperda* to emamectin benzoate was established using populations collected in 2002, and a diagnostic concentration for resistance monitoring was defined. Resistance to emamectin benzoate was monitored in field-collected populations of *S. frugiperda* in Brazil from 2003 to 2004 and 2019 to 2021 using different methods, as follows: (i) by estimating concentration-mortality curves; (ii) by determining larval survival at

a diagnostic concentration; and (iii) by assessing its survival rate on maize leaves sprayed with emamectin benzoate.

## **2.2. Material and Methods**

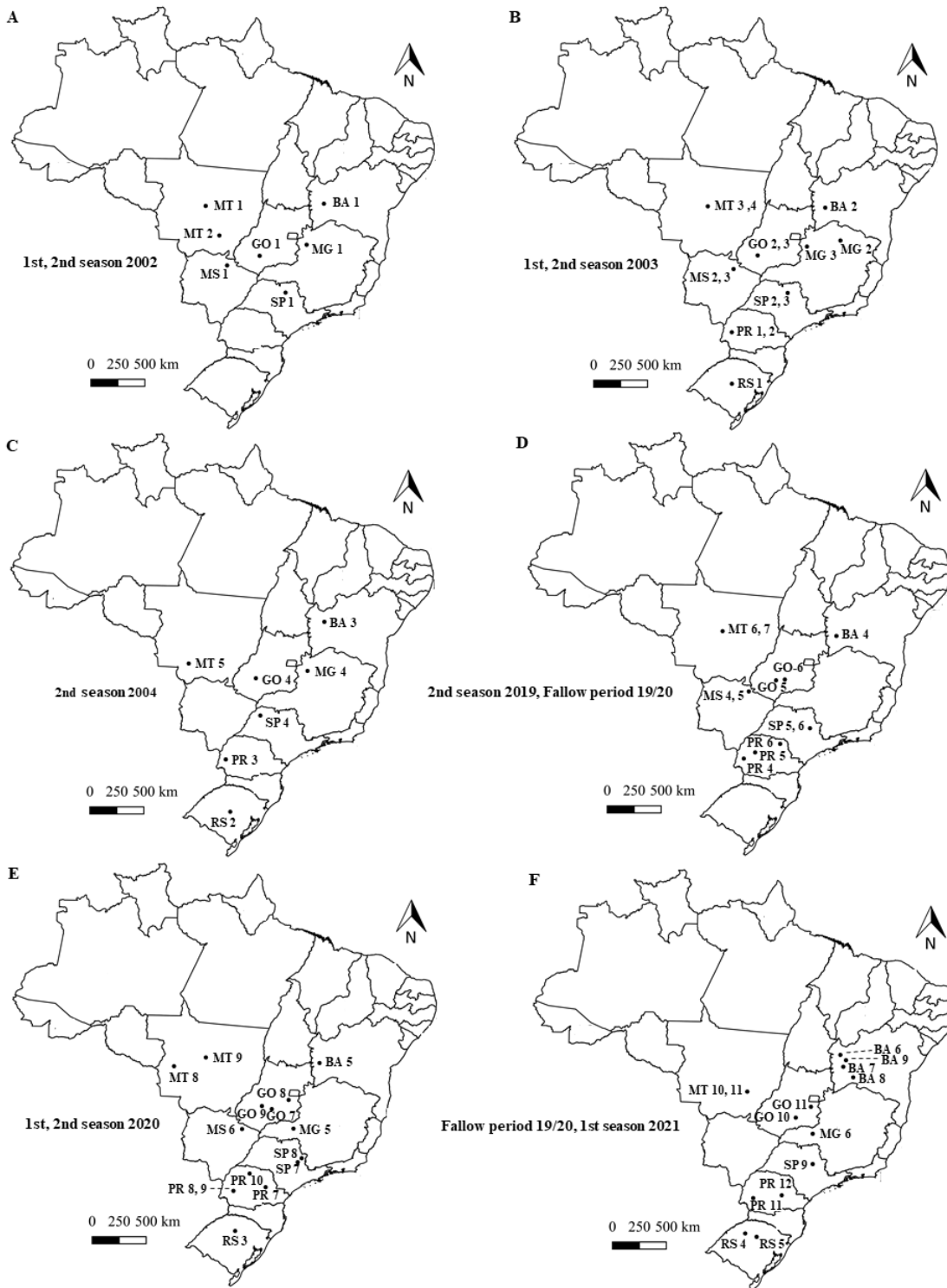
### **2.2.1. Insects**

For characterizing baseline susceptibility and monitoring resistance to emamectin benzoate, field populations of *S. frugiperda* were collected on non-Bt maize, non-Bt cotton, or sorghum fields (~1,000 larvae/location) in different geographic regions of Brazil from 2002 to 2004 (Data collected by Arthropod Resistance Laboratory USP/ESALQ) and then from 2019 to 2021 (Table 1 and Figure 1). After collections, larvae were transported to the laboratory and transferred to an artificial diet proposed by Kasten et al. (1978). In addition, a *S. frugiperda* population, maintained in the laboratory for >20 years free of exposure to insecticides or Bt proteins, was used as a susceptible reference for comparison with the field populations. All populations were maintained at  $25 \pm 2^\circ\text{C}$ ,  $70 \pm 10\%$  RH, and a 14:10 h (L:D) photoperiod.

**Table 1.** Brazilian populations of the *Spodoptera frugiperda* collected from 2002 to 2004 and from 2019 to 2021.

Population code	City, State	Host	Latitude (S)	Longitude (W)	Date
<b>First season 2002</b>					
GO-1	Rio Verde, GO	Maize	17°44'20"	50°57'53"	Nov. 2001
SP-1	Guaíra, SP	Maize	20°13'39"	48°26'50"	Nov. 2001
<b>Second season 2002</b>					
BA-1	Barreiras, BA	Maize	12°06'05"	44°54'15"	Jan. 2002
MS-1	Chapadão do Sul, BA	Cotton	18°49'51"	52°32'28"	Mar. 2002
MT-1	Primavera do Leste, MT	Cotton	14°45'47"	54°05'35"	Apr. 2002
MT-2	Rondonópolis, MT	Cotton	16°34'32"	54°50'20"	Feb. 2002
MG-1	Unaí, MG	Cotton	15°54'40"	46°40'35"	Feb. 2002
<b>First season 2003</b>					
GO-2	Rio Verde, GO	Maize	17°39'29"	51°19'18"	Dec. 2002
SP-2	Guaíra, SP	Maize	20°17'06"	48°24'57"	Dec. 2002
MG-2	Janaúba, MG	Maize	15°47'04"	43°17'06"	Dec. 2002
MS-2	Chapadão do Sul, MS	Cotton	18°53'14"	52°32'38"	Dec. 2002
MT-3	Lucas do Rio Verde, MT	Maize	13°10'21"	54°08'28"	Dec. 2002
PR-1	Cascavel, PR	Maize	24°53'44"	53°29'05"	Nov. 2002
RS-1	Santa Maria, RS	Maize	29°43'56"	53°36'47"	Dec. 2002
<b>Second season 2003</b>					
BA-2	Barreiras, BA	Maize	12°03'38"	45°03'13"	May 2003
GO-3	Rio Verde, GO	Maize	17°24'57"	51°35'36"	Apr. 2003
SP-3	Guaíra, SP	Maize	20°12'39"	48°29'16"	Apr. 2003
MG-3	Unaí, MG	Maize	16°18'06"	46°56'11"	May 2003
MS-3	Chapadão do Sul, MS	Maize	19°07'40"	52°54'47"	Mar. 2003
MT-4	Lucas do Rio Verde, MT	Cotton	13°01'25"	55°51'48"	Apr. 2003
PR-2	Cascavel, PR	Maize	24°58'22"	53°32'03"	Apr. 2003
<b>Second season 2004</b>					
BA-3	Barreiras, BA	Cotton	12°08'37"	44°57'51"	Feb. 2004
GO-4	Montividiu, GO	Maize	17°28'51"	51°11'59"	Mar. 2004
SP-4	Jales, SP	Maize	20°10'00"	50°34'02"	Mar. 2004
MG-4	Unaí, MG	Maize	16°22'13"	46°55'57"	Mar. 2004
MT-5	Rondonópolis, MT	Maize	16°33'32"	54°39'12"	Apr. 2004
PR-3	Marechal Cândido Rondon, PR	Maize	24°31'40"	54°00'25"	May 2004
RS-2	Santa Maria, RS	Maize	29°45'34"	53°32'37"	Mar. 2004
<b>Second season 2019</b>					
GO-5	Rio Verde, GO	Maize	17°48'24"	50°48'12"	May 2019
MS-4	Chapadão do Sul, MS	Maize	18°44'14"	52°32'09"	Apr. 2019
MT-6	Lucas do Rio Verde, MT	Maize	12°55'36"	56°03'13"	Mar. 2019
PR-4	Toledo, PR	Maize	24°38'52"	53°42'39"	Mar. 2019
PR-5	Campo Mourão, PR	Maize	24°01'41"	52°19'06"	Mar. 2019
PR-6	Londrina, RS	Maize	24°38'52"	53°42'39"	Mar. 2019
SP-5	Casa Branca-SP	Maize	21°45'01"	47°08'10"	Mar. 2019
<b>Fallow period 2019/20</b>					
BA-4	Correntina-BA	Maize	13°23'57"	45°52'50"	June 2019
GO-6	Santa Helena de Goiás, GO	Maize	17°45'10"	50°32'52"	June 2019
MS-5	Chapadão do Sul, MS	Maize	18°46'48"	55°52'24"	Aug. 2019
MT-7	Lucas do Rio Verde, MT	Maize	12°58'44"	55°52'24"	June 2019
SP-6	Casa Branca, SP	Maize	21°54'43"	47°08'29"	June 2019
<b>First season 2020</b>					
BA-5	Luis Eduardo Magalhães, BA	Maize	11°31'16"	45°44'17"	Jan. 2020
GO-7	Santa Helena de Goiás, GO	Sorghum	17°45'13"	50°35'12"	Nov. 2019

GO-8	Cristalina-GO	Maize	16°27'07"	47°37'48"	Nov. 2019
MG-5	Uberlândia-MG	Sorghum	19°10'49"	48°09'16"	Nov. 2019
PR-7	Tibagi-PR	Maize	24°27'09"	50°11'24"	Oct. 2019
PR-8	Cascavel, PR	Maize	25°50'46"	53°19'10"	Nov. 2019
SP-7	Pirassununga, SP	Sorghum	21°55'56"	47°21'10"	Oct. 2019
RS-3	Selbach, RS	Maize	28°38'45"	53°00'17"	Oct. 2019
<b>Second season 2020</b>					
GO-9	Rio Verde, GO	Maize	17°52'23"	50°50'11"	Mar. 2020
MS-6	Chapadão do Sul, MS	Maize	18°57'10"	52°47'48"	Mar. 2020
MT-8	Sapezal, MT	Maize	12°58'44"	55°52'24"	Mar. 2020
MT-9	Lucas do Rio Verde, MT	Maize	15°19'01"	54°56'24"	Mar. 2020
PR-9	Cascavel, PR	Maize	21°47'11"	49°36'52"	Mar. 2020
PR-10	Londrina, PR	Maize	24°38'52"	53°42'39"	Mar. 2020
SP-8	Casa Branca, SP	Maize	21°45'41"	46°59'45"	Feb. 2020
<b>Fallow period 2020/21</b>					
BA-6	Barreiras, BA	Cotton	11°41'47"	46°00'17"	May 2020
BA-7	São Desidério, BA	Cotton	12°40'33"	45°58'00"	May 2020
BA-8	Jaborandi, BA	Cotton	14°14'33"	45°26'32"	May 2020
MT-10	Primavera do Leste, MT	Cotton	13°25'29"	57°57'17"	Apr. 2020
MT-11	Primavera do Leste, MT	Cotton	15°19'01"	54°56'24"	May 2020
<b>First season 2021</b>					
BA-9	Luis Eduardo Magalhães, BA	Maize	12°05'40"	45°42'41"	Jan. 2021
GO-10	Santa Helena de Goiás, GO	Maize	17°41'21"	50°39'09"	Nov. 2020
GO-11	Cristalina, GO	Maize	16°28'08"	47°39'09"	Dec. 2020
MG-6	Araguari, MG	Maize	18°43'54"	47°58'41"	Dec. 2020
PR-11	Cascavel, PR	Maize	25°42'39"	53°04'12"	Nov. 2020
PR-12	Ponta Grossa, PR	Maize	24°27'40"	50°10'38"	Nov. 2020
SP-9	Casa Branca, SP	Maize	21°48'05"	46°55'54"	Oct. 2020
RS-4	Selbach, RS	Maize	28°38'70"	52°57'05"	Oct. 2020
RS-5	Santo Ângelo, RS	Maize	28°28'50"	54°20'16"	Oct. 2020



**Figure 1.** Collection sites of Brazilian *Spodoptera frugiperda* populations used in this study: (A) 1st and 2nd season in 2002; (B) 1st and 2nd season in 2003; (C) 2nd season in 2004; (D) 2nd season in 2019 and fallow period 2019/20; (E) 1st and 2nd season in 2020; (F) fallow period in 2020/21 and 1st season in 2021.

### 2.2.2. Baseline susceptibility

To characterize the baseline susceptibility of the *S. frugiperda* to emamectin benzoate, seven field populations collected in 2002 were used (Table 1 and Figure 1). In the bioassays, the artificial diet proposed by Kasten et al. (1978) was prepared and poured in 24-well acrylic plates (Costar<sup>®</sup>, Sigma-Aldrich Brasil Ltda, São Paulo, Brazil) at a volume of 1.5 mL/well. Then, the insecticide emamectin benzoate (Proclaim<sup>®</sup> 50 g a.i. (active ingredient) kg<sup>-1</sup>, Syngenta Crop Protection, São Paulo, Brazil) was diluted in distilled water to prepare four to six concentrations (0.10 to 3.2 µg a.i. mL<sup>-1</sup>). A nonionic surfactant at 0.1% (Triton X-100, Sigma-Aldrich Brasil Ltda) was added to each concentration to spread the solution over the diet surface. The control treatment contained distilled water and surfactant. A volume of 30 µL/well of emamectin benzoate was applied to the diet surface using an electronic micropipette (Repetman<sup>®</sup>, Gilson, Illinois, US). The diet surface area in each well was 1.90 cm<sup>2</sup>. After drying, a single third-instar larva was placed in each well. Plates were sealed and maintained in a chamber at 25 ± 2°C, 70 ± 10% RH, and a 14:10 h (L:D) photoperiod. The bioassays were conducted in a completely randomized design with four replicates (each replicate was composed of 24 larvae), totaling 96 larvae tested per concentration. Mortality was assessed at 96 h after insecticide exposure. Larvae that did not show coordinated movement after a light touch were considered dead. The concentration-mortality data were submitted to a generalized linear model (GLM). For these data, binomial or quasibinomial distributions using the probit link function were adjusted. A half-normal plot with a simulated envelope was also used with the hnp package to evaluate the goodness-of-fit of the model (Moral et al., 2017). Then, to estimate the insecticide toxicity, data were submitted to Probit analysis to estimate LC<sub>50</sub> values (concentration required to kill 50% of larvae tested) (Finney, 1953), confidence intervals (95% CIs), and slope of log concentration-mortality regression lines using R software (R Development Core Team, 2020). Tests for parallelism and equality were also performed to compare the angular and linear coefficients of the regression lines for the tested populations (Robertson et al., 2007). For estimating the diagnostic concentration of emamectin benzoate to be used in resistance monitoring, a joint analysis of the baseline susceptibility data was performed to estimate the LC<sub>99</sub> value (concentration required to kill 99% of larvae tested), 95% CI, and slope following the same statistical procedure described previously.



### **2.2.3. Resistance monitoring**

#### **2.2.4. Estimating LC<sub>50</sub> values to emamectin benzoate**

To estimate LC<sub>50</sub> to emamectin benzoate, larvae at F<sub>1</sub> or F<sub>2</sub> generations from 65 populations of the *S. frugiperda* collected from 2003 to 2004 and 2019 to 2021 (Table 1 and Figure 1) were exposed in four to nine logarithmically spaced concentrations of emamectin benzoate following the same methodology, environmental conditions, experimental design, and statistical procedures described above. The LC<sub>50</sub> values were considered significantly different when the 95% CIs among populations did not overlap. Resistance ratios were calculated by dividing the LC<sub>50</sub> values of the field populations by the corresponding parameter of the susceptible reference population.

#### **2.2.5. Exposure to a diagnostic concentration of emamectin benzoate**

Using a diagnostic concentration of emamectin benzoate applied to the diet surface, as described in the baseline study, 240 to 720 third-instar larvae in the F<sub>1</sub> or F<sub>2</sub> generations (10–30 replicates of 24 larvae) from 65 field populations collected from 2003 and 2004 and from 2019 to 2021 were monitored. In the bioassays, the same methodology, environmental conditions, and mortality criterion were used as described previously. Data were analyzed using a non-parametric bootstrap technique, with 100,000 pseudoreplications to estimate the 95% CIs with the boot package (Canty et al., 2019). Survival data of *S. frugiperda* populations exposed to emamectin benzoate were compared within each season and were grouped per Brazilian state and season to compare survival in different years. Survival rates were considered significantly different when the 95% CIs did not overlap. All analyses were performed in R software (R Development Core Team, 2020).

#### **2.2.6. Survival rate on non-Bt maize leaves sprayed with emamectin benzoate**

On bioassays with maize leaves, 96 third-instar larvae (6 replicates of 16 larvae) from F<sub>1</sub> or F<sub>2</sub> generations of seven field populations of *S. frugiperda* collected in the first season of 2021 were exposed to non-Bt maize (30A37, Dow AgroSciences, Jardinópolis, SP, Brazil) leaves sprayed with the field rate of emamectin benzoate (15 g a.i. ha<sup>-1</sup> diluted in 150 L of water) or non-sprayed (control). Maize leaves were placed over a 3% mixture of water-agar in

16-well plastic plates (CM&CM Comércio de Plásticos, São Paulo, SP, Brazil). The survival data of *S. frugiperda* populations exposed to non-Bt maize leaves sprayed with emamectin benzoate were corrected based on the survival on non-sprayed leaves using Abbott's formula (Abbott, 1925). Subsequently, data were adjusted to a GLM for quasibinomial distribution to analyze larval survival. The goodness-of-fit of the model was analyzed using the *hnp* package with a simulated envelope (Moral et al., 2017). To evaluate the interaction between main factors (*S. frugiperda* populations and sprayed or non-sprayed maize leaves), a deviance analysis ( $P = 0.05$ ) was performed. Statistical differences were determined using the function *glht* (Hothorn et al., 2008) in R software (R Development Core Team, 2020).

## 2.3. Results

### 2.3.1. Baseline susceptibility

The  $LC_{50}$  values to emamectin benzoate for Brazilian populations of the *S. frugiperda* collected in 2002 ranged from 0.25 to 0.44  $\mu\text{g a.i. mL}^{-1}$ , not differing from the  $LC_{50}$  value of the susceptible reference population (0.20  $\mu\text{g a.i. mL}^{-1}$ ) (Table 2). These LC values indicated a resistance ratio  $< 2.2$ -fold. The similar susceptibility to emamectin benzoate in field populations of the *S. frugiperda* was also confirmed by equality ( $\chi^2 = 0.53$ ;  $df = 6$ ;  $P = 0.426$ ) and parallelism tests ( $\chi^2 = 0.62$ ;  $df = 5$ ;  $P = 0.382$ ), which showed that mortality curves had similar parameters (slopes and intercepts).

To define a diagnostic concentration of emamectin benzoate for resistance monitoring, the concentration-mortality data of the baseline susceptibility study were grouped and analyzed jointly. In the joint analysis, the estimated  $LC_{99}$  of emamectin benzoate was 2.31 (95% CI [1.62–2.67])  $\mu\text{g a.i. mL}^{-1}$  ( $n = 5030$ ; slope [ $\pm$  SE] = 3.18 [ $\pm$  0.32];  $\chi^2 = 9.26$ ;  $df = 5$ ). Based on the upper limit of the 95% CI of the  $LC_{99}$  value, the diagnostic concentration of 3.2  $\mu\text{g a.i. mL}^{-1}$  was defined for resistance monitoring of *S. frugiperda* to emamectin benzoate in Brazil.

**Table 2.** Baseline susceptibility of Brazilian populations of the *Spodoptera frugiperda* to emamectin benzoate in diet-overlay bioassays performed in 2002.

Pop. code	Generation	n <sup>a</sup>	Slope (± SE)	LC <sub>50</sub> (95% CI) <sup>b</sup>	χ <sup>2</sup> (df) <sup>c</sup>	RR <sup>d</sup>
Sus	-	480	2.31 (± 0.16)	0.20 (0.12–0.27) a	10.84 (4)	-
<b>First season 2002</b>						
GO-1	F <sub>1</sub>	480	4.11 (± 0.49)	0.41 (0.26–0.46) a	8.11 (4)	2.1
SP-1	F <sub>1</sub>	480	3.89 (± 1.13)	0.44 (0.21–0.63) a	8.95 (4)	2.2
<b>Second season 2002</b>						
BA-1	F <sub>1</sub>	384	4.31 (± 0.45)	0.40 (0.26–0.44) a	4.27 (3)	2.0
MS-1	F <sub>1</sub>	384	5.22 (± 0.58)	0.39 (0.26–0.43) a	2.00 (3)	1.9
MT-1	F <sub>1</sub>	384	4.14 (± 0.68)	0.25 (0.20–0.28) a	5.30 (3)	1.3
MT-2	F <sub>2</sub>	576	2.97 (± 0.21)	0.25 (0.22–0.28) a	2.05 (5)	1.3
MG-1	F <sub>1</sub>	384	1.91 (± 0.40)	0.25 (0.06–0.47) a	9.20 (3)	1.3

<sup>a</sup>Number of insects tested.

<sup>b</sup>LC<sub>50</sub> values (µg a.i. mL<sup>-1</sup>) followed by the same letter did not differ significantly due to non-overlap of 95% confidence intervals (95% CIs).

<sup>c</sup>df = degrees of freedom.

<sup>d</sup>Resistance Ratio (RR) = LC<sub>50</sub> of field populations/LC<sub>50</sub> of susceptible (Sus) population.

### 2.3.2. Resistance monitoring

#### 2.3.3. Comparing LC<sub>50</sub> values to emamectin benzoate

Quando The LC<sub>50</sub> values to emamectin benzoate for field populations of µg a.i. mL<sup>-1</sup> monitored in 2003 (first and second seasons) and 2004 (second season) were similar, ranging from 0.13 to 0.43, not differing from the LC<sub>50</sub> value of the susceptible population (0.22 µg a.i. mL<sup>-1</sup>) (Table 4). These results indicated a resistance ratio < 2.0-fold. In contrast, in 2019 (second season) and 2019/20 (fallow period), susceptibility to emamectin benzoate in most *S. frugiperda* field populations decreased (Table 3). In these periods, the LC<sub>50</sub> values ranged from 0.78 to 7.26 µg a.i. mL<sup>-1</sup>, with most field populations differing from the susceptible population (0.25 µg a.i. mL<sup>-1</sup>), indicating a significant increase on resistance ratio from 3.1- to 29.0-fold.

In *S. frugiperda* populations collected in 2020 (first and second seasons), 2020/21 (fallow period), and 2021 (first season), the susceptibility to emamectin benzoate continues to decrease, mainly in populations from Bahia, Goiás, Mato Grosso, and Mato Grosso do Sul states (Table 3). In these seasons, all field populations differed significantly from the susceptible population regarding their susceptibility to emamectin benzoate. The LC<sub>50</sub> values in field populations ranged from 0.78 to 158.0 µg a.i. mL<sup>-1</sup>, whereas the LC<sub>50</sub> value for the susceptible population remained 0.25 µg a.i. mL<sup>-1</sup>. These results indicated that resistance

ratios increased from 3.1- to 632-fold, suggesting a significant increase on the magnitude of resistance to emamectin benzoate in *S. frugiperda* in Brazil.

**Table 3.** Susceptibility monitoring of Brazilian populations of the *Spodoptera frugiperda* to emamectin benzoate in diet-overlay bioassays from 2003 to 2004 and from 2019 to 2021.

Pop. code	Fit of probit lines					Diagnostic concentration	
	$n^a$	Slope $\pm$ SE	LC <sub>50</sub> (95% CI) <sup>b</sup>	$\chi^2$ (df) <sup>c</sup>	RR <sup>d</sup>	$n^a$	% survival (95% CI) <sup>b</sup>
<b>First season 2003</b>							
Sus	721	1.72 ( $\pm$ 0.13)	0.22 (0.15–0.31) a	11.22 (5)	-	480	0.09 (0.03–0.22) a
GO-2	455	4.87 ( $\pm$ 1.17)	0.38 (0.16–0.52) a	11.13 (3)	1.5	480	0.15 (0.04–0.34) a
SP-2	562	3.56 ( $\pm$ 0.32)	0.37 (0.20–0.73) a	10.42 (4)	1.4	720	1.74 (0.21–3.10) a
MG-2	218	2.67 ( $\pm$ 0.62)	0.17 (0.08–0.21) a	5.81 (3)	0.8	480	1.32 (0.20–2.66) a
MS-2	736	3.56 ( $\pm$ 0.32)	0.38 (0.24–0.43) a	4.20 (4)	1.5	480	1.50 (0.19–3.08) a
MT-3	369	3.32 ( $\pm$ 0.12)	0.27 (0.11–0.21) a	5.81 (3)	1.1	408	0.20 (0.09–0.45) a
PR-1	323	2.28 ( $\pm$ 0.32)	0.28 (0.12–0.32) a	8.81 (4)	1.2	480	0.0 (0.00–0.00) *
RS-1	359	2.67 ( $\pm$ 0.62)	0.13 (0.15–0.23) a	5.81 (3)	0.6	288	0.28 (0.16–0.63) a
<b>Second season 2003</b>							
Sus	721	1.72 ( $\pm$ 0.13)	0.22 (0.15–0.31) a	11.22 (5)	-	480	0.09 (0.03–0.22) a
BA-2	323	2.15 ( $\pm$ 0.12)	0.24 (0.16–0.28) a	8.21 (3)	1.0	480	0.21 (0.06–0.54) a
GO-3	415	2.21 ( $\pm$ 0.24)	0.16 (0.12–0.26) a	1.81 (3)	0.7	480	0.0 (0.00–0.00) *
SP-3	412	3.22 ( $\pm$ 0.21)	0.36 (0.20–0.42) a	9.97 (4)	1.4	480	1.59 (0.20–2.55) a
MG-3	316	1.67 ( $\pm$ 0.32)	0.22 (0.16–0.33) a	8.33 (4)	1.0	480	0.25 (0.10–0.76) a
MS-3	328	3.27 ( $\pm$ 0.27)	0.23 (0.15–0.27) a	6.81 (3)	1.0	480	0.21 (0.12–0.88) a
MT-4	330	2.67 ( $\pm$ 0.41)	0.26 (0.18–0.23) a	1.81 (3)	1.0	480	1.51 (0.22–2.16) a
PR-2	418	3.02 ( $\pm$ 0.20)	0.16 (0.08–0.26) a	9.22 (4)	0.7	480	1.60 (0.10–3.55) a
<b>Second season 2004</b>							
Sus	721	1.72 ( $\pm$ 0.13)	0.22 (0.15–0.31) a	11.22 (5)	-	480	0.09 (0.03–0.22) a
BA-3	981	3.22 ( $\pm$ 0.14)	0.43 (0.31–0.56) a	9.24 (5)	1.7	480	1.79 (0.20–3.20) a
GO-4	432	2.76 ( $\pm$ 0.21)	0.23 (0.11–0.27) a	11.81 (4)	1.0	480	0.0 (0.00–0.00) *
SP-4	421	2.65 ( $\pm$ 0.42)	0.31 (0.12–0.36) a	7.21 (3)	1.2	480	0.74 (0.18–1.95) a
MG-4	652	3.07 ( $\pm$ 0.51)	0.32 (0.19–0.43) a	8.33 (3)	1.3	480	1.10 (0.23–2.67) a
MT-5	537	2.33 ( $\pm$ 0.18)	0.29 (0.18–0.36) a	8.44 (3)	1.2	480	0.11 (0.04–1.03) a
PR-3	348	2.12 ( $\pm$ 0.42)	0.40 (0.13–0.43) a	9.12 (3)	1.6	480	0.0 (0.00–0.00) *
RS-2	762	1.33 ( $\pm$ 0.33)	0.28 (0.22–0.33) a	6.86 (3)	1.1	480	1.39 (0.20–2.05) a
<b>Second season 2019</b>							
Sus	742	2.83 ( $\pm$ 0.30)	0.25 (0.19–0.32) a	9.52 (4)	-	240	0.04 (0.02–0.06) a
GO-5	997	1.43 ( $\pm$ 0.08)	1.32 (1.09–1.58) c	7.56 (7)	5.2	480	28.75 (19.17–39.02) d
MS-4	940	1.05 ( $\pm$ 0.09)	2.27 (1.21–3.67) c	5.77 (6)	9.0	480	33.75 (28.21–36.18) d
MT-6	990	1.30 ( $\pm$ 0.09)	4.64 (3.76–5.65) d	10.31 (8)	18.5	480	36.67 (22.09–48.46) d
PR-4	996	1.19 ( $\pm$ 0.12)	2.29 (1.55–3.10) c	8.75 (8)	9.1	480	24.58 (16.62–40.19) d
PR-5	840	1.69 ( $\pm$ 0.12)	1.87 (1.53–2.21) c	11.05 (7)	7.4	480	6.04 (4.27–12.47) c
PR-6	640	1.23 ( $\pm$ 0.13)	1.98 (1.62–2.58) c	10.12 (6)	7.9	504	1.90 (0.92–3.15) b
SP-5	624	2.46 ( $\pm$ 0.24)	0.88 (0.65–1.12) b	4.65 (4)	3.5	408	1.87 (0.72–2.32) b
<b>Fallow period 2019/20</b>							
Sus	742	2.83 ( $\pm$ 0.30)	0.25 (0.19–0.32) a	9.52 (4)	-	240	0.04 (0.02–0.06) a
BA-4	672	0.99 ( $\pm$ 0.19)	7.26 (5.13–17.90) d	9.19 (6)	29.0	480	49.17 (36.33–66.49) c
GO-6	912	1.44 ( $\pm$ 0.13)	1.20 (0.82–1.62) b	9.42 (6)	4.8	480	27.08 (22.15–38.17) c
MS-5	723	1.23 ( $\pm$ 0.12)	2.82 (1.31–4.29) bc	7.98 (6)	11.3	480	45.42 (32.08–52.12) c
MT-7	872	1.13 ( $\pm$ 0.19)	3.13 (2.32–6.22) cd	11.09 (7)	12.5	480	43.33(20.46–55.87) c
SP-6	610	1.63 ( $\pm$ 0.22)	0.78 (0.44–2.55) bc	10.33 (5)	3.1	480	0.83 (0.65–1.23) b
<b>First season 2020</b>							
Sus	742	2.83 ( $\pm$ 0.30)	0.25 (0.19–0.32) a	9.52 (4)	-	240	0.04 (0.02–0.06) a
BA-5	821	1.27 ( $\pm$ 0.12)	11.52 (4.59–16.82) e	10.21 (6)	46.1	480	55.42 (42.03–60.28) d
GO-7	552	1.23 ( $\pm$ 0.26)	2.33 (1.60–8.19) cde	11.01 (5)	4.9	480	53.54 (36.22–66.31) d

GO-8	478	1.45 ( $\pm$ 0.16)	2.12 (1.17–3.29) bcd	1.82 (5)	8.5	480	33.34 (26.14–43.19) d
MG-5	684	1.16 ( $\pm$ 0.14)	0.96 (0.75–1.45) b	4.35 (4)	3.8	456	11.67 (9.49–14.22) c
PR-7	623	0.94 ( $\pm$ 0.31)	5.29 (2.90–9.75) de	7.41 (6)	21.1	456	11.18 (8.17–18.23) c
PR-8	840	1.35 ( $\pm$ 0.11)	1.32 (0.84–1.88) bc	10.60 (6)	5.2	480	14.59 (9.14–19.59) c
SP-7	624	2.18 ( $\pm$ 0.22)	0.92 (0.68–1.20) b	3.71 (5)	3.7	600	5.01 (2.43–7.22) b
RS-3	552	2.22 ( $\pm$ 0.24)	0.94 (0.70–1.17) b	3.36 (4)	3.8	480	20.21 (17.21–25.18) c
<b>Second season 2020</b>							
Sus	742	2.83 ( $\pm$ 0.30)	0.25 (0.19–0.32) a	9.52 (4)	-	240	0.04 (0.02–0.06) a
GO-9	984	1.04 ( $\pm$ 0.10)	4.28 (2.73–6.53) cd	14.25 (8)	17.1	480	40.83 (36.12–44.15) d
MS-6	648	1.27 ( $\pm$ 0.12)	11.52 (4.59–16.82) de	10.21 (6)	46.1	600	51.04 (41.26–56.33) d
MT-8	696	1.10 ( $\pm$ 0.09)	11.18 (8.53–14.57) e	8.10 (7)	46.0	480	42.08 (34.51–62.21) d
MT-9	648	1.09 ( $\pm$ 0.12)	8.22 (2.44–12.82) cde	12.46 (6)	32.9	480	47.29 (36.87–58.12) d
PR-9	600	1.33 ( $\pm$ 0.14)	2.16 (1.38–3.26) c	11.60 (6)	8.64	432	14.35 (6.34–24.19) c
PR-10	627	1.22 ( $\pm$ 0.21)	2.63 (1.49–3.92) c	10.20 (6)	10.5	432	19.25 (8.67–29.22) c
SP-8	514	3.68 ( $\pm$ 0.39)	0.79 (0.71–0.88) b	3.61 (3)	3.1	480	3.59 (2.25–4.32) b
<b>Fallow period 2020/21</b>							
Sus	742	2.83 ( $\pm$ 0.30)	0.25 (0.19–0.32) a	9.52 (4)	-	240	0.04 (0.02–0.06) a
BA-6	816	1.02 ( $\pm$ 0.15)	2.84 (0.59–4.62) b	8.41 (6)	11.4	480	45.62 (20.41–50.93) b
BA-7	532	1.32 ( $\pm$ 0.13)	102.0 (72.23–182.12) d	8.23 (6)	408.0	480	71.25 (64.37–81.76) c
BA-8	664	1.59 ( $\pm$ 0.22)	158.0 (98.78–215.28) d	5.56 (6)	632.0	480	80.42 (65.81–93.44) c
MT-10	526	1.12 ( $\pm$ 0.10)	8.22 (7.23–10.22) c	9.15 (6)	32.9	600	52.90 (42.54–55.25) b
MT-11	484	1.02 ( $\pm$ 0.11)	12.28 (8.26–15.32) c	10.11 (6)	49.1	480	59.17 (50.03–62.51) b
<b>First season 2021</b>							
Sus	742	2.83 ( $\pm$ 0.30)	0.25 (0.19–0.32) a	9.52 (4)	-	240	0.04 (0.02–0.06) a
BA-9	768	1.25 ( $\pm$ 0.11)	42.89 (31.69–55.25) d	4.99 (7)	171.5	672	72.42 (58.94–84.36) d
GO-10	320	1.11 ( $\pm$ 0.14)	55.21 (32.87–66.34) d	9.29 (6)	220.8	480	76.43 (60.55–82.27) d
GO-11	384	1.45 ( $\pm$ 0.27)	85.43 (80.30–152.21) e	8.46 (5)	341.7	502	81.41 (71.66–92.53) d
MG-6	432	1.89 ( $\pm$ 0.24)	21.30 (15.76–27.28) c	3.22 (6)	85.2	480	49.36 (32.49–55.43) c
PR-11	600	1.42 ( $\pm$ 0.19)	1.81 (1.49–2.03) b	11.92 (6)	7.2	480	34.77 (20.81–42.31) c
PR-12	317	1.82 ( $\pm$ 0.17)	1.74 (1.59–1.85) b	6.97 (6)	6.9	480	38.13 (19.63–56.72) c
SP-9	696	1.14 ( $\pm$ 0.17)	0.92 (0.25–1.72) ab	11.22 (7)	1.1	480	8.25 (4.82–12.93) b
RS-4	600	1.33 ( $\pm$ 0.18)	1.97 (1.15–1.85) b	13.29 (6)	7.9	552	43.58 (30.23–55.66) c
RS-5	624	2.38 ( $\pm$ 0.76)	1.62 (1.11–2.31) b	12.21 (6)	6.5	480	43.31 (20.21–52.52) c

<sup>a</sup>Number of insects tested.

<sup>b</sup>LC<sub>50</sub> values ( $\mu\text{g a.i. mL}^{-1}$ ) or percent survival at the diagnostic concentration ( $3.2 \mu\text{g a.i. mL}^{-1}$ ) followed by the same letter in each season did not differ significantly due to non-overlap of 95% CIs.

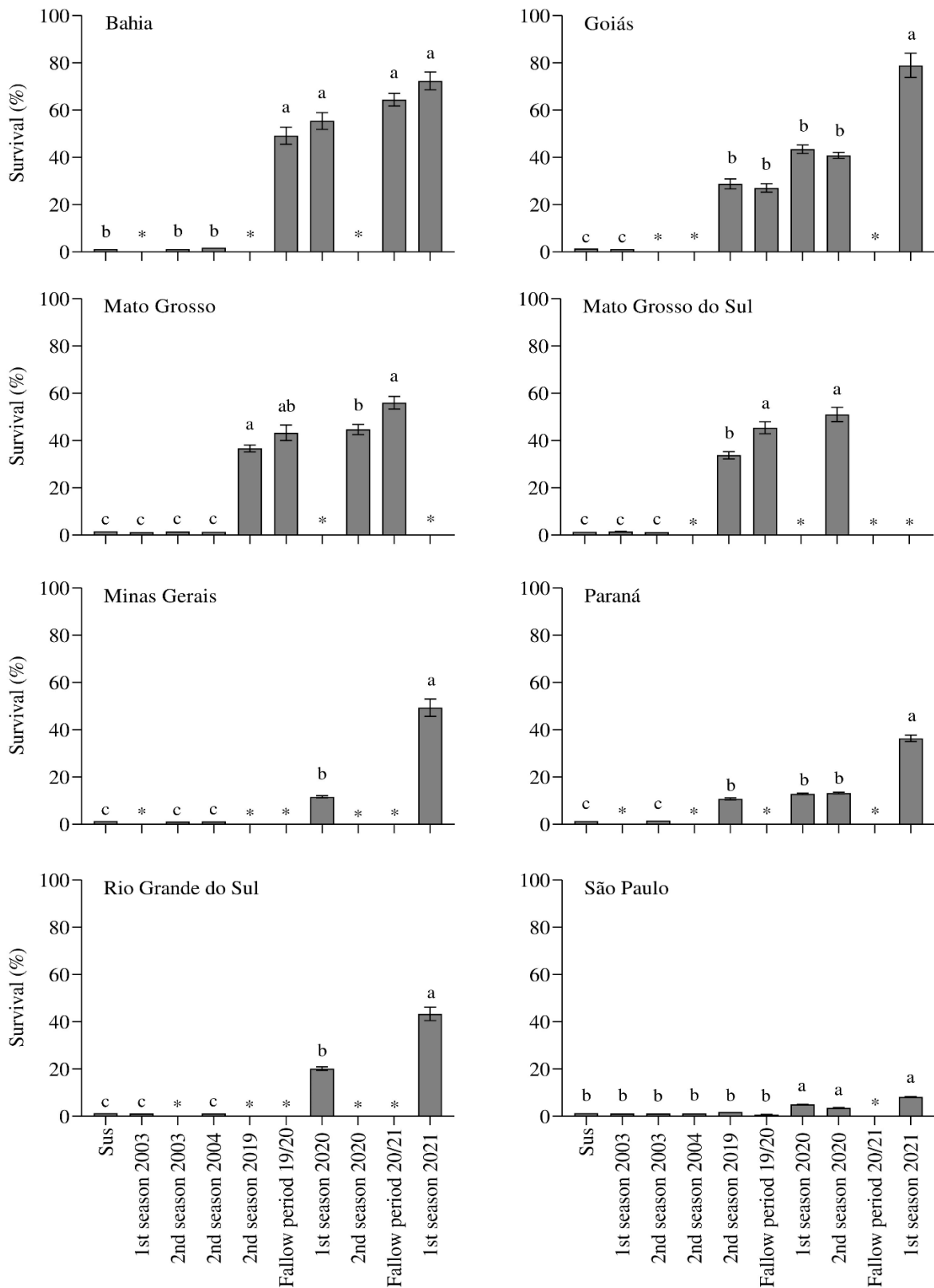
<sup>c</sup>df = degrees of freedom.

<sup>d</sup>Resistance Ratio (RR) = LC<sub>50</sub> of field populations/LC<sub>50</sub> of susceptible (Sus) population.

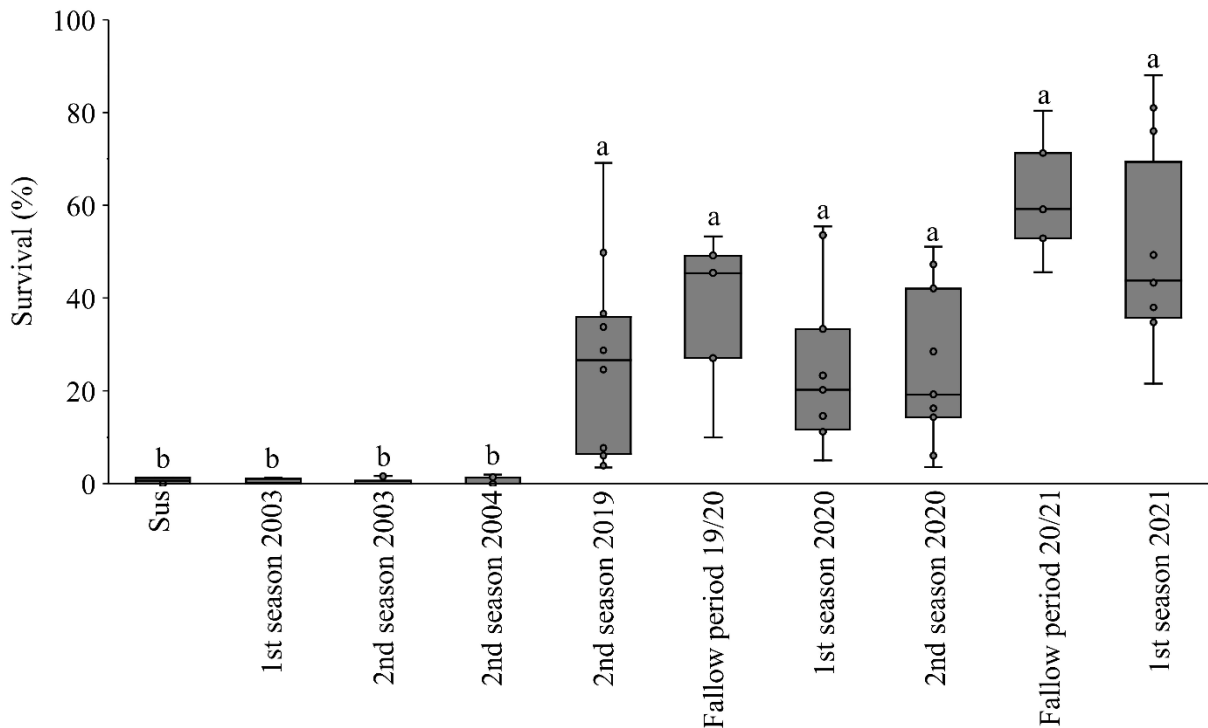
### 2.3.4. Exposure at a diagnostic concentration of emamectin benzoate

The survival rate of field populations of *S. frugiperda* collected in 2003 (first and second seasons) and 2004 (second season) at the diagnostic concentration of emamectin benzoate was lower than 1.79%, not differing from those of the susceptible population (Table 4). In contrast, from 2019 to 2021, there was a significant increase in the larval survival of field populations (up to 81.4% survival), whereas the larval survival of the susceptible population was < 1% (Table 4).

From 2003 to 2021, there was also an increase in larval survival at the diagnostic concentration of emamectin benzoate in all Brazilian states where *S. frugiperda* populations were sampled, namely Bahia (0.21 to 80.42%), Goiás (0.15 to 81.42%), Mato Grosso (0.11 to 59.17%), Mato Grosso do Sul (0.21 to 51.04%), Minas Gerais (0.25 to 49.30%), Paraná (0.0 to 38.11%), Rio Grande do Sul (0.28 to 43.50%), and São Paulo (0.74 to 8.22%) (Figure 2). Considering all populations together, it was verified that from 2003 to 2004 and from 2019 to 2021, larval survival at a diagnostic concentration of emamectin benzoate increased substantially from 0.48 to 61.87% (Figure 3). These results revealed significant shifts in the susceptibility indicating increase in frequency of resistant individuals to emamectin benzoate in field populations of *S. frugiperda* from Brazil.



**Figure 2.2.** Larval survival of Brazilian *Spodoptera frugiperda* populations at a diagnostic concentration of 3.2 µg of emamectin benzoate mL<sup>-1</sup> in 2003 and 2004 and from 2019 to 2021. Bars (± SE) with the same letter are not significantly different by non-overlapping 95% CIs. An asterisk (\*) indicates that 95% CIs were not estimated because no variability existed.

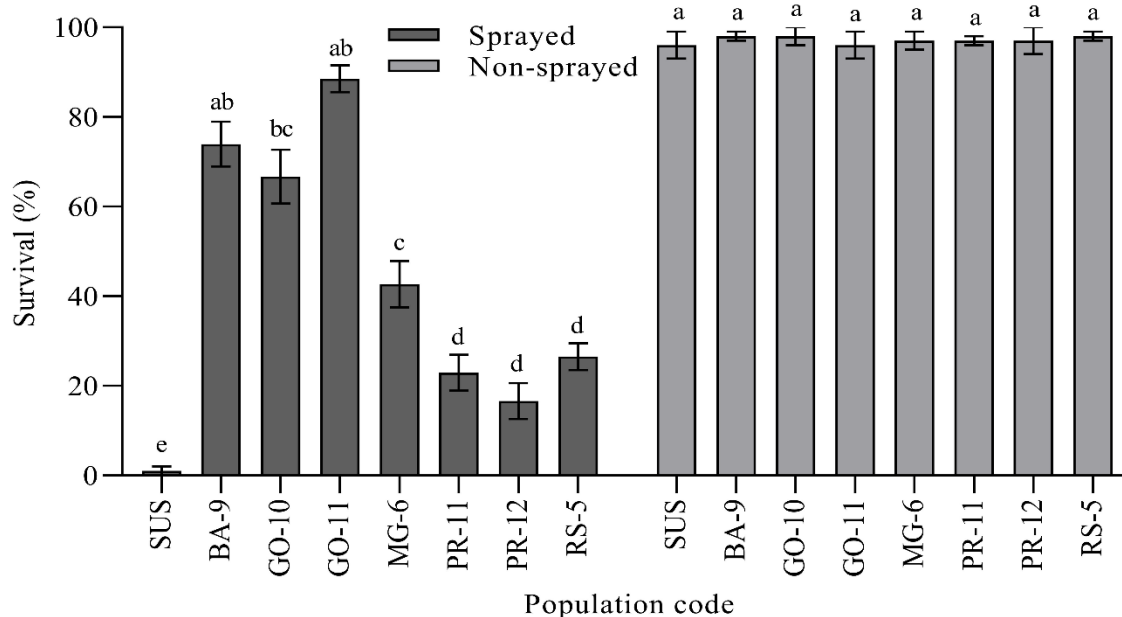


**Figure 3.** Variation in temporal susceptibility of Brazilian *Spodoptera frugiperda* populations exposed to a diagnostic concentration of emamectin benzoate ( $3.2 \mu\text{g a.i. mL}^{-1}$ ). Box plots with the same letter are not significantly different by non-overlapping 95% CIs.

### 2.3.5. Survival rate on non-Bt maize leaves sprayed with emamectin benzoate

There was a significant interaction between the *S. frugiperda* population  $\times$  maize treatment (sprayed and non-sprayed) for the percentage of survival ( $df = 7$ ;  $F = 352.25$ ;  $P < 0.001$ ). The main effects were also significant for population ( $df = 7$ ;  $F = 13.13$ ;  $P < 0.001$ ) and maize treatment ( $df = 1$ ;  $F = 5.47$ ;  $P < 0.001$ ). The *S. frugiperda* populations from Bahia (BA) and Goiás (GO) had a significantly high larval survival (66.6 to 88.5%) on maize leaves sprayed with the field rate of emamectin benzoate compared to populations from Minas Gerais (MG), Paraná (PR), and Rio Grande do Sul (RS) (survival from 16.6 to 42.7%) (Figure 4). All field populations showed higher survival on maize leaves treated with emamectin benzoate than the susceptible population (0.2% survival). On non-sprayed maize leaves, all populations presented similar survival rate (96 to 98%). These results provide robust evidence of a genetically based decrease in susceptibility to emamectin benzoate in *S. frugiperda* populations from Brazil, indicating that control failures are likely to occur in the field.





**Figure 4.** Brazilian *Spodoptera frugiperda* populations collected in 2021 and exposed to non-Bt maize leaves sprayed and non-sprayed with the field rate of emamectin benzoate. Bars ( $\pm$  SE) with the same letter are not significantly different ( $P > 0.05$ ).

## 2.4. Discussion

Understanding the susceptibility of target pests to insecticides is essential to support resistance monitoring programs and IRM practices. In this study, the baseline susceptibility was established, and a diagnostic concentration of emamectin benzoate was defined for Brazilian *S. frugiperda* populations collected in 2002, and in subsequent years, resistance was monitored. From 2002 to 2004, a similar susceptibility to emamectin benzoate was verified in field *S. frugiperda* populations, with resistance ratios  $< 2.2$ -fold and a survival rate at the diagnostic concentration of  $< 1.79\%$ . A low resistance ratio to emamectin benzoate was also reported in *S. frugiperda* populations from Puerto Rico (7.0-fold) (Gutiérrez-Moreno et al., 2019), *Spodoptera litura* (Lepidoptera: Noctuidae) in Pakistan ( $< 4.8$ -fold) (Rehan et al., 2011), and *H. armigera* and *Helicoverpa punctigera* (Lepidoptera: Noctuidae) in Australia ( $< 2.3$ -fold) (Bird 2015; Bird and Walker 2019). In Brazil, a laboratory-selected resistant strain of *S. frugiperda* to emamectin benzoate showed resistance ratio  $> 2,000$ -fold (Muraro et al., 2021).

The susceptibility of Brazilian populations of *S. frugiperda* to emamectin benzoate significantly decreased from 2019 to 2021 when compared with its susceptibility verified 20

years ago. In recent years, the resistance ratios to emamectin benzoate increased significantly, reaching up to 632-fold, and larval survival at a diagnostic concentration reached 81.4% survival. Previous studies also documented a high resistance ratio to emamectin benzoate in field populations of *P. xylostella* from the United States (60.5-fold) (Zhao et al., 2006), *S. litura* from Pakistan (80.0-fold) (Shad et al., 2010), *S. exigua* from China (36.0-fold) (Che et al., 2015), and *H. armigera* from Pakistan (52.0-fold) (Ahmad et al., 2019).

Our results also indicated that, over 20 years, the susceptibility to emamectin benzoate decreased more strongly in *S. frugiperda* populations from central Brazil, mainly in Bahia, Goiás, Mato Grosso, and Mato Grosso do Sul. However, the same trend seemed to occur for *S. frugiperda* populations from Paraná, São Paulo, and Rio Grande do Sul. In central Brazil, favorable climatic conditions and an intensive agricultural system favor the survival and rapid infestation of the *S. frugiperda* in cultivated hosts (e.g., maize, cotton, sorghum, soybean, and millet), increasing the use of insecticides, including applications of emamectin benzoate for its control. The environmental conditions and agricultural practices in these locations also contributed to increasing the frequency of resistance in *S. frugiperda* populations to chlorantraniliprole, organophosphates, carbamates (Boaventura et al., 2020 a, b), and Bt maize products that express Cry1F and Cry1Ab toxins (Farias et al., 2014, 2016; Omoto et al., 2016).

Field populations of *S. frugiperda* collected in 2021 were also exposed to maize leaves sprayed with the field rate of emamectin benzoate. In all populations, a relative high survival rate, ranging from 16.63 to 87.5%, was found. These results confirmed the shifts in the susceptibility to emamectin benzoate detected in concentration-mortality curves (increasing in  $LC_{50}$  values) and at a diagnostic concentration (increasing in larval survival rate). Overall, these findings indicated a genetically based decrease in the susceptibility to emamectin benzoate as a response to its intensive use for *S. frugiperda* control, providing robust evidence of field-evolved resistance in field populations of this pest in Brazil. This study corroborates with the high risk of resistance evolution to this insecticide based on incomplete dominant inheritance (Muraro et al., 2021).

The present study reports the evidence of field-evolved resistance to emamectin benzoate in the *S. frugiperda* in Brazil. This finding highlights the importance of considering other control tactics for managing the *S. frugiperda* and the rotation of insecticides with different modes of action as one of the principal IRM strategies to delay resistance to insecticides, mainly in an intensive agricultural system, such as that in Brazil, where resistance is already widespread.

## 2.5. Conclusions

- A significant shift towards decrease in the susceptibility to emamectin benzoate was detected in *Spodoptera frugiperda* populations from 2002 to 2021.
- Field-evolved resistance to emamectin benzoate was confirmed in *Spodoptera frugiperda* populations in Brazil.

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### 3. INHERITANCE PATTERNS, CROSS-RESISTANCE AND SYNERGISM IN *Spodoptera frugiperda* (LEPIDOPTERA: NOCTUIDAE) RESISTANT TO EMAMECTIN BENZOATE

#### Abstract

Fall armyworm, *Spodoptera frugiperda* (J. E. Smith), is a relevant global pest due to severe damage caused on agricultural crops and its capacity to evolve resistance to insecticides. Here, we selected a strain of *S. frugiperda* resistant to emamectin benzoate under laboratory conditions to understand the inheritance patterns, cross-resistance and synergism involved in the resistance. The emamectin benzoate-resistant (Ben-R) strain was isolated by using F<sub>2</sub> screen in a field population collected in Lucas do Rio Verde, Mato Grosso state, Brazil. After 10 generations of selection pressure with emamectin benzoate, the estimated LC<sub>50</sub> of the Ben-R strain was 678.38 µg a.i. mL<sup>-1</sup> whereas of the susceptible (Sus) strain was 0.29 µg a.i. mL<sup>-1</sup>, resulting in a resistance ratio of ~2,340-fold. The LC<sub>50</sub> values of the offspring from reciprocal crosses of Sus and Ben-R strains were 93.37 and 105.32 µg a.i. mL<sup>-1</sup>, suggesting that resistance is autosomal incompletely dominant trait. The high survival of heterozygous and Ben-R strains (> 92%) on non-Bt maize sprayed with the field rate of emamectin benzoate confirmed that resistance is functionally dominant. The minimum number of segregations influencing resistance was 3.55, suggesting a polygenic effect. Low cross-resistance was detected between emamectin benzoate and the insecticides methomyl, chlorpyrifos, lambda-cyhalothrin, spinetoram, indoxacarb and chlorantraniliprole (resistance ratio < 5.75-fold). There was no effect of synergists piperonyl butoxide, diethyl maleate and S, S, S-tributyl phosphorotrithiotate on Ben-R strain, suggesting a minor role of metabolic resistance. Our results showed a high risk of resistance evolution of *S. frugiperda* to emamectin benzoate, based on incompletely dominant inheritance. Rotation of insecticides with different mode of action can be one of the resistance management strategies to be implemented to delay the evolution of resistance of *S. frugiperda* to emamectin benzoate in Brazil.

Keywords: Fall armyworm; Inheritance pattern; Synergist; Resistance management

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#### 3.1. Introduction

The evolution of insect resistance to insecticides represents a great threat to integrated pest management (IPM) programs (Bass et al., 2015; Hawkins et al., 2019). Cases of



resistance have increased worldwide since the discovery of the first insecticide molecules, causing economic losses and environmental and social impacts on agricultural production systems (Sparks et al., 2015). Conversely, the discovery of insecticides with new mode of action to deploying insect resistance management (IRM) programs has decreased, and the resources and time have increased considerably (Sparks et al., 2013; Sparks et al., 2017). Therefore, the implementation of effective IRM strategies is necessary to preserve the effectiveness of old and new chemistries.

Fall armyworm, *Spodoptera frugiperda* (J. E. Smith, 1797) (Lepidoptera: Noctuidae), has high capacity to evolve resistance to insecticides and transgenic plants expressing *Bacillus thuringiensis* Berliner (Bt) proteins. According to Arthropod Pesticide Resistance Database, there are more than 140 reported cases of resistance of *S. frugiperda* to 40 different active ingredients. This species has also evolved resistance to several Bt maize technologies (Storer et al., 2010; Farias et al., 2014; Huang et al., 2014; Omoto et al., 2015; Chandrasena et al., 2018). This insect pest is native of the Americas, but it was recently reported as invasive pest in Africa (Goergen et al., 2016; Day et al., 2017), Asia (Kallethwaraswamy et al., 2018; Sidana et al., 2018; Gilal et al., 2020; Jing et al., 2020) and Oceania (Piggott et al., 2021), becoming a pest of global relevance.

Among the insecticides registered to control *S. frugiperda* in Brazil, emamectin benzoate – glutamate-gated chloride channel allosteric modulator – is an active ingredient used since 2017, although this insecticide has been registered to control *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) since 2014. So far, no cases of resistance to emamectin benzoate were reported in *S. frugiperda* worldwide. However, other species of *Spodoptera* genus has evolved resistance to this active ingredient, such as in *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) and *Spodoptera litura* (F.) (Lepidoptera: Noctuidae) in China and Pakistan (Su et al., 2014; Ishtiaq et al., 2014; Saleem et al., 2016; Wang et al., 2018; Wang et al., 2019). The resistance to emamectin benzoate was also documented in other Lepidoptera species such as in *H. armigera* in Pakistan (Qayyum et al., 2015), and *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) in Australia, United States, Mexico, and Pakistan (Shelton et al., 2000; Sayyed et al., 2005; Rahman et al., 2010; Zhao et al., 2016).

In Brazil, resistance of *S. frugiperda* has already been reported to lambda-cyhalothrin (Diez-Rodríguez et al., 2001), chlorpyrifos (Carvalho et al., 2001; Garlet et al., 2021), lufenuron (Nascimento et al., 2016), spinosad (Okuma et al., 2018), chlorantraniliprole (Bolzan et al., 2019), spinetoram (Lira et al., 2021), teflubenzuron (Nascimento et al., 2021), and Bt proteins Cry1F (Farias et al., 2014), and Cry1Ab (Omoto et al., 2016). Given the

history of resistance of *S. frugiperda* to insecticides and Bt plants in the current Brazilian crop production system, it is important to understand the risk of resistance evolution of *S. frugiperda* to emamectin benzoate in order to improve IPM and IRM programs. Based on this, we initially conducted selection studies to isolate a strain of *S. frugiperda* resistant to emamectin benzoate from a field-collected population. Then, we characterized the inheritance of resistance, evaluated cross-resistance to other insecticides and tested some synergists.

## **3.2. Material and Methods**

### **3.2.1. Selection of *S. frugiperda* resistant to emamectin benzoate**

The *S. frugiperda* strain resistant to emamectin benzoate was selected from a field-collected population in non-Bt maize in Lucas do Rio Verde, Mato Grosso, Brazil (12°55'36" S and 56°03'13" W) in 2019. The F<sub>2</sub> screen technique developed by Andow and Alstad (1998) was used to select resistant individuals to emamectin benzoate. We initially separated field adults into pairs (each pair represent an isofamily) and with the F<sub>1</sub> generation progeny, endogamic crosses were performed to obtain the F<sub>2</sub> progeny of each isofamily. Third-instar larvae from each isofamily (F<sub>2</sub> generation) were tested with a high concentration of emamectin benzoate (320 µg a.i. mL<sup>-1</sup>) in diet-overlay bioassays described below. After 96 h post-exposure, isofamilies that presented survival rate higher than 50% was considered positive for resistance. All larvae from the positive isofamilies were transferred to 50-mL plastic cups containing 20 mL of artificial diet, proposed by Kasten et al. (1978). Then, all emerged adults were grouped for further selection with emamectin benzoate. From F<sub>3</sub> to F<sub>10</sub> generations, larvae were exposed to selection at 1,000 µg a.i. mL<sup>-1</sup> of emamectin benzoate to produce our emamectin benzoate-resistant (Ben-R) strain.

### **3.2.2. Bioassays**

Diet-overlay bioassays were conducted to select and evaluate resistance patterns. The bioassays were performed in 24-well acrylic plates (Costar<sup>®</sup>, Sigma-Aldrich Ltda, São Paulo, Brazil), containing approximately 1 mL of artificial diet, proposed by Kasten et al. (1978) in each well. The diet surface area per well was 1.90 cm<sup>2</sup>. Concentrations of emamectin benzoate (Proclaim<sup>®</sup> 50 g a.i. (active ingredient) kg<sup>-1</sup>, Syngenta Crop Protection, São Paulo,

Brazil) were diluted in water and a nonionic surfactant (Triton X-100, Sigma-Aldrich Brazil Ltda) at 0.1% was added in each concentration to spread the solution over the diet surface. The control treatment consisted of water + surfactant. A 30  $\mu\text{L}$  of different emamectin benzoate concentrations was applied on diet surface in each well using an electronic micropipette (Repetman<sup>®</sup>, Gilson, Illinois, US). After drying, a single third-instar larva was added in each well. The acrylic plates were closed and placed on a chamber at  $25 \pm 2^\circ\text{C}$ ,  $70 \pm 10\%$  RH and a 14:10 h photoperiod (light: dark). The mortality was evaluated at 96 h after infestation, and larvae without coordinated movement when touched with brush were considered dead.

### 3.2.3. Characterization of *S. frugiperda* resistance to emamectin benzoate

To characterize the *S. frugiperda* resistance to emamectin benzoate, we used the population obtained from Embrapa Milho e Sorgo (Sete Lagoas, State of Minas Gerais, Brazil) as our susceptible (Sus) strain. This population has been maintained since 1996 in laboratory conditions, without selection pressure by insecticides or Bt proteins. Third instar larvae from Sus and Ben-R strains were subjected to diet-overlay bioassays as previously described. Six to eight concentrations of emamectin benzoate were used to obtain concentration-mortality curves of the Sus ( $0.18$  to  $1.8 \mu\text{g a.i. mL}^{-1}$ ) and Ben-R ( $100$  to  $3,200 \mu\text{g a.i. mL}^{-1}$ ) strains. To convert concentrations expressed as  $\mu\text{g mL}^{-1}$  to  $\mu\text{g cm}^{-2}$  of artificial diet, the values in  $\mu\text{g mL}^{-1}$  should be divided by 64. The experimental design was completely randomized with four to five replicates ( $24$  larvae replicate<sup>-1</sup>), totaling 96 to 120 larvae tested concentration<sup>-1</sup>. Mortality was assessed at 96 h after insecticide exposure. Larvae that did not show coordinated movement were considered dead. A generalized linear model (GLM) for binomial or quasibinomial distributions with probit link function were adjusted to analyze the concentration-mortality data. We used a half-normal plot with a simulated envelope with the hnp package to assess the goodness-of-fit of the model (Moral et al., 2017). Then, concentration-mortality data were submitted to Probit analysis to estimate  $\text{LC}_{50}$  (concentration of insecticide required to kill 50% of larvae tested) values (Finney, 1953), confidence intervals (95% CIs) and slope of log concentration-mortality regression lines using the R software (R Development Core Team, 2017). Resistance ratios (RR) was determined by dividing  $\text{LC}_{50}$  value of Ben-R strain by the  $\text{LC}_{50}$  value of the Sus strain. Tests for parallelism

and equality were also performed to compare the angular and linear coefficients of the regression lines (Robertson et al., 2007).

### 3.2.4. Degree of dominance of the emamectin benzoate resistance in *S. frugiperda*

To perform this study, reciprocal crosses (20 pairs cross<sup>-1</sup>) were performed to obtain the heterozygous strains: Ben-R♀ × Sus♂ and Ben-R♂ × Sus♀. The progenies of reciprocal crosses (F<sub>1</sub> generation) were maintained in artificial diet until the third instar (Kasten et al., 1978). Subsequently, diet-overlay bioassays were performed using Ben-R, Sus and heterozygous strains, as previous described.

The concentration–mortality data of Sus, Ben-R and heterozygous were used to estimate the dominance degree of resistance, using the method proposed by Bourguet et al. (2000) (Equation 1):

$$D_{ML} = (ML_H - ML_{SS}) / (ML_{RR} - ML_{SS}) \quad (1)$$

where,  $ML_H$ ,  $ML_{SS}$ , and  $ML_{RR}$  are the mortalities of the heterozygous, Sus and Ben-R strains, respectively. Values for  $D_{ML}$  close to 0 indicate resistance effectively recessive, whereas values close to 1 indicate resistance effectively dominant, and intermediate values indicate effectively incomplete.

The degree of dominance ( $D_{LC}$ ) was also determined by Stone (1968) method, which uses the LC<sub>50</sub> values of emamectin benzoate against heterozygous, Sus and Ben-R strains estimated by Probit analysis. For this estimate were used the Equation 2:

$$D_{LC} = (2\log LC_H - \log LC_R - \log LC_S) / (\log LC_R - \log LC_S) \quad (2)$$

where  $LC_H$ ,  $LC_R$  and  $LC_S$  are the LC<sub>50</sub> of heterozygous, Ben-R and Sus strains, respectively.  $D_{LC} = 1$  indicates complete dominance,  $0 < D_{LC} < 1$  indicates incomplete dominance,  $-1 < D_{LC} < 0$  indicates incompletely recessive, and  $D_{LC} = -1$  indicates completely recessive.

### 3.2.5. Number of genes involved in emamectin benzoate resistance in *S. frugiperda*

To quantify genes associated with emamectin benzoate resistance in *S. frugiperda* it was used the methods described by Tsukamoto (1983) and Roush and Daly (1990). Heterozygous were backcrossed (30 pairs backcross<sup>-1</sup>) with the Sus strain in PVC cages (20 cm diameter × 10 cm high). The backcross performed were:  $R_1 = \text{♀Sus} \times (\text{Ben-R♀} \times \text{Sus♂})$ ;

$R_2 = \text{♂Sus} \times (\text{Ben-R♀} \times \text{Sus♂})$ ;  $R_3 = \text{♀Sus} \times (\text{Ben-R♂} \times \text{Sus♀})$ ;  $R_4 = \text{♂Sus} \times (\text{Ben-R♂} \times \text{Sus♀})$ . Then, third instar larvae from these backcrosses were exposed to diet-overlay bioassays as described above. Mortality data were used to test the hypothesis of monogenic inheritance, using the chi-square ( $\chi^2$ ) test proposed by Sokal and Rohlf (1995) (Equation 3):

$$\chi^2 = ((Ni - pni)^2)/pqni \quad (3)$$

where  $Ni$  is the mortality observed for the backcrossing progeny for concentration  $i$  and  $p$  is the expected proportion of mortality calculated from the Mendelian model proposed by Georghiou (1977) (Equation 4):

$$p = (a + b)/2 \quad (4)$$

where  $a$  is the mortality in the parental strain used,  $b$  is the mortality of the heterozygous derived from the reciprocal crosses,  $ni$  is the number of individuals tested, and  $q = 1 - p$ . The hypothesis of monogenic inheritance was rejected when calculated  $\chi^2 \geq$  tabulated  $\chi^2$  with one degree of freedom ( $P < 0.05$ ). The number of loci associated with resistance was also estimated using the method proposed by Lande (1981) (Equation 5):

$$n_E = (\log_{RR} - \log_{SS})^2 / (8\sigma_S^2) \quad (5)$$

where  $RR$  and  $SS$  are the  $LC_{50}$  of the Ben-R and Sus strains respectively,  $\sigma_S^2 = \sigma_{B_1}^2 + \sigma_{B_2}^2 - [\sigma_H^2 + 0.5\sigma_{RR}^2 + 0.5\sigma_{SS}^2]$ ,  $\sigma_{B_1}^2$ ,  $\sigma_{B_2}^2$ ,  $\sigma_H^2$ ,  $\sigma_{RR}^2$  and  $\sigma_{SS}^2$  correspond to the phenotypic variances of the heterozygous, Ben-R and Sus strains respectively, estimated by the inverse of the slope squared.

### 3.2.6. Functional dominance of *S. frugiperda* resistance to emamectin benzoate

In this study, non-Bt maize seeds (3700RR2, Agrocere, Rio Claro, SP, Brazil) were sown in 6-L plastic pots (two seeds pot<sup>-1</sup>) and maintained in a greenhouse. At V<sub>4</sub> growth stage, plants were sprayed with emamectin benzoate (Proclain<sup>®</sup> 50 g a.i. kg<sup>-1</sup>) at the recommended field rate of 15 g a.i. ha<sup>-1</sup> diluted in 150 L of water. The control treatment consisted of unsprayed plants. After drying, leaves were removed from the maize whorls and placed on a 3% mixture of water-agar (15 mL well<sup>-1</sup>) in plastic plates (CM&CM Comércio de Plásticos, São Paulo, SP, Brazil) containing 16 wells. Maize leaves were separated from the water-agar layer by a filter paper. Then, a single third-instar larva was added on each well. A total of 160 larvae strain<sup>-1</sup> were tested for each treatment (each replicate consisted of 16 larvae). Plates were sealed and placed in a chamber at 25 ± 2°C, 70 ± 10% RH and a 14:10 h (light: dark) photoperiod. The experimental design was completely randomized and

distributed in a  $4 \times 2$  factorial design. The first factor (A) was represented by Ben-R, heterozygous ( $\text{Ben-R}^{\ominus} \times \text{Sus}^{\ominus}$  and  $\text{Ben-R}^{\ominus} \times \text{Sus}^{\ominus}$ ) and Sus strains. The second factor (B) was represented by maize plants sprayed with emamectin benzoate and unsprayed plants. Larval survival was evaluated 96 h after insecticide exposure. A GLM for quasibinomial distribution was adjusted to analyze the data on larval survival. Good adjustment of the data to the model was obtained by using an hnp package with a simulated envelope (Moral et al., 2017). To assess the occurrence of a significant interaction between factors, a deviance analysis ( $P = 0.05$ ) was performed. Statistical differences were determined with the Tukey's honestly significant difference test (HSD). All analyses were performed using the R software (R Development Core Team, 2017).

### 3.2.7. Cross-resistance between emamectin benzoate and other insecticides

To assess patterns of cross-resistance, third-instar larvae from Ben-R and Sus strains were exposed to insecticides with different modes of action. In diet-overlay bioassays as described above, the insecticides lambda-cyhalothrin (Kaiso<sup>®</sup>, 250 g a.i. L<sup>-1</sup>, Nufarm A/S, Maracanaú, CE, Brazil), spinetoram (Exalt<sup>®</sup>, 120 g a.i. L<sup>-1</sup>, Dow AgroSciences Industrial Ltda., São Paulo, SP, Brazil), indoxacarb (Avatar<sup>®</sup>, 150 g a.i. L<sup>-1</sup>, FMC, São Paulo, SP, Brazil) and chlorantraniliprole (Premio<sup>®</sup>, 200 g a.i. L<sup>-1</sup>, FMC, São Paulo, SP, Brazil) were tested. Using topical bioassays, we also test methomyl (Lannate<sup>®</sup>, 215 g a.i. L<sup>-1</sup>, FMC, São Paulo, SP, Brazil) and chlorpyrifos (Lorsban<sup>®</sup>, 480 g a.i. L<sup>-1</sup>, Dow AgroSciences Industrial Ltda., São Paulo, SP, Brazil). Topical bioassays consist of diluted the technical grade insecticide in acetone (99.5% purity; Sigma-Aldrich, São Paulo, SP, Brazil) and then applied 1  $\mu\text{L}$  of the solution on the pronotum of third instar larvae. Concentration-mortality data were submitted to the same statistical procedures described in inheritance resistance study.

### 3.2.8. Synergist bioassays

Bioassays with the synergists piperonyl butoxide (PBO, 90% purity, Sigma Aldrich, São Paulo, SP, Brazil), diethyl maleate (DEM, 97% purity, Sigma Aldrich, São Paulo, SP, Brazil) and S, S, S-tributyl phosphorotrithiotate (DEF, 97.2% purity, Chem Service, São Paulo, SP, Brazil) were carried out to investigate possible metabolic resistance involved in the resistance to emamectin benzoate in *S. frugiperda*. Initially, synergists were diluted in acetone

(99.5% purity, Sigma Aldrich, São Paulo, SP, Brazil) at concentrations of 0.1, 1.0 and 0.32  $\mu\text{g larva}^{-1}$  of PBO, DEM and DEF, respectively, and then applied topically (1  $\mu\text{L larva}^{-1}$ ) in the pronotum of third-instar larvae. The control treatment was composed by larvae treated with 1  $\mu\text{L}$  acetone only. Two-hour post-exposure to the synergists, larvae were exposed to different concentrations of emamectin benzoate in diet-overlay bioassays as previously described. The larval mortality was evaluated at 96 h after exposure to insecticide. Concentration-mortality data were submitted to the same statistical procedures described in the inheritance of resistance study.

### 3.3. Results

#### 3.3.1. Selection of *S. frugiperda* resistant to emamectin benzoate

The  $\text{LC}_{50}$  value of emamectin benzoate of the field-collected population of *S. frugiperda* after one generation in laboratory was 4.64  $\mu\text{g a.i. mL}^{-1}$  (Table 1), whereas for the Sus strain was 0.25  $\mu\text{g a.i. mL}^{-1}$ . These values indicate that the field population of *S. frugiperda* presented a resistance ratio to emamectin benzoate of 18.5-fold. Using the  $F_2$  screen method described to Andow and Alstad (1998) to identify larvae capable of surviving at a high concentration of emamectin benzoate (320  $\mu\text{g a.i. mL}^{-1}$ ); a total of 62 isofamilies (~7740 larvae tested) were obtained from the field population mentioned above. Resistance alleles were found in 32 isofamilies (~2280 larvae survived at 96 h and originated more than 1550 adults). These adults were used to establish the emamectin benzoate-resistant (Ben-R) strain. The progeny of these adults (now  $F_3$  generation) presented  $\text{LC}_{50}$  value of emamectin benzoate of 255.91  $\mu\text{g a.i. mL}^{-1}$ , indicating an increase of resistance ratio of 1,023.6-fold relative to the Sus strain. From  $F_4$  to  $F_{10}$  generation, the concentration of emamectin benzoate used in the selection process was increased to 1,000  $\mu\text{g a.i. mL}^{-1}$ , and this also increased significantly the  $\text{LC}_{50}$  of emamectin benzoate against Ben-R strain, which ranged from 380.41 ( $F_4$  generation) to 570.86  $\mu\text{g a.i. mL}^{-1}$  ( $F_{10}$  generation), resulting in a resistance ratio > 2,280-fold at  $F_{10}$  generation (Table 1).

**Table 1.** Response to selection by *Spodoptera frugiperda* exposed to emamectin benzoate in diet-overlay bioassays during 10 generations.

<i>S. frugiperda</i> strain (generation)	<i>n</i>	Fit of probit lines			LC <sub>50</sub> (95% CI) <sup>b</sup>	RR <sub>50</sub> <sup>c</sup>
		Slope ± SE	χ <sup>2</sup> ( <i>df</i> )	<i>P</i>		
Sus	742	2.83 ± 0.30	9.52 (4)	0.32	0.25 (0.19–0.32)	-
Ben-R (F <sub>1</sub> )	990	1.30 ± 0.09	10.31 (8)	0.38	4.64 (3.76–5.65)	18.5
Ben-R (F <sub>3</sub> )	978	2.51 ± 0.25	4.77 (4)	0.31	255.91 (214.53–293.25)	1023.64
Ben-R (F <sub>4</sub> )	686	3.31 ± 0.31	5.26 (4)	0.26	380.41 (340.38–422.43)	1521.70
Ben-R (F <sub>5</sub> )	841	3.01 ± 0.27	6.92 (5)	0.22	418.66 (368.25–427.59)	1674.60
Ben-R (F <sub>6</sub> )	866	2.68 ± 0.24	6.54 (5)	0.26	442.06 (386.52–496.53)	1768.24
Ben-R (F <sub>7</sub> )	864	2.41 ± 0.21	7.42 (6)	0.28	593.73 (511.50–576.59)	2374.91
Ben-R (F <sub>8</sub> )	504	2.02 ± 0.40	8.43 (5)	0.18	619.40 (238.41–656.92)	2477.60
Ben-R (F <sub>9</sub> )	722	2.42 ± 0.31	8.32 (6)	0.22	562.22 (422.22–622.23)	2248.88
Ben-R (F <sub>10</sub> )	896	2.22 ± 0.32	6.92 (6)	0.32	570.86 (412.96–552.94)	2283.44

<sup>a</sup>*df* = degrees of freedom.

<sup>b</sup>LC<sub>50</sub> values (µg a.i. mL<sup>-1</sup>) followed by the same letter do not differ significantly due to non-overlap of 95% confidence intervals (CIs).

<sup>c</sup>Resistance Ratio (RR) = LC<sub>50</sub> of resistant strain/LC<sub>50</sub> of susceptible strain.

### 3.3.2. Characterization of *S. frugiperda* resistance to emamectin benzoate

The LC<sub>50</sub> value of emamectin benzoate against third-instar larvae of Ben-R strain at F<sub>11</sub> generation post-selection was 678.38 µg a.i. mL<sup>-1</sup>, while for the Sus strain was 0.29 µg a.i. mL<sup>-1</sup>, resulting in a maximum resistance ratio of 2,339.2-fold (Table 2). Third-instar larvae from reciprocal crosses (heterozygous) had similar response to emamectin benzoate with LC<sub>50</sub> values ranging from 93.37 to 105.32 µg a.i. mL<sup>-1</sup>, indicating resistance ratios of 321.96 and 363.17-fold, based on Sus strain (Table 2). All *S. frugiperda* strains presented a non-significant chi-square in the goodness-of-fit test ( $P > 0.05$ ), suggesting that mortality data adjusted the Probit model. However, concentration-mortality curves of Ben-R, Sus, and heterozygous strains showed distinct parameters (slopes and/or intercepts) as indicated by equality ( $\chi^2 = 0.11$ ;  $df = 6$ ;  $P < 0.001$ ) and parallelism ( $\chi^2 = 69.63$ ;  $df = 3$ ;  $P < 0.001$ ) tests (Table 2 and Figure 1).



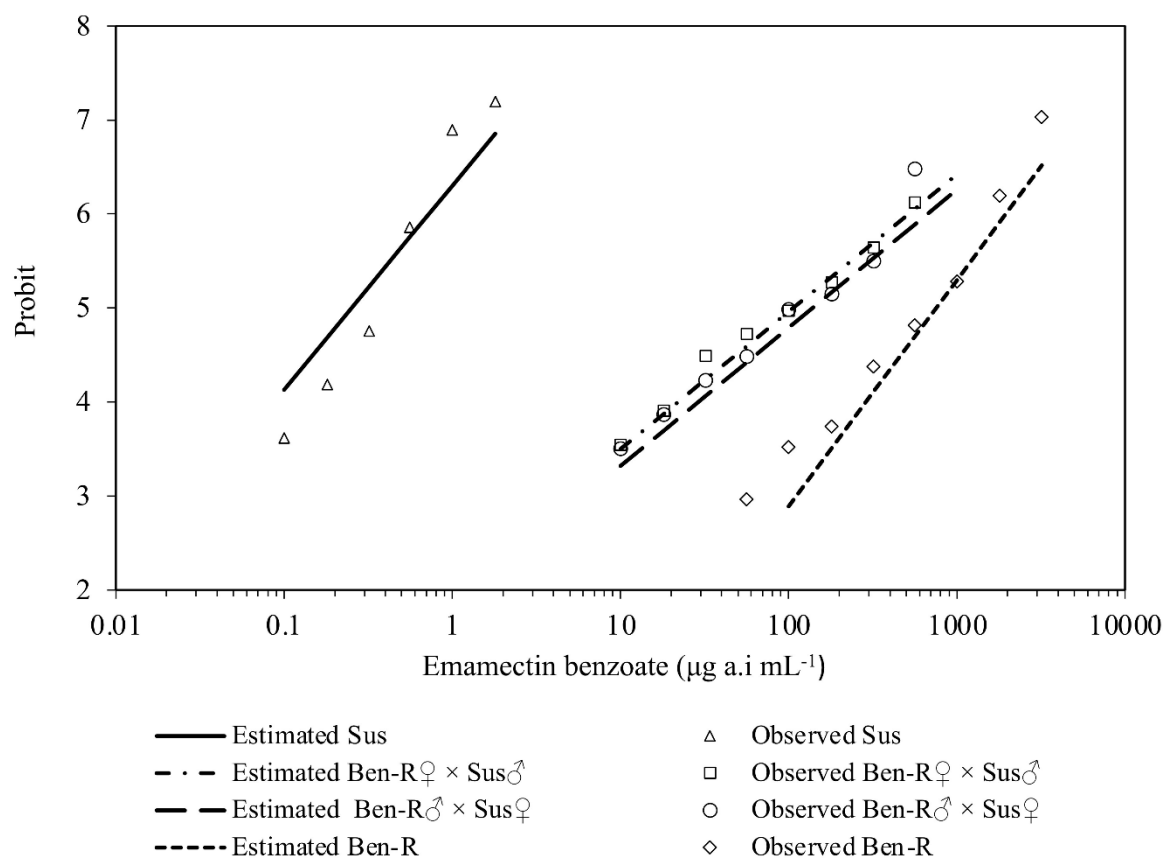
**Table 2.** Concentration-mortality ( $\mu\text{g a.i. mL}^{-1}$ ) to emamectin benzoate in susceptible (Sus), resistant (Ben-R), and  $F_1$  progenies of reciprocal crosses.

Strain	<i>n</i>	Fit of probit lines			LC <sub>50</sub> (95% CI) <sup>b</sup>	RR <sub>50</sub> <sup>c</sup>
		Slope $\pm$ SE	$\chi^2$ ( <i>df</i> )	<i>P</i>		
Sus	453	1.27 $\pm$ 0.16	6.55 (3)	0.08	0.29 (0.07–0.46) a	-
Ben-R	1008	2.41 $\pm$ 0.21	7.25 (6)	0.07	678.38 (495.25–841.0) c	2,339.2
Ben-R $\sigma$ $\times$ Sus $\phi$	1056	1.47 $\pm$ 0.20	13.80 (7)	0.06	105.32 (55.94–156.18) b	363.17
Ben-R $\phi$ $\times$ Sus $\sigma$	984	1.46 $\pm$ 0.23	7.44 (7)	0.38	93.37 (72.27–120.64) b	321.96

<sup>a</sup>*df* = degrees of freedom.

<sup>b</sup>LC<sub>50</sub> values ( $\mu\text{g a.i. mL}^{-1}$ ) followed by the same letter do not differ significantly due to non-overlap of 95% confidence intervals (CIs).

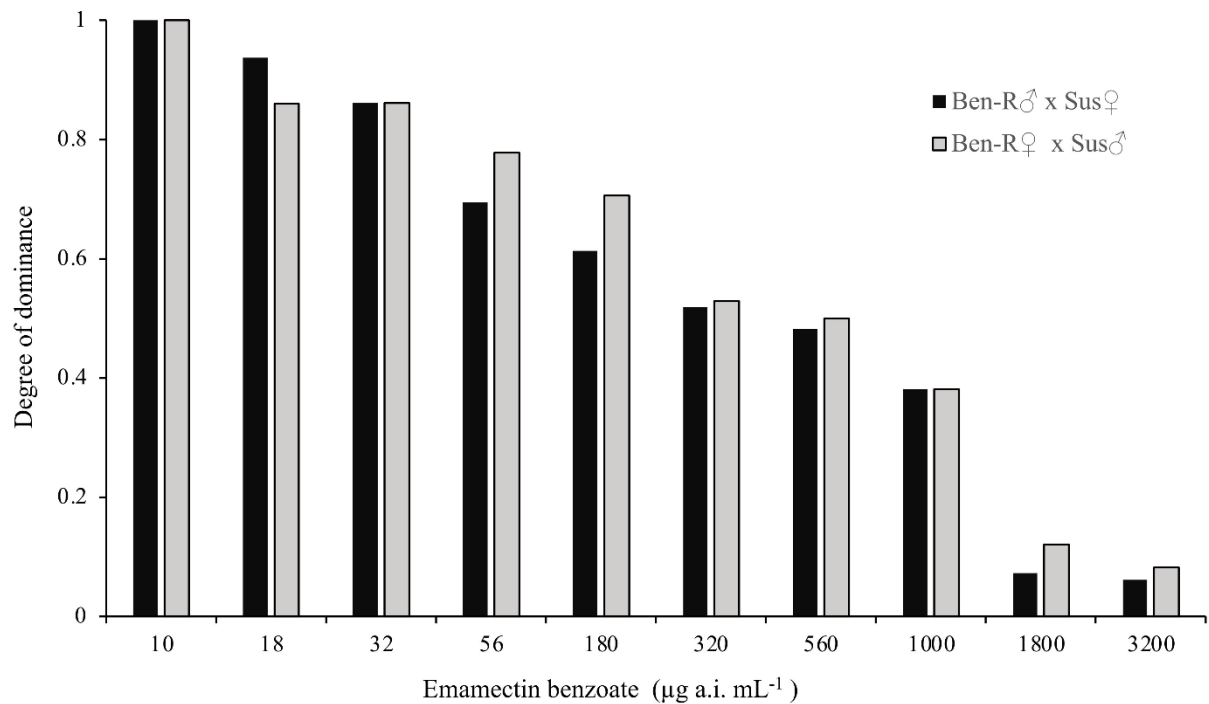
<sup>c</sup>Resistance Ratio (RR) = LC<sub>50</sub> of resistant strain or heterozygous/LC<sub>50</sub> of susceptible strain.

**Figure 1.** Mortality probability response of *Spodoptera frugiperda* strains to emamectin benzoate concentrations.

### 3.3.3. Inheritance of *S. frugiperda* resistance to emamectin benzoate

The mortality-response curves of heterozygous showed that the 95% confidence intervals (CIs) of the LC<sub>50</sub> values overlapped (Table 2 and Figure 1), suggesting that genes related to the resistance of *S. frugiperda* to emamectin benzoate might be in the autosomal regions of the karyotype. It was also observed that the dominance level ( $D_{ML}$ ) calculated

according to Bourguet et al. (2000). method decreased as the emamectin benzoate concentrations increased (Figure 2). In the concentrations of emamectin benzoate lowest than  $320 \mu\text{g a.i. mL}^{-1}$ , the resistance inheritance was incompletely dominant ( $D_{ML} = 0.51$  and  $0.52$ ), while in concentrations higher than  $560 \mu\text{g a.i. mL}^{-1}$  the inheritance pattern was incompletely recessive ( $D_{ML} = 0.48$  and  $0.50$ ). Close to the recommended field rate of emamectin benzoate against *S. frugiperda* ( $15 \text{ g a.i. ha}^{-1}$  diluted in  $150 \text{ L}$  of water – equivalent to approximately  $9.5 \mu\text{g a.i. mL}^{-1}$ ) the resistance can be considered a dominant trait ( $D_{ML} = 1$ ). The degree of dominance ( $D_{LC}$ ) by Stone's (1968) method also indicated that resistance to emamectin benzoate in *S. frugiperda* is incompletely dominant inherited, with  $D_{LC}$  values of  $0.49$  and  $0.52$  for progenies from  $\text{Ben-R}^{\sigma} \times \text{Sus}^{\text{f}}$  and  $\text{Ben-R}^{\text{f}} \times \text{Sus}^{\sigma}$ , respectively.



**Figure. 2.** Degree of dominance to emamectin benzoate in *Spodoptera frugiperda* as a function of concentration.

### 3.3.4. Number of genes influencing resistance of *S. frugiperda* to emamectin benzoate

Third-instar larvae of *S. frugiperda* from backcrosses exposed to distinct concentrations of emamectin benzoate showed a significant deviation between observed and expected mortality concentrations tested (Table 3). At the lowest concentrations, the

mortalities differed significantly by the chi-square test, indicating that resistance of *S. frugiperda* to emamectin benzoate is polygenic. However, in the highest concentrations, the observed and expected mortalities did not differ in most concentrations and the monogenic inheritance hypothesis was not rejected. The number of independent segregations was 3.55, suggesting that resistance is influenced by multiple genes.

**Table 3.** Chi-square analysis ( $\chi^2$ ) of the mortality data from the backcrosses between the susceptible strain (Sus) and F<sub>1</sub> progeny of the reciprocal crosses (H<sub>1</sub> = Ben-R<sub>♀</sub> × Sus<sub>♂</sub>; H<sub>2</sub> = Ben-R<sub>♂</sub> × Sus<sub>♀</sub>) exposed to different concentrations of emamectin benzoate.

Concentration ( $\mu\text{g a.i. mL}^{-1}$ )	R <sub>1</sub> = Sus <sub>♂</sub> × H <sub>1</sub> <sub>♀</sub>			R <sub>2</sub> = Sus <sub>♀</sub> × H <sub>1</sub> <sub>♂</sub>			R <sub>3</sub> = Sus <sub>♂</sub> × H <sub>2</sub> <sub>♀</sub>			R <sub>4</sub> = Sus <sub>♀</sub> × H <sub>2</sub> <sub>♂</sub>		
	Obs <sup>a</sup>	Exp <sup>b</sup>	$\chi^2$ <sup>c</sup>	Obs <sup>a</sup>	Exp <sup>b</sup>	$\chi^2$ <sup>c</sup>	Obs <sup>a</sup>	Exp <sup>b</sup>	$\chi^2$ <sup>c</sup>	Obs <sup>a</sup>	Exp <sup>b</sup>	$\chi^2$ <sup>c</sup>
1	28.2	48.5	12.9*	27.1	48.6	14.4*	25.0	48.5	17.7*	27.1	48.5	12.9*
1.8	28.2	50.0	15.0*	28.2	50.0	15.0*	30.3	50.0	12.0*	32.3	50.0	6.0
3.2	42.1	50.0	1.5	45.4	50.0	0.4	40.0	50.0	1.3	42.1	50.0	1.5
5.6	57.1	50.0	2.0	60.7	50.0	8.0*	50.0	50.0	0.1	52.9	50.0	0.5
10	50.0	53.1	0.1	49.3	53.1	0.3	57.1	54.0	0.7	61.0	54.0	2.1
18	55.7	56.9	0.0	57.1	56.9	0.1	68.9	55.0	8.5*	53.7	55.0	0.0
32	47.1	65.3	8.8*	62.8	65.3	0.1	60.4	61.1	0.0	61.4	61.1	0.1
56	68.6	70.0	0.0	65.7	70.0	0.7	61.6	65.4	0.4	59.3	65.4	1.1

<sup>a</sup>Observed mortality.

<sup>b</sup>Expected mortality, based on Mendelian inheritance.

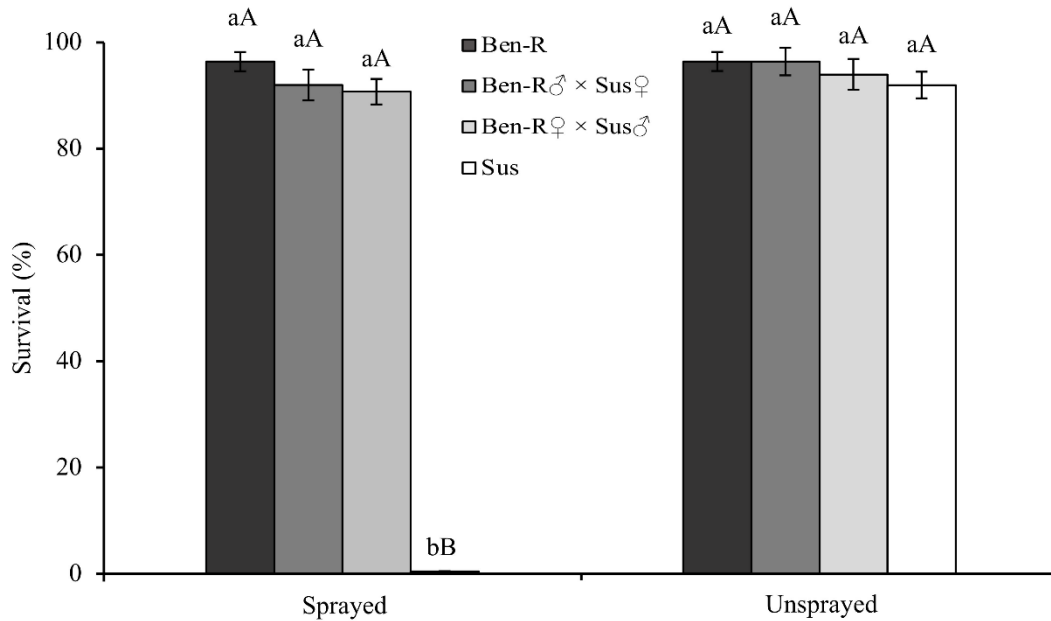
<sup>c</sup>Chi-square analysis.

\*Significant difference ( $P < 0.05$ ,  $df = 1$ ) between observed and expected mortalities.

### 3.3.5. Functional dominance of *S. frugiperda* resistance to emamectin benzoate

There was significant interaction between *S. frugiperda* strains (Ben-R, heterozygous and Sus) and maize leaves (sprayed and unsprayed) for the percentage of survival ( $df = 3$ ; deviance = 42.20;  $P < 0.001$ ). Significant effect was also observed for *S. frugiperda* strain ( $df = 3$ ; deviance = 409.27;  $P < 0.001$ ) and maize leaves source ( $df = 1$ ; deviance = 376.11;  $P < 0.001$ ). The Ben-R and heterozygous strains presented similar survival (>91%) on emamectin benzoate-sprayed leaves (Figure 3). In contrast, the Sus strain had a significantly lower survival (0.62%) than previous strains on maize leaves treated with emamectin benzoate ( $df = 3$ ; deviance = 602.56;  $P < 0.001$ ). On unsprayed maize leaves, all strains presented similar survival (>98%) ( $df = 3$ ; deviance = 0.685;  $P = 0.905$ ) (Fig. 3). The high survival of heterozygous on emamectin benzoate-sprayed leaves indicates that the resistance

in the field is an incompletely dominant trait ( $D_{ML} = 0.95$  according to Bourguet et al. (2000) method).



**Figure 3.** Larval survival of resistant (Ben-R), heterozygous (Ben-R♂ × Sus♀ and Ben-R♀ × Sus♂) and susceptible (Sus) strains in non-Bt maize leaves sprayed and unsprayed with emamectin benzoate. Groups of bars ( $\pm$  SE) with same lowercase letters in and bars with the same uppercase letters are not significantly different ( $P > 0.05$ ).

### 3.3.6. Cross-resistance between emamectin benzoate and other insecticides

The Ben-R strain exposed to distinct mode-of-action insecticides such as lambda-cyhalothrin, spinetoram, indoxacarb, chlorantraniliprole, methomyl and chlorpyrifos presented resistance ratios ranging from 1.4 to 5.7-fold, relative to the Sus strain, indicating low cross-resistance (Table 4). However, concentration-mortality curves of Ben-R and Sus had distinct intercepts, according to the results of equality (chlorantraniliprole:  $\chi^2 = 212.12$ ;  $df = 2$ ;  $P < 0.001$ , indoxacarb:  $\chi^2 = 220.25$ ;  $df = 2$ ;  $P < 0.001$ , methomyl:  $\chi^2 = 8.86$ ;  $df = 2$ ;  $P = 0.012$ ), lambda-cyhalothrin:  $\chi^2 = 27.71$ ;  $df = 2$ ;  $P < 0.001$ ; and spinetoram:  $\chi^2 = 11.99$ ;  $df = 2$ ;  $P < 0.001$ ) and intercepts and slopes according to parallelism (chlorantraniliprole:  $\chi^2 = 34.42$ ;  $df = 1$ ;  $P < 0.001$ ; and indoxacarb:  $\chi^2 = 7.36$ ;  $df = 1$ ;  $P < 0.001$ ) tests. In contrast, concentration-mortality curves of Ben-R and Sus strains had similar intercepts when exposed to chlorpyrifos ( $\chi^2 = 0.77$ ;  $df = 2$ ;  $P = 0.680$ ), and intercepts and slopes when exposed to methomyl ( $\chi^2 = 3.21$ ;

$df = 1$ ;  $P = 0.073$ ), lambda-cyhalothrin ( $\chi^2 = 0.99$ ;  $df = 1$ ;  $P = 0.319$ ), and chlorpyrifos ( $\chi^2 = 0.72$ ;  $df = 1$ ;  $P = 0.397$ ).

**Table 4.** Concentration-mortality response of resistant and susceptible *Spodoptera frugiperda* strains exposed to different insecticides.

<i>S. frugiperda</i> strains	<i>n</i>	Fit of probit lines			LC <sub>50</sub> (95% CI) <sup>c</sup>	RR <sub>50</sub> <sup>d</sup>
		Slope ± SE <sup>a</sup>	$\chi^2$ ( <i>df</i> <sup>b</sup> )	<i>P</i>		
<b>Methomyl</b> – Acetylcholinesterase inhibitor						
Ben-R	432	1.68 ± 0.15	7.22 (5)	0.20	16.03 (12.60–20.03) a	1.40
Sus	359	2.16 ± 0.22	2.26 (4)	0.68	11.37 (9.26–13.89) a	-
<b>Chlorpyrifos</b> – Acetylcholinesterase inhibitor						
Ben-R	504	2.07 ± 0.19	4.40 (4)	0.35	295.54 (248.06–346.53) a	1.16
Sus	648	2.29 ± 0.17	3.32 (5)	0.65	253.81 (223.81–335.47) a	-
<b>Lambda-cyhalothrin</b> – Sodium channel modulator						
Ben-R	576	2.36 ± 0.29	6.72 (5)	0.24	13.80 (11.36–16.27) a	2.60
Sus	648	3.64 ± 0.41	3.51 (4)	0.47	5.29 (4.59–5.92) b	-
<b>Spinetoram</b> – Nicotinic acetylcholine receptor allosteric modulator						
Ben-R	528	1.69 ± 0.22	1.62 (5)	0.89	12.03 (6.70–17.63) a	1.72
Sus	576	1.64 ± 0.17	3.05 (5)	0.69	6.99 (4.29–10.12) a	-
<b>Indoxacarb</b> – voltage dependent sodium channel blocker						
Ben-R	528	2.30 ± 0.25	3.42 (5)	0.63	22.04 (17.90–26.37) a	5.75
Sus	672	1.71 ± 0.24	10.88 (6)	0.09	3.83 (1.83–5.97) b	-
<b>Chlorantraniliprole</b> – ryanodine receptor modulator						
Ben-R	432	2.62 ± 0.37	3.03 (3)	0.38	96.70 (74.32–118.41) a	5.39
Sus	600	1.61 ± 0.21	3.80 (5)	0.57	17.93 (11.14–25.02) b	-

<sup>a</sup>*df* = degrees of freedom.

<sup>b</sup>LC<sub>50</sub> values (µg a.i. mL<sup>-1</sup>) followed by the same letter in for each Ben-R and Sus strains in each insecticide do not differ significantly for the confidence intervals (95% CIs).

<sup>c</sup>Resistance Ratio (RR) = LC<sub>50</sub> of resistant strain/LC<sub>50</sub> of susceptible strain.

### 3.3.1. Synergist bioassays

The LC<sub>50</sub> values of emamectin benzoate was similar for the Ben-R strain when previous exposed to the synergists PBO (845.36 µg a.i. mL<sup>-1</sup>), DEM (581.02 µg a.i. mL<sup>-1</sup>), and DEF (835.50 µg a.i. mL<sup>-1</sup>) and without exposure (951.69 µg a.i. mL<sup>-1</sup>), with a synergistic ratio <1.13-fold (Table 5). Similar results were observed for the Sus strain exposed to PBO (0.75 µg a.i. mL<sup>-1</sup>), DEM (0.73 µg a.i. mL<sup>-1</sup>), and DEF (1.03 µg a.i. mL<sup>-1</sup>). In addition,

concentration-mortality curves of the Ben-R and Sus strains also presented similar parameters when exposed and not exposed to synergists as indicated by equality (Ben-R:  $\chi^2 = 10.32$ ;  $df = 6$ ;  $P = 0.112$  and Sus:  $\chi^2 = 15.85$ ;  $df = 6$ ;  $P = 0.065$ ) and parallelism (Ben-R:  $\chi^2 = 4.87$ ;  $df = 3$ ;  $P = 0.182$  and Sus:  $\chi^2 = 2.09$ ;  $df = 3$ ;  $P = 0.560$ ) tests. These findings suggested a minor role for metabolic resistance to emamectin benzoate in *S. frugiperda*.

**Table 5.** Concentration-mortality of *Spodoptera frugiperda* strains exposed to emamectin benzoate and synergists.

Strains	Treatment	Fit of probit lines			LC <sub>50</sub> (95% CI) <sup>b</sup>	SR <sup>c</sup>
		Slope ± SE	$\chi^2$ (df <sup>a</sup> )	P		
Sus	Emamectin benzoate	1.86 ± 0.42	8.66 (4)	0.06	0.90 (0.28–1.68) a	-
	+ PBO	2.25 ± 0.31	7.68 (4)	0.16	0.75 (0.51–0.96) a	0.83
	+ DEF	2.24 ± 0.30	7.56 (4)	0.11	1.03 (0.83–1.27) a	1.14
	+ DEM	1.92 ± 0.22	6.53 (4)	0.10	0.73 (0.59–0.90) a	0.81
Ben-R	Emamectin benzoate	2.95 ± 0.48	5.32 (5)	0.37	951.69 (700.89–1167.0) a	-
	+ PBO	2.38 ± 0.44	7.14 (5)	0.21	845.36 (580.85–998.56) a	1.12
	+ DEF	2.68 ± 0.36	6.62 (5)	0.25	835.50 (664.07–996.94) a	1.13
	+ DEM	2.47 ± 0.36	8.44 (4)	0.07	581.02 (379.40–875.45) a	0.61

<sup>a</sup>df = degrees of freedom.

<sup>b</sup>LC<sub>50</sub> values ( $\mu\text{g a.i. mL}^{-1}$ ) followed by the same letter for each strain do not differ significantly due to non-overlap of 95% confidence intervals (CIs).

<sup>c</sup>Synergistic Ratio (SR) = LC<sub>50</sub> of emamectin benzoate without synergist/LC<sub>50</sub> of emamectin benzoate + synergist.

### 3.4. Discussion

A significant selection response to emamectin benzoate was observed in a field-collected population of *S. frugiperda* in this study. The resistance ratio increased from 18.5 to 1,023.64 after F<sub>2</sub> screening. After 10 generations of selection by increasing concentration of emamectin benzoate from 320  $\mu\text{g a.i. mL}^{-1}$  to 1,000  $\mu\text{g a.i. mL}^{-1}$ , the resistance ratio of emamectin benzoate-resistant (Ben-R) strain was >2,300-fold. The resistance ratio of *S. frugiperda* to emamectin benzoate detected in our study was greater than the ones reported in *P. xylostella* (80-fold) (Rahman et al., 2010), *S. litura* (730-fold) (Shad et al., 2010), *S. exigua* (1,110-fold) (Che et al., 2015), and *H. armigera* (94-fold) (Ahmad et al., 2019).

The inheritance of *S. frugiperda* resistance to emamectin benzoate is an autosomal incompletely dominant trait with polygenic effect. Similar inheritance pattern of emamectin benzoate resistance was also reported in *S. litura* (Shad et al., 2010), *S. exigua* (Che et al., 2015), and *Phenacoccus solenopsis* (Tinsley) (Hemiptera: Pseudococcidae) (Afzal et al.,

2015). Dominant and polygenic resistance was verified in *P. xylostella* resistant to abamectin, another glutamate-gated chloride channel allosteric modulator (Pu et al., 2010; Wang et al., 2016). The high survival of the heterozygous individuals (F<sub>1</sub> progeny from reciprocal crosses) at the recommended field rate of emamectin benzoate against *S. frugiperda* showed that the resistance is functionally dominant under field conditions, favoring the evolution of resistance because the heterozygous ones are the main carriers of the resistance alleles at the beginning of resistance evolution (Tabashnik and Croft, 1982; Roush and Mckenzi, 1987). This result explains some of field failures due to resistance with the use of emamectin benzoate in some locations in Brazil, particularly where Ben-R strain was collected.

The absence or low cross-resistance between insecticides used in rotation strategy is one of the key assumptions for the success of IRM programs. The Ben-R strain presented low cross-resistance to other insecticides (lambda-cyhalothrin, spinetoram, indoxacarb, chlorantraniliprole, methomyl, and chlorpyrifos) with different modes of action. A low cross-resistance between emamectin benzoate and spinosad, indoxacarb, chlorantraniliprole, chlorfenapyr, tebufenozide, chlorpyrifos, and chlorfluazuron was also verified in *S. exigua* (Che et al., 2015).

The resistance of *S. frugiperda* to emamectin benzoate was not associated with detoxification enzymes. Previous studies evaluating mechanisms of resistance to emamectin benzoate in *S. exigua* indicated that the overexpression of some specific ABC transporters are responsible for inhibiting the accumulation of intracellular pesticides and their metabolites, exporting conjugated toxins out of the cell (Roush and Mckenzi, 1987; Zuo et al., 2018). The resistance to abamectin, another glutamate-gated chloride channel allosteric modulator, was associated with reduced cuticular penetration in *Musca domestica* (L.) (Diptera: Muscidae) (Konno and Scott, 1991), alterations in target sites in *Tetranychus urticae* (Koch) (Acari: Tetranychidae) (Dermauw et al., 2012), and *P. xylostella* (Liu et al., 2014), metabolic detoxification in *T. urticae* and *Bemisia tabaci* (Genn.) (Hemiptera: Aleyrodidae) (Riga et al., 2014; Wang and Wu, 2007), and overexpression of ABC transporters in *Tetranychus cinnabarinus* (Boisduval) (Acari: Tetranychidae) (Xu et al., 2016). Further studies will need to be conducted to identify major resistance mechanisms as well as fitness costs associated to emamectin benzoate in *S. frugiperda*.

Our results showed a high risk of resistance evolution of *S. frugiperda* to emamectin benzoate based on incompletely dominant inheritance. Therefore, rotation of insecticides with different modes of action can be one of the IRM strategies to be implemented to delay the evolution of resistance of *S. frugiperda* to emamectin benzoate in Brazil.

### 3.5. Conclusions

- The inheritance of *S. frugiperda* resistance to emamectin benzoate is an autosomal incompletely dominant trait with polygenic effect.
- There is low cross-resistance between emamectin benzoate and other insecticides (methomyl, chlorpyrifos, lambda-cyhalothrin, spinetoram, indoxacarb and chlorantraniliprole) in *S. frugiperda*.

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#### 4. INVESTIGATING BIOLOGICAL AND BEHAVIORAL FITNESS COSTS OF EMAMECTIN BENZOATE RESISTANCE IN *Spodoptera frugiperda* (LEPIDOPTERA: NOCTUIDAE)

##### Abstract

Evidence of field-evolved resistance to emamectin benzoate (glutamate-gated chloride channel allosteric modulator) has already been reported in *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae) in Brazil. Thus, we selected strains of *S. frugiperda* resistant (Ben-R) and susceptible (Ben-S) to emamectin benzoate to investigate fitness costs associated with the resistance. Fitness costs was quantified by comparing biological (survival rate, developmental time, reproduction, and population growth) and behavioral (ability to escape from predation by *Podisus nigrispinus* (Dallas) (Heteroptera: Pentatomidae)) parameters of Ben-R, Ben-S, and heterozygote strains. Concentration-mortality response indicated that the Ben-R strain presented ~2,445-fold resistance to emamectin benzoate relative to Ben-S strain. Results also revealed that Ben-R strain had lower survival rate and longer developmental time and lower larval and pupal weights than Ben-S strain in non-Bt maize and artificial diet. Fertility life table parameters showed that the Ben-R strain increased the mean length of a generation up to 5 days and reduced ~35% in the intrinsic rate of population increase in both food sources compared to Ben-S strain. In contrast, lack of fitness cost was found in behavioral parameters of larvae of Ben-R strain exposed to the predation by *P. nigrispinus*. Our findings suggest the presence of strongly fitness costs associated with the resistance to emamectin benzoate in *S. frugiperda* based on life history traits, whereas no fitness costs are linked with defensive response to predation by *P. nigrispinus*.

Keywords: Fall armyworm; Resistance; Glutamate-gated chloride channel allosteric modulator; Life history traits

##### 4.1. Introduction

The fall armyworm, *Spodoptera frugiperda* (J. E. Smith, 1797) (Lepidoptera: Noctuidae), is a polyphagous pest that causes damage to several economically important crops worldwide (Pogue, 2002; Ayala et al., 2013; Adhikari et al., 2020; Wang et al., 2020). This species is native from the Americas and has recently been reported in Africa, Asia, and Oceania continents (Goergen et al., 2016; Day et al., 2017; Sharanabasappa et al., 2018; Sidana et al., 2018; Gilal et al., 2020; Jing et al., 2020; Piggott et al., 2021).

In Brazil, the management of *S. frugiperda* has been accomplished mainly by the use of chemical insecticides and genetically modified plants that express *Bacillus thuringiensis* Berliner (Bt) proteins (Burtet et al., 2017; Muraro et al., 2019, Moscardini et al., 2020). Cases of resistance of *S. frugiperda* to Bt maize led to increase the use of insecticides for its control



in maize, cotton, and soybean (Farias et al., 2014; Omoto et al., 2016; Burtet et al., 2017; Muraro et al., 2019). Among insecticides used for controlling *S. frugiperda*, emamectin benzoate (glutamate-gated chloride channel allosteric modulator) has been registered for its control since 2017 in Brazil. The widespread use of this insecticide against *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) in Brazil since 2014 contributed to the exposure of *S. frugiperda* to emamectin benzoate because these species share the same ecological niche, attacking same crops, including cotton, maize, and soybean (Bentivenha et al., 2017; Malaquias et al., 2021). Recently, field-evolved resistance to emamectin benzoate in *S. frugiperda* was reported (Muraro et al., 2021; 2022).

The evolution of resistance to insecticides can result in deleterious changes in biological, physiological, and behavioral parameters in the absence of the selective agent (Freeman et al., 2021). These effects are linked with fitness costs because resistant insects may have disadvantages in their development in relation to susceptible ones in the absence of the selective agent (Georghiou, 1972; Roush and McKenzie, 1987; Gassmann et al., 2009; Tabashnik et al., 2005; Kliot and Ghanim, 2012; Freeman et al., 2021). Understanding fitness costs associated with resistance is important for improving insect resistance management (IRM) strategies and help to reset the susceptibility, once selection pressure is removed (Carrière and Tabashnik, 2001; Carrière et al., 2010; ffrench-constant and Bass, 2017).

Most fitness cost studies have focused on biological parameters to evaluate disadvantages of resistant insects in relation to susceptible ones (Gassmann et al., 2009; Kliot and Ghanim, 2012; Freeman et al., 2021). However, behavioral and defensive responses of resistant insects to natural enemies are also important parameters that should be investigated. Previous studies reported that *S. frugiperda* larvae with resistance to lambda-cyhalothrin was most preyed by *Podisus nigrispinus* (Dallas, 1851) (Hemiptera: Pentatomidae) than susceptible ones (Malaquias et al., 2014). A similar study demonstrated that *H. armigera* larvae with resistance to flubendiamide had less ability to escape of predation by *P. nigrispinus* (Abbate Neto, 2021). Thus, the objective of this study was to investigate fitness costs associated with emamectin benzoate resistance in *S. frugiperda* by measuring biological parameters and behavioral response to the predator *P. nigrispinus*, a natural enemy of this pest in maize and cotton fields in Brazil.

## 4.2. Material and Methods

### 4.2.1. Insect sources

The emamectin benzoate-resistant strain (Ben-R) was selected from a field-collected population in Lucas do Rio Verde, Mato Grosso, Brazil (12°55'36''S; 56°03'13''W), during the 2019 maize season. The susceptible strain (Ben-S) was also obtained from this same field population. For the selection of both strains, it was used the F<sub>2</sub> screen technique as described by Muraro et al. (2021). Adults from the field-collected population were single-pair mated into cages and each pair represented an isofamily, and a total of 62 isofamilies were established. The F<sub>1</sub> progeny from each isofamily was used to establish endogamic crosses to obtain the F<sub>2</sub> progeny. Third-instar larvae at F<sub>2</sub> generation were exposed to a discriminating concentration (320 µg a.i. (active ingredient) mL<sup>-1</sup>) of emamectin benzoate (Proclaim® 50 g a.i. kg<sup>-1</sup>, Syngenta Crop Protection, São Paulo, Brazil) to select the Ben-R strain and to the discriminatory concentration of 3.2 µg emamectin benzoate a.i. mL<sup>-1</sup> to select the Ben-S strain. The diet-overlay bioassays technique was used to select both strains as described in detail by Muraro et al., 2022. After 4 d post-exposure to emamectin benzoate, 32 isofamilies presented surviving larvae, being considered positive for the presence of resistant alleles. These larvae were transferred to 50-mL plastic pots containing an artificial diet proposed by Kasten et al. (1978), where remained until pupal stage. Then, emerged adults were grouped to establish the Ben-R colony. On the contrary, eggs from 12 isofamilies that presented complete larval mortality when exposed to emamectin benzoate were separated. Then, neonates of these isofamilies were transferred to artificial diet. All emerged adults were grouped to establish the Ben-S colony.

Fitness cost studies were performed at the 13 generations of the establishment of Ben-R and Ben-S colonies under laboratory. Larvae of Ben-R strain from F<sub>3</sub> to F<sub>12</sub> generations were exposed to selection pressure at 1,000 µg of emamectin benzoate mL<sup>-1</sup>. In contrast, Ben-S strain were maintained during this period without exposure to selection pressure. At F<sub>13</sub> generation and before starting fitness cost studies, concentration-mortality studies were carried out to characterize the susceptibility of Ben-R and Ben-S strains to emamectin benzoate using diet-overlay bioassays as previous described. To test heterozygotes strains (F<sub>1</sub> hybrids), the Ben-R strain was crossed with the Ben-S strain (resistant♀ × susceptible♂) with at least 50 pairs. As the resistance are autosomally inherited (Muraro et al., 2021), only one side of crossing was used.

#### 4.2.2. Assessing fitness costs of emamectin benzoate resistance in *S. frugiperda*

To perform fitness cost studies, neonates (<24 h old) from Ben-R, Ben-S and F<sub>1</sub> hybrids of the resistant and susceptible strain (heterozygote) at F<sub>13</sub> generation were fed on leaves of non-Bt maize (30A37, Dow AgroSciences, Jardinópolis, SP, Brazil) and an artificial diet proposed by Kasten et al. (1978). Maize leaves (V<sub>4</sub> to V<sub>6</sub> growth stages) were cut into pieces (~5 cm<sup>2</sup>) and placed over a 3% agar-water mixture (10 mL well<sup>-1</sup>) in 16-well plastic plates (CM&CM Comércio de Plásticos, São Paulo, SP, Brazil). Maize leaves were separated from the agar-water layer by a filter paper. The same plastic plates were also used in the bioassays with artificial diet (20 mL of diet well<sup>-1</sup>). Then, a single neonate was placed in each well. Leaves were replaced every 24 h until pupation, while the diet was the same during the larval development phase. Plastic plates were sealed and maintained in a room at 25 ± 2°C, 60 ± 10% RH, and a 14:10 h light:dark photoperiod. The experimental design was completely randomized with three *S. frugiperda* strains (Ben-R, Ben-S, and heterozygote) and two food sources (non-Bt maize and artificial diet), distributed in 10 replicates of 16 larvae strain<sup>-1</sup> and food source.

The following life history traits were evaluated: larval survival and developmental time of neonate-to-pupae, pupae-to-adult, and neonate-to-adult periods; larval weight at 10 d; pupal weight 24 h after pupal formation; and number of eggs per female. Larval survival and developmental time were determined by daily observations, whereas the number of eggs was assessed daily from 20 pairs (one pair cage<sup>-1</sup>) kept in polyvinyl chloride tube cages (10-cm in diameter × 23-cm in height) internally coated with a paper towel and closed at the top with a plastic plate.

To evaluate behavioral parameters, third-instar larvae (12 mm length) of Ben-R, Ben-S and heterozygote strains reared on the same food sources previously described were individualized on glass plates (14 cm in diameter × 2 cm in height) in which was introduced a single adult of *P. nigrispinus*. This predator was fed with *S. frugiperda* until adult stage, but adults were maintained without feeding for 24 h before the experiment. The experimental design was completely randomized with 15 replicates (larvae) strain<sup>-1</sup> and food source. Right after the release of the predator, larval movements were recorded for 15 min using a video camera (Iphone 8, HD resolution 1080p at 60 fps). Larval speed, distance traveled, and mobility time was calculated using an automated motion tracking software (EthoVision®) (Noldus et al., 2002). The predation rate by *P. nigrispinus* was recorded during 15 min.

### 4.2.3. Data analysis

The concentration-mortality data were adjusted by a generalized linear model (GLM) with a quasibinomial distributions, using probit link function. We used a half-normal plot with a simulated envelope with the *hnp* package to assess the goodness-of-fit of the model (Moral et al., 2017). Then, concentration-response data were submitted to Probit analysis using the R software (R Development Core Team, 2021). Resistance ratios was determined by dividing LC<sub>50</sub> value of Ben-R and heterozygote strains by the LC<sub>50</sub> value of the Ben-S strain.

Biological (larval survival and developmental time) and behavioral (larval speed, distance traveled, mobility time, and predation rate) data of *S. frugiperda* strains were analyzed using a generalized linear model (GLM) according to the distribution of each data (gamma or gaussian distribution for continuous data and binomial or quasi-binomial for discrete data). The number of eggs.female<sup>-1</sup>, larval and pupal weights, and *P. nigrispinus* predation rate was transformed into *log* function. The appropriate distribution was determined based on the goodness-of-fit of the model using the *hnp* package (Moral et al., 2017). Then, data were subjected to one-way analysis of variance (ANOVA) using the F test to verify significant the differences between strains. Means were compared by the Tukey test using the *lsmeans* package (Lenth, 2016).

Larval survival, developmental time, and reproduction data were also used to estimate fertility life table parameters, including mean length of a generation (*T*), net reproductive rate (*R<sub>0</sub>*; average number of female offspring that would be born to a cohort of females), and intrinsic rate of population increase (*rm*; daily production of females per parental female). Fertility life table parameters were estimated by the *jackknife* technique developed by Maia et al. (2014). All analyses were performed with the R software (R Development Core Team, 2021).

## 4.3. Results

### 4.3.1. Concentration-mortality responses

The Ben-R, Ben-S and heterozygote strains presented a non-significant chi-square in the goodness-of-fit test ( $P > 0.05$ ), suggesting that data adjusted the Probit model (Table 1). The LC<sub>50</sub> of emamectin benzoate against third-instar larvae of Ben-R strain was 562.14 µg a.i. (active ingredient) mL<sup>-1</sup>, while for the Ben-S strain was 0.23 µg a.i. mL<sup>-1</sup>, indicating a

resistance ratio of ~2445-fold (Table 1). Third-instar larvae from heterozygote showed LC<sub>50</sub> of emamectin benzoate of 88.28 µg a.i. mL<sup>-1</sup>, resulting in a resistance ratio of 383-fold, in relation to Ben-S strain. These results indicate a genetically based decrease in susceptibility to emamectin benzoate in Ben-R strain.

**Table 1.** Concentration-mortality (µg a.i. mL<sup>-1</sup>) of emamectin benzoate in resistant (Ben-R), susceptible (Ben-S), and F<sub>1</sub> progeny of reciprocal cross (Ben-R♀ × Ben-S♂).

<i>S. frugiperda</i> strain	<i>n</i>	Fit of probit lines			LC <sub>50</sub> (95% CI) <sup>b</sup>	RR <sub>50</sub> <sup>c</sup>
		Slope ± SE	χ <sup>2</sup> ( <i>df</i> )	<i>P</i>		
Ben-R	935	2.36 ± 0.32	7.31 (6)	0.17	562.14 (455.12–745.7)	2,444.8
Ben-R♀ × Ben-S♂	845	1.22 ± 0.14	6.12 (6)	0.22	88.28 (65.43–109.22)	383.82
Ben-S	322	1.43 ± 0.13	7.22 (3)	0.12	0.23 (0.10–0.32)	-

<sup>a</sup>*df* = degrees of freedom.

<sup>b</sup>LC<sub>50</sub> values followed by the same letter do not differ significantly due to non-overlap of 95% confidence intervals (CIs).

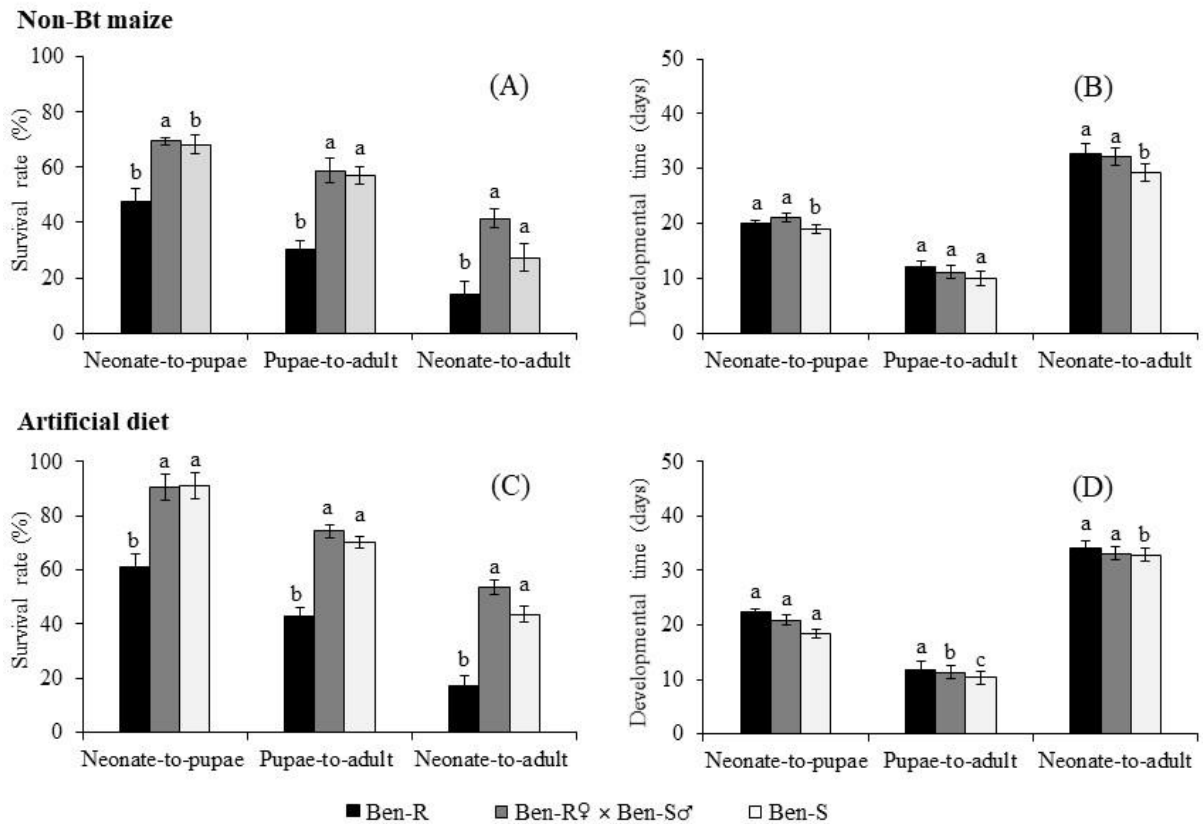
<sup>c</sup>Resistance Ratio (RR) = LC<sub>50</sub> of Ben-R or heterozygote strains/LC<sub>50</sub> of Ben-S strain.

#### 4.3.2. Survival rate of *S. frugiperda* strains on non-Bt maize and artificial diet

The survival rate of neonate-to-pupae differed significantly between *S. frugiperda* strains developed on non-Bt maize (*df* = 2, 27; *F* = 4.23; *P* < 0.001) and artificial diet (*df* = 2, 27; *F* = 5.69; *P* < 0.001) (Figure 1 A, C). The Ben-R strain presented lower neonate-to-pupae survival on non-Bt maize (47%) and artificial diet (61%) than the Ben-S and heterozygote strains (>68% on non-Bt maize and >90% on artificial diet), which did not differ from each other.

The survival rate of pupae-to-adult was also varied significantly different among *S. frugiperda* strains on non-Bt maize (*df* = 2, 27; *F* = 3.28; *P* < 0.001) and artificial diet (*df* = 2, 27; *F* = 19.86; *P* < 0.001) (Figure 1 A, C). The Ben-R strain had lower pupae-to-adult survival on both food sources (30% on non-Bt maize and 43% on artificial diet) than Ben-S and heterozygote strain (>56%). The last two strains had similar pupae-to-adult survival rate, independently of the food source.

The survival rate of neonate-to-adult also differed significantly between *S. frugiperda* strains developed on non-Bt maize (*df* = 2, 27; *F* = 6.07; *P* < 0.001) and artificial diet (*df* = 2, 27; *F* = 12.39; *P* < 0.001) (Figure 1 A, C). The Ben-R strain showed lower neonate-to-adult survival rate (<20% on both food sources) than Ben-S (34% and 42% on non-Bt maize and artificial diet, respectively) and heterozygote (42% on non-Bt maize and 57% on artificial diet) strains, which do not differ.



**Figure 1.** Survival rate and developmental time of *S. frugiperda* strains on leaves of non-Bt maize and artificial diet. Groups of bars ( $\pm$  SE) with the same letter are not significantly different (Tukey test;  $P > 0.05$ ).

#### 4.3.3. Developmental time of *S. frugiperda* strains on non-Bt maize and artificial diet

The neonate-to-pupae developmental time of *S. frugiperda* strains varied significantly on non-Bt maize ( $df = 2, 27$ ;  $F = 3.99$ ;  $P = 0.030$ ) and artificial diet ( $df = 2, 27$ ;  $F = 27.07$ ;  $P < 0.001$ ) (Fig. 1 B, D). The Ben-R and heterozygote strains had a longer larval developmental time on non-Bt maize and artificial diet (20–22 d) than the Ben-S strain (18–19 d). Contrary to previous results, the pupae-to-adult developmental time of *S. frugiperda* strains on non-Bt maize ( $df = 2, 27$ ;  $F = 6.27$ ;  $P = 0.082$ ) and artificial diet ( $df = 2, 27$ ;  $F = 5.18$ ;  $P = 0.148$ ) did not differ between the *S. frugiperda* strains (Fig. 1 B, D). The average pupal developmental time on both food sources was 11 d.

The neonate-to-adult developmental time of *S. frugiperda* strains varied significantly according to the food source ( $df = 2, 27$ ;  $F = 16.22$ ;  $P < 0.001$  on non-Bt maize;  $df = 2, 27$ ;  $F$

= 26.95;  $P < 0.001$  on artificial diet) (Fig. 2 B, D). On both food sources, the Ben-R and heterozygote strains (30–32 d) showed longer neonate-to-adult developmental time than the Ben-S strain (26–29 d).

#### 4.3.4. Larval and pupal weights and reproduction of *S. frugiperda* strains on non-Bt maize and artificial diet

Larval weights of *S. frugiperda* strains differed significantly when its development occurred on non-Bt maize ( $df = 2, 27; F = 13.69; P < 0.001$ ) and artificial diet ( $df = 2, 27; F = 106.43; P < 0.001$ ) (Table 2). Larvae from Ben-R strain fed on both food sources had lower larval weights (60.7 and 65.3 mg.larva<sup>-1</sup>) than Ben-S and heterozygote strains (101.8 and 108 mg.larva<sup>-1</sup> on non-Bt maize; 199.2 and 157.7 mg.larva<sup>-1</sup> on artificial diet), indicating >59% reduction on larval weight of resistant insects (Table 2). In contrast, the pupal weight did not vary significantly among strains when larvae developed on non-Bt maize ( $df = 2, 27; F = 1.23; P = 0.308$ ) and artificial diet ( $df = 2, 27; F = 0.70; P = 0.504$ ) (Table 2). The average pupal weights ranged from 175.8 to 229.9 mg.pupa<sup>-1</sup>.

The average eggs oviposited per female differ significantly between *S. frugiperda* strains developed on non-Bt maize ( $df = 2, 57; F = 8.77; P = 0.003$ ) and artificial diet ( $df = 2, 57; F = 7.91; P = 0.009$ ) (Table 2). Heterozygote females oviposited more eggs in both food sources (~2200 eggs.female<sup>-1</sup>) than Ben-R and Ben-S females (1156–1524 eggs.female<sup>-1</sup>).

**Table 2.** Eggs per female, larval and pupal weights of *S. frugiperda* strains fed on leaves of non-Bt maize and artificial diet.

<i>S. frugiperda</i> strain	Larval weight (mg)	Pupal weight (mg)	Mean eggs per female
<b>Non-Bt maize</b>			
Ben-R	60.7 ± 5.2 b	183.9 ± 3.7 a	1203.4 ± 137.1 b
Ben-R♀ × Ben-S♂	101.8 ± 7.1 a	178.9 ± 3.5 a	2115.5 ± 104.3 a
Ben-S	108.0 ± 6.2 a	175.8 ± 3.8 a	1524.5 ± 136.2 b
<b>Artificial diet</b>			
Ben-R	55.38 ± 5.4 b	228.1 ± 3.0 a	1156.7 ± 179.2 b
Ben-R♀ × Ben-S♂	199.2 ± 5.9 a	229.9 ± 4.1 a	2216.6 ± 152.0 a
Ben-S	157.7 ± 4.2 a	227.0 ± 2.1 a	1346.4 ± 213.0 b

<sup>a</sup>Means ± SE within a column followed by the same letter are not significantly different (Tukey test,  $P > 0.05$ ).

#### 4.3.5. Life table parameters of *S. frugiperda* strains on non-Bt maize and artificial diet

The life table parameters (Table 3) indicated that the Ben-R and heterozygote strains had a higher mean length of a generation ( $T$ ) and Ben-R strain low population growth ( $R_o$  and  $r_m$ ) on both food sources than Ben-S strain (Table 3). These findings indicated that Ben-R females from both food sources generated fewer than 90 females.female.generation<sup>-1</sup> ( $R_o$ ), in up to 42 d, whereas the Ben-S strain in the same food sources generated more than 200 females.female.generation<sup>-1</sup> up to 37 d, revealing that Ben-R females produced 61% and 65% fewer females than Ben-S females per generation.

The mean time of a generation of heterozygote strain was up to 4 d longer on non-Bt maize and artificial diet than Ben-S strain, but this time was similar for Ben-R and Ben-S strains (Table 3). However, Ben-S females produced a significant high number of females.female<sup>-1</sup> in both food sources than Ben-R and heterozygote strains (Table 3). The intrinsic rate of population increase ( $r_m$ ) of Ben-R strain was ~35% lower than Ben-S and heterozygote strains, indicating that resistant insects have less potential for population growth.

**Table 3.** Fertility life table parameters of *S. frugiperda* resistant (Ben-R) and susceptible (Ben-S) heterozygotes strains fed on leaves of non-Bt maize and artificial diet.

<i>S. frugiperda</i> strain	Fertility life table parameters <sup>a,b</sup>		
	$T$ (days)	$R_o$ (♀/♀)	$r_m$ (♀/♀*day)
<b>Non-Bt maize</b>			
Ben-R	38.75 ± 0.10 a	89.28 ± 12.58 c	0.10 ± 0.004 b
Ben-R♀ × Ben-S♂	39.23 ± 0.25 a	441.55 ± 28.91 a	0.15 ± 0.004 a
Ben-S	35.44 ± 0.19 b	229.12 ± 38.44 b	0.14 ± 0.002 a
<b>Artificial diet</b>			
Ben-R	42.57 ± 0.28 a	69.06 ± 10.56 c	0.09 ± 0.003 b
Ben-R♀ × Ben-S♂	40.79 ± 0.17 a	398.83 ± 28.77 a	0.14 ± 0.002 a
Ben-S	37.05 ± 0.15 b	204.96 ± 31.70 b	0.14 ± 0.003 a

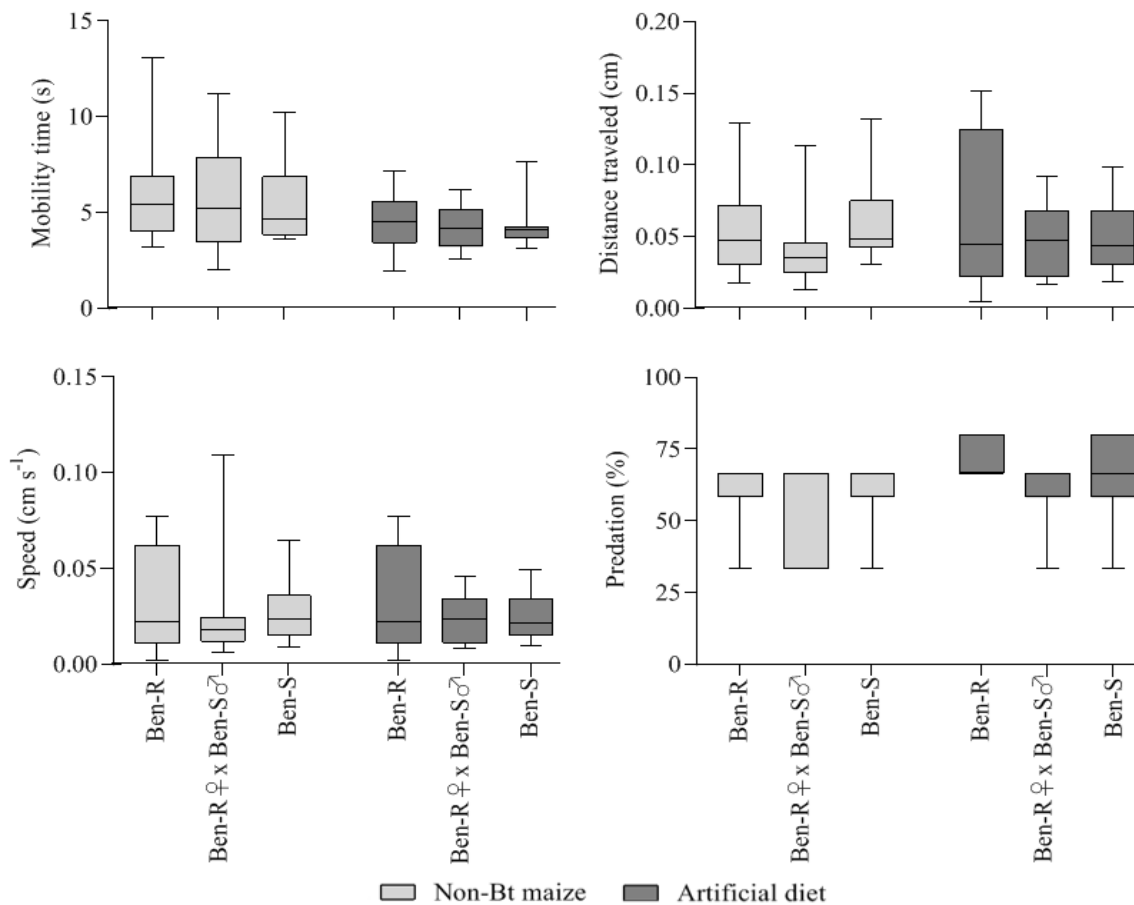
<sup>a</sup> $T$  = mean length of a generation (days);  $R_o$  = net reproductive rate (females per female per generation);  $r_m$  = intrinsic rate of population increase (per day).

<sup>b</sup>Means within a column followed by the same letter are not significantly different ( $t$ -tests for pairwise group comparisons,  $P > 0.05$ ).



#### 4.3.6. Behavioral parameters of *S. frugiperda* strains in the presence of *P. nigrispinus*

Third-instar larvae of Ben-R, Ben-S and heterozygote strains developed on non-Bt maize and artificial diet showed similar mobility time (4.23–6.04 s), distance traveled (0.049–0.066 cm), and speed (0.007–0.074 cm.s<sup>-1</sup>), when exposed to the predation by *P. nigrispinus* ( $df = 2, 42; P > 0.05$  for all comparison) (Fig. 2). Regarding of the food source, the predation rate of Ben-R, Ben-S, and heterozygote strains by *P. nigrispinus* was also similar, ranging from 46% to 72% ( $df = 2, 42; F = 1.06; P = 0.352$  for non-Bt maize;  $df = 2, 42; F = 0.90; P = 0.411$  for artificial diet) (Fig. 2).



**Figure 2.** Behavioral parameters of *S. frugiperda* strains fed on non-Bt maize and artificial diet exposed to predation by the natural enemy *P. nigrispinus*.

#### 4.4. Discussion

In this study, resistant (Ben-R) and susceptible (Ben-S) strains of *S. frugiperda* to emamectin benzoate were selected from the same field-collected population and used to investigate the magnitude of fitness costs on two food sources (non-Bt maize and artificial diet). It was founded that Ben-R strain had reduced larval survival and longer developmental time of neonate-to-pupae and neonate-to-adult periods, and lower population growth potential in both food sources compared to Ben-S strain, suggesting the presence of strongly fitness costs of the resistance to emamectin benzoate in *S. frugiperda*. Regardless of the food source, a lack of fitness cost was observed in behavioral parameters of resistant larvae when exposed to the predator *P. nigrispinus*. This suggests that the resistance to emamectin benzoate in *S. frugiperda* does not affect its defensive response by the predation to *P. nigrispinus*.

Fitness costs of insecticide resistance affecting survival rate, developmental time, and population growth potential of *S. frugiperda* has been previously reported. Using maize, cotton, soybean, and oats, Garlet et al. (2021) reported fitness costs of the resistance to chlorpyrifos-resistant in *S. frugiperda* affecting survival of pupa-to-adult and egg-to-adult period and reducing larval weights on oats; prolonging neonate-to-pupa and egg-to-adult periods on maize; affecting pupal weights on soybean; and reducing fecundity on cotton. The fitness costs of resistance to spinosad in *S. frugiperda* quantified on an artificial diet also reduced survival rate to adulthood and reproductive rate of resistant insects (Okuma et al., 2018). In contrast, *S. frugiperda* resistance to chlorantraniliprole was not linked with relevant fitness costs (Padovez et al., 2022). In other *Spodoptera* species, fitness costs of resistance to emamectin benzoate also affected larval survival, the duration of larval and pupal stages, pupal weights, number of eggs, and insects that emerged into adults in *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) and *Spodoptera litura* (Fab.) (Lepidoptera: Noctuidae) (Ishtiaq et al., 2014; Zaka et al., 2014).

Fitness cost studies associated with resistance to insecticides are also expanding beyond biological development and reproduction of resistant insects (Freeman et al., 2021). Behavioral studies demonstrated that larvae of *S. frugiperda* resistant to lambda-cyhalothrin was most preyed by *P. nigrispinus* than susceptible larvae (Malaquias et al., 2014). A similar behavior was observed for *H. armigera* larvae resistant to flubendiamide, which also showed lower mobility and speed to escape of predation by *P. nigrispinus* (Abbade Neto, 2021). In contrast, our results indicated that the resistance to emamectin benzoate in *S. frugiperda* did

not affect its defensive response by the predation to *P. nigrispinus*, suggesting that under field conditions resistant and susceptible strains will be predated at similar rates.

The fitness costs of emamectin benzoate resistance in *S. frugiperda* here reported indicate that reductions in resistance alleles frequency may be achieved in the absence of the selective agent, restoring the susceptibility to this mode-of-action insecticide. However, the resistance of *S. frugiperda* to emamectin benzoate has an incompletely dominant trait at the recommended field rate of emamectin benzoate (15 g a.i. ha<sup>-1</sup>), indicating that heterozygotes will survive in field conditions, increasing changes of resistance evolution (Muraro et al., 2021). Recently, evidence of field resistance of *S. frugiperda* to emamectin benzoate was reported from main maize, soybean and cotton growing regions in Brazil (Muraro et al., 2022). Therefore, the presence of fitness costs associated with the resistance to emamectin benzoate in *S. frugiperda* should be exploited in IRM programs, since fitness costs reduce resistance alleles frequency and the spread of resistance evolution, if there is a reduction in selection pressure.

From an insect resistance management perspective, it is important to highlight that the adoption of insecticides with different modes-of-action in combination with other available control tactics such as Bt plants, baculovirus-based biopesticides – *Spodoptera frugiperda* multiple nucleopolyhedrovirus–SfMNPV, and natural enemies are essential to prolong the useful life of all insecticides mainly in a “tropical agriculture” as in Brazil, where *S. frugiperda* had several generations per year and are being exposed to same mode-of-action in distinct crops (e.g. maize, soybean, cotton, sorghum, and millet) throughout the seasons.

#### 4.5. Conclusions

- There are fitness costs of emamectin benzoate resistance in *S. frugiperda* affecting survival rate, developmental time, and population growth;
- No fitness costs are linked with defensive response to predation by *P. nigrispinus*.

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## 5. FINAL CONSIDERATIONS

The susceptibility of the populations of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) to emamectin benzoate in Brazil decreased significantly from 2019 and 2021 compared to the susceptibility observed in 2003 and 2004. In recent years, a significant increase in the frequency of resistance to emamectin benzoate in populations of *S. frugiperda* was observed. Field populations presented a resistance ratio up to 632-fold and a survival up to 81% at the diagnostic concentration in some locations from 2019 to 2021. These results corroborate with high survival of larvae (up to 88.5%) from field-collected populations of *S. frugiperda* in 2021 when exposed to maize leaves sprayed with a recommended commercial rate of emamectin benzoate. Additionally, the lowest susceptibilities and greater survival rates of *S. frugiperda* populations to emamectin benzoate were observed mainly in the states of Bahia, Goiás, Mato Grosso, and Mato Grosso do Sul. These results confirmed the evolution of resistance in *S. frugiperda* populations in Brazil.

The intense selection pressure of *S. frugiperda* in the field to emamectin benzoate resulted in the selection of a strain with a resistance ratio of 2,322-fold under laboratory conditions. This strain showed an autosomal inheritance, incompletely dominant, and polygenic resistance to emamectin benzoate. Additionally, no effect of synergists piperonyl butoxide, diethyl maleate and S, S, S-tributyl phosphorotrithiotate on resistant strain was observed, suggesting a minor role of metabolic resistance. Furthermore, low cross-resistance was detected between emamectin benzoate and the insecticides methomyl, chlorpyrifos, lambda-cyhalothrin, spinetoram, indoxacarb and chlorantraniliprole. The dominant inheritance pattern of resistance to the insecticide emamectin benzoate in *S. frugiperda* can favor the evolution of resistance if resistance management strategies are not implemented.

Fitness costs were quantified by comparing biological and behavioral parameters on non-Bt maize and artificial diet, using strains with similar genetic background. We concluded that resistant strain had significant lower survival rate and longer developmental time of neonate-to-pupae and neonate-to-adult periods, and lower larval and pupal weights than susceptible strain in both food sources. Fitness costs also affected fertility life table parameters of the resistant strain, reducing the potential for population growth on both feed sources. In contrast, lack of fitness cost was detected in the behavioral parameters of larvae and predation rate of *Podisus nigrispinus* (Heteroptera: Pentatomidae) in larvae reared non-Bt maize and artificial diet. These results suggest relevant fitness costs associated with the resistance of *S. frugiperda* to emamectin benzoate.



The information presented in this thesis showed the importance of implementing resistance management strategies to delay resistance of *S. frugiperda* to emamectin benzoate in order to reduce the frequency of resistant alleles and prolonging the lifetime of this insecticide in the management of *S. frugiperda* in Brazil. Furthermore, with advances in molecular tools, RNA/DNA sequencing studies should be performed to understanding the mechanisms of resistance to emamectin benzoate in *S. frugiperda*.