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

Resistance to pyrethroid and oxadiazine insecticides in *Helicoverpa armigera* (Lepidoptera: Noctuidae) populations in Brazil

Mariana Regina Durigan

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

Piracicaba
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versão revisada de acordo com a resolução CoPGr 6018 de 2011

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

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Dados Internacionais de Catalogação na Publicação
DIVISÃO DE BIBLIOTECA – DIBD/ESALQ/USP

Durigan, Mariana Regina

Resistance to pyrethroid and oxadiazine insecticides in *Helicoverpa armigera* (Lepidoptera: Noctuidae) populations in Brazil / Mariana Regina Durigan. - - versão revisada de acordo com a resolução CoPGr 6018 de 2011. - - Piracicaba, 2018.

99 p.

Tese (Doutorado) - - USP / Escola Superior de Agricultura “Luiz de Queiroz”.

1. Manejo da resistência de insetos 2. *Helicoverpa armigera* 3. Inseticidas que atuam no canal de sódio 4. Mecanismos de resistência 5. Indoxacarb 6. Fenvalerato 7. Deltametrina I. Título

I dedicate this work to my parents Carlos Alberto Durigan and Solange Aparecida da Silva, to my brother, Carlos Alberto Durigan Junior, and to my boyfriend Alvaro Ramirez for all the support, incentive, comprehension and for always believe in my capacity.

ACKNOWLEDGMENTS

To God for allow me to live this amazing experience and for all the opportunities and challenges provided during this period.

To the Graduate Program in Entomology at USP-ESALQ for the chance to perform a very important part of my career, for all the knowledge, structure and all the opportunities provided during my journey as a doctoral student.

To Professor Celso Omoto for all the advices, patience, help, trustiness, friendship and all the opportunities provided during my time under his advisor.

To Professor Alberto Soares Corrêa for all the support, discussions, teachings and friendship. His collaboration was essential for the execution of this work.

To Dr. David G. Heckel for all the learning, support, patience and for providing me the opportunity to work in his laboratory at the Max Planck Institute for Chemical Ecology.

To Dr. Juil Kim from South Korea for all his help, patience, friendship and everything that he taught me which were extremely important for this work.

To the Arthropod Resistance Laboratory ESALQ-USP for everything that I had experienced and learned while I was there, in special to Oderlei Bernardi, Daniel Bernardi, Daniela Okuma, Renato Horikoshi, Rebeca Ribeiro, Fernando Amaral, Ingrid Kaiser, Anderson Bolzan, Rubens Kano, Ewerton Lira, José Paulo Bentivenha, Patrick Dourado, Antônio Rogério Nascimento, Osmar Arias, Leonardo Miraldo, Yasmin Barmejo, Juliana Rodrigues and Fernando Padovez. Thank you for all the friendship and for everything I learned not only in the professional but also in the personal aspect.

To Dr. Eloisa Salmeron and the staff Janice Soares for all the support and for taking care of every detail, organization and for providing all the necessary things in the Arthropod Resistance Laboratory.

To the Helicoverpa team Rogério Machado, Natália Leite, Douglas Amado, Sandy Spinelli and Dyrson Abbade for everything we have learned and experienced together and for our friendship.

To the trainees Rodrigo Franciscatti, Marcelo Mocheti, Alessandra Tokarski, Luís Perazollo, Natasha Umezu, Alexandre Colli, Mariana Colli and Guilherme Picarelli, for all the support and friendship.

To my dear friends that I made during this time who were responsible for many great moments Dayana Sousa, Bianca Carbogim, Renato Horikoshi, Daniela Okuma, Ana Flávia Martinez, Aline Franco, Laura Pantoja, Sandy Spineli, Cleane Souza and Beatriz Lima.

To the friends and colleges who made my time in Germany very special Camila Plata, Kanjamas Malipohl, Pauline Sell, Franziska Beran, Sabine Hänniger, Domenica Schnabelrauch, Astrid Grot, Antje Schmaltz, Melaine Unbehend, Regina Seibt, Zhi-Ling Yang, Theresa Sporer, Katrin Böhmer, Henriette Beckstein, Shantanu Shukla and Roy Kirsch.

To the Professors Fernando Luis Cônsoli, Roberto Antonio Zucchi, José Roberto Postali Parra and José Djair Vendramim for all the learning provided.

To all the staffs from the Department of Entomology and Acarology in special to Regina Botequio, Marta Colletti, Andrea Sintoni and Solange Barros for all their support, attention and assistance.

To Eliana Maria Garcia from the main library at USP-ESALQ for the format revision of this thesis.

To my parents Carlos Alberto Durigan and Solange Aparecida da Silva, to my brother Carlos Alberto Durigan Junior and to my boyfriend Alvaro Ramirez for all the support, incentive, love and for vibrating with every achievement.

To the Conselho Nacional de Desenvolvimento Tecnológico (CNPq), to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and to IRAC Brazil committee for the scholarships and funding provided during my PhD and also for the grant provided to realize the training in Germany.

To all those who were involved during this journey, thank you very much!

CONTENTS

RESUMO

Resistência de *Helicoverpa armigera* (Lepidoptera: Noctuidae) a inseticidas dos grupos piretroides e oxadiazinas no Brasil

Helicoverpa armigera (Hübner) foi reportada oficialmente no Brasil em 2013, ano em que causou grandes perdas em lavouras de soja e algodão no país. Devido ao ataque severo de *H. armigera* e por ser mais tolerante do que as demais pragas que ocorriam no Brasil, houve um aumento significativo da pressão de seleção com inseticidas no campo. Inúmeros casos de resistência desta praga a inseticidas do grupo dos piretroides já havia sido reportado em alguns países do Velho Mundo. Dentro desse contexto o objetivo desse trabalho foi caracterizar a suscetibilidade e investigar possíveis mecanismos de resistência a piretroides bem como indoxacarb no Brasil. A mortalidade das populações de *H. armigera* foi menor do que 50 % quando tratadas com a dose máxima de 10 µg i.a./lagarta de 3° instar para fenvalerato e deltametrina. As populações de campo de *H. armigera* monitoradas entre os anos de 2013 a 2016 na dose diagnóstica de 10 µg i.a./lagarta de 3º instar apresentaram mortalidade de 10 a 40%. A frequência do gene P450 *CYP337B3* foi maior do que 0,95 em 33 populações testada. Além disso, as bases genéticas da resistência de *H. armigera* a piretroides foram investigadas e a razão de resistência com a linhagem suscetível foi de 780 vezes. O grau de dominância variou de 0,66 a 0,92, incompletamente e completamente dominante e a resistência foi caracterizada como autossômica e poligênica. Adicionalmente investigou-se a presença de possíveis mutações no canal de sódio bem como a expressão de outros genes P450 em uma linhagem resistente a piretroides. Foi possível detectar duas mutações não-sinonímias V937G, e Q960H no canal de sódio e os genes *CYP6AB10, CYP301A, CYP4S13 e CYP321A5* foram super expressos na linhagem resistente. A suscetibilidade de populações de *H. armigera* para o inseticida indoxacarb foi caracterizada a partir de bioensaios de ingestão com lagartas de 3° instar. Os valores de CL_{50} variaram de 0,22 (0,16 – 0,28) µg i.a./cm^2 até 0,57 (0,41 – 0,82) µg i.a./cm^2 variando em 2,6 vezes. As populações foram monitoradas ao longo das safras agrícolas entre 2013 e 2017 com a concentração diagnóstica de 6,1 µg i.a./cm^2 e observou-se uma diminuição na suscetibilidade da praga a indoxacarb. Uma linhagem resistente a indoxacarb foi selecionada em laboratório e comparada com uma linhagem suscetível de referência, apresentando uma razão de resistência de 297,5 vezes. Os resultados obtidos são extremamente importantes e poderão contribuir na tomada de decisões bem como na implementação de programas de manejo da resistência de insetos (MRI) no Brasil.

Palavras-chave: Manejo da resistência de insetos; *Helicoverpa armigera*; Inseticidas que atuam no canal de sódio; Mecanismos de resistência; Indoxacarb; Fenvalerato; Deltametrina

ABSTRACT

Resistance to pyrethroid and oxadiazine insecticides in *Helicoverpa armigera* (Lepidoptera: Noctuidae) populations in Brazil

Helicoverpa armigera (Hübner) was officially reported in Brazil in 2013 causing serious damage to several crops, especially soybean and cotton crops. Because of this severe damage and also because *H. armigera* is more tolerant to insecticides in compare to other lepidopteran pests in Brazil, there was a significant increase of selection pressure with insecticides in the field. Many cases of insecticide resistance, especially to pyrethroids, have been reported in some countries of the Old World. The main objective of the present study was to characterize the susceptibility of *H. armigera* and to investigate the mechanisms of its resistance to pyrethroids and indoxacarb in Brazilian populations. Mortality of *H. armigera* populations was less than 50% when treated with the highest dose of 10 µg a.i./3rd-instar larva of fenvalerate and deltamethrin. Field populations of *H. armigera* monitored from 2013 to 2016 growing seasons showed mean mortalities of 10 to 40% at the diagnostic dose of 10 µg a.i./3rd-instar larva. The resistance ratio to pyrethroid was 780-fold. The frequency of the chimeric P450 *CYP337B3* gene was above 0.95 in all 33 populations screened. The genetic basis of *H. armigera* resistance to pyrethroids was also investigated. The dominance degree varied from 0.66 to 0.92, i.e., incompletely to completely dominant, and resistance was characterized as autosomal and polygenic. Possible mutations in the sodium channel were investigated, as well as the expression of other P450 genes via RT-qPCR. Two non-synonymous mutations, V937G and Q960H were found, and the genes *CYP6AB10, CYP301A, CYP4S13* and *CYP321A5* were up-regulated in the Brazilian pyrethroid-resistant strain compared to the susceptible strain. The susceptibility of *H. armigera* populations to indoxacarb was characterized with a diet overlay bioassay in 3rd-instar larvae. LC_{50} values ranged from 0.22 (0.16–0.28) µg a.i./cm^2 to 0.57 (0.41–0.82) µg a.i./cm^2, varying 2.6-fold. The populations were monitored through the 2013–2017 growing seasons, with the diagnostic dose of 6.1 µg a.i./cm^2; during the period, the susceptibility to indoxacarb decreased. An indoxacarb-resistant strain was selected under laboratory conditions and showed a resistance ratio of 297.5-fold. These results will contribute to decision-making and implementation of insect resistance-management (IRM) programs in Brazil and other recently invaded countries in Brazil.

Keywords: Resistance management; *Helicoverpa armigera*; Sodium channel; Mechanisms of resistance; Indoxacarb; Fenvalerate; Deltamethrin

1. INTRODUCTION

Insecticide resistance is one of the main problems for the success of pest control worldwide (Sparks and Nauen, 2015; Sparks and Lorsbach, 2017). Insect resistance was first documented in the United States in 1914 for a strain of San José scale, where sulphur, an inorganic insecticide, was no longer controlling this insect (Melander, 1914). From this first documentation until 2017, more than 15,000 cases of insecticide resistance have been reported for different pest species all over the world (Sparks and Lorsbach, 2017).

Resistance is defined as "the development of an ability in a strain of insects to tolerate doses of toxicants which would prove lethal to the majority of individuals in a normal population of the same species" (World Health Organization, 1957). The IRAC committee defined resistance as "a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species" (Insecticide Resistance Action Committee, 2018).

Resistance is a heritable genetic characteristic that can enable an insect and its descendants to tolerate a higher dose of insecticide than others in a population. The evolution of resistance in a population starts with the genetic variability among individuals, where some can be more adaptable to changes than others (Georghiou, 1972; Georghiou and Taylor, 1977a). This means that under high selection pressure from a specific insecticide, the individuals able to tolerate the high amount will survive. Furthermore, these pre-adapted individuals have some mechanisms which allow them to tolerate and survive the exposure (Georghiou, 1969; Georghiou, 1972; Roush and McKenzie, 1987). If their descendants possess this same characteristic, it can be termed resistance.

Selection pressure is a key factor in the rate of resistance evolution, which is an external factor that most of the time is determined by farmers at the time of insecticide spraying on their crops (Georghiou, 1972; Roush and McKenzie, 1987). Spraying insecticides with the same mode of action (MoA), with a high dose or for a long period, can create a situation where the pre-adapted individuals will have advantages over non-adapted individuals, and as a consequence the frequency of resistant individuals may increase (Roush and McKenzie, 1987; Tabashnik et al., 2003).

Resistance is governed by three major factors, genetic, biological and operational (Georghiou and Taylor, 1977a; Georghiou and Taylor, 1977b). Genetic factors are associated with intrinsic characteristics, which involves the frequency of the resistant allele (R) in a

population as well as the number of R alleles, inheritance of resistance such as the degree of dominance, past selection by other chemicals, and the combination of R with other fitness factors in the genome (Georghiou and Taylor, 1977a). Biological factors involve biotic characteristics such as generation turnover; offspring per generation; reproductive type (e.g., sexual, parthenogenetic); behavioral characteristics such as migration, dispersal or isolation; and monophagy or polyphagy. Operational factors are those associated with external factors, which can be manipulated by humans, involving the characteristics of the chemical, such as the mode of action, persistence, and formulation, as well as the characteristics of application in the field (Georghiou and Taylor, 1977a; Georghiou and Taylor, 1977b).

Resistance is always associated with mechanisms that enable an individual to tolerate more chemicals than the others (Roush and McKenzie, 1987). These mechanisms can be i) metabolic, associated with the detoxification of the toxic compound by enzymes such as P450, esterase, or glutathione-S-transferase, or the excretion rate due to ABC transporters; ii) target-site mutations, based on changes in the structure of the site or the number of sites where the pesticide binds to cause toxicity to the insect; iii) penetration, when the insecticide takes more time or cannot cross the cuticle of the insect; and iv) behavioral, when the insect is able to evade contact with the toxic compound. Metabolic and target-site mechanisms are considered to be the most important and have been associated with many cases of resistance in the literature (Joußen and Heckel, 2016).

Only the operational factor can be managed and changed by farmers, and also recommended by the companies who offer the technologies. The evolution of insect resistance to insecticides can force companies and growers to spend large sums once a pest-control tactic becomes inefficient and it becomes necessary to develop or adopt a new technology to control pests in the field (Sparks and Lorsbach, 2017). Furthermore, the development of a new technology is costly in time and money. Currently it is necessary to test around 159 574 compounds for each product developed, and on average, 10 years of research with a total cost of U$286 million are needed to find and develop a new agrochemical (Sparks and Lorsbach, 2017).

Thus, when an insecticide becomes ineffective because of resistance, most of the time and money invested during this discovery process is lost. In the United States, estimated crop losses in 2005 due to pesticide resistance were about U$1.5 billion year^{-1}(Pimentel, 2005). In 1990 and 1991, Australia suffered losses of around A$150 million due to *Helicoverpa armigera* management in cotton fields after this pest evolved resistance to several insecticides, including pyrethroids (Fitt, 1994).

In the 1940s, the first class of synthetic organic insecticides, such as DDT, was introduced. However, a case of resistance in houseflies was reported only a few years later (Keiding and Van Deurs, 1949). Nowadays it is possible to find different groups of insecticides with different modes of action, including pyrethroids, oxadiazines, neonicotinoids, carbamates, spinosyns, growth regulators, and the recently introduced diamides. Although many different classes of insecticides are currently available on the market, many of them target the nervous and muscular systems, and some of the compounds affect different sites in the same target (Sparks and Nauen, 2015). The classical example is the sodium channel, a transmembrane protein with four domains, each one containing six subunits (Dong, 2007). The sodium channel is the target of pyrethroids, a sodium-channel modulator, and oxadiazines such as indoxacarb and metaflumizone, which are sodium-channel blockers (Lapied et al., 2001; Dong, 2007). Cases of resistance for most of the classes of insecticides, especially pyrethroids, have been reported for different pest species worldwide (Dong, 2007).

Members of the order Lepidoptera are responsible for 4,425 cases of resistance reported among all the insect orders; *Plutella xylostela* has the most, 862 cases, followed by the cotton bollworm *Helicoverpa armigera* with 856 cases (Arthropod Pesticide Resistance Database, 2018). The cotton bollworm is a generalist and worldwide-distributed pest, and causes serious damage to many economically important crops around the world (Fitt, 1989). In many countries of Oceania, Asia, Africa and Europe, this is a major pest attacking cotton, chickpea, tomato and sorghum (Anderson et al., 2016).

In 2013 *H. armigera* was detected for the first time in the Americas, causing serious damage to soybean and cotton crops in Bahia and Goiás states in Brazil (Czepak et al., 2013; Specht et al., 2013). However, some records confirm that this pest has been present in Brazil since 2008 (Sosa-Gómez et al., 2016). After that, this pest spread rapidly in a short period of time, invading other countries in South and Central America including Argentina, Paraguay, Uruguay and Puerto Rico (Leite et al., 2014; Mastrangelo et al., 2014; Murúa et al., 2014; Kriticos et al., 2015; Arnemann et al., 2016; Pearce et al., 2017). In 2015 a single moth was detected in a tomato plantation in Florida, causing a pest alert in the USA (Hayden and Bambila, 2015).

Brazilian crops are attacked by several economically important lepidopteran pests, e.g. *Spodoptera frugiperda*, *Helicoverpa zea*, *Heliothis virescens* and *Chrysodeixis includens*. However, *H. armigera* has become one of the most destructive and difficult pests to control, mainly because most of the insecticides recommended by Brazilian government agencies for

emergency use against this pest have cases of resistance reported in the countries where this species originated. Furthermore, the cotton bollworm has proved to be more tolerant to conventional insecticides than the other lepidopteran pests in Brazil (Durigan et al., 2017).

H. armigera populations in Brazil arrived in multiple invasions from Pakistan, India, China and some European countries, and in these countries, resistance evolved rapidly to most of the insecticides recommended to control *H. armigera* in Brazil (Tay et al., 2013; Leite et al., 2014; Anderson et al., 2016; Tay et al., 2017; Pearce et al., 2017). Resistance of *H. armigera* to pyrethroids has been reported in Australia, China, India and Pakistan, and recently in Brazil (Gunning et al., 1984; Forrester, 1990; Pittendrigh, 1997; McCaffery, 1998; Martin et al., 2002; Grubor and Heckel, 2007; Djihinto et al., 2009; Joußen et al., 2012; Rasool et al., 2014; Qayyum et al., 2015; Bird, 2015; Anderson et al., 2016; Durigan et al., 2017; Bird, 2018). A total of 18 cases of *H. armigera* resistance to indoxacarb have been reported in the Old World, in Australia, China and Pakistan (Aheer et al., 2009; Qayyum et al., 2015; Bird, 2015; Bird, 2017; Wang et al., 2017).

Considering that *H. armigera* is an invasive, polyphagous and destructive pest and also has a background indicating a high capacity to evolve resistance to many insecticides, becoming difficult to control, the main objectives of this study were to characterize and monitor the susceptibility to pyrethroids and indoxacarb in populations of *H. armigera* in Brazil, in addition to investigating and elucidating possible mechanisms of resistance associated with pyrethroid resistance, as well as inheritance patterns. The results provided enough information to manage this pest, to implement an insect resistance-management (IRM) program, and also to prevent the evolution of *H. armigera* resistance to insecticides in Brazil.

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2. HIGH FREQUENCY OF *CYP337B3* GENE ASSOCIATED WITH CONTROL FAILURES OF *Helicoverpa armigera* WITH PYRETHROID INSECTICIDES IN BRAZIL

Abstract

Control failures with the use of pyrethroid insecticides have been reported frequently for populations of *Helicoverpa armigera* (Hübner) in Brazil, since its detection in 2013. Here, we confirmed and investigated the metabolic mechanisms of pyrethroid resistance in *H. armigera* populations from Brazil. Mortality of *H. armigera* populations was lower than 50% at the highest dose (10 μg a.i./3rd instar larva) of the pyrethroids deltamethrin and fenvalerate in dose-response bioassays. Very low mortality (10 to 40%) was obtained at a diagnostic dose of 10 μg a.i./larva for each pyrethroid in *H. armigera* populations collected from different agricultural regions in Brazil, from 2013 to 2016. In synergist bioassays, when larvae were treated with PBO synergist, the mortality of all populations tested was 100%. The frequency of the cytochrome P450 *CYP337B3* gene was above 0.95 in all populations of *H. armigera*. We found only fourteen heterozygous *H. armigera* out of 497 individuals tested for this gene subfamily. Our results indicated that *H. armigera* populations from Brazil have different degrees of susceptibility to deltamethrin and fenvalerate, but all populations can be considered tolerant to pyrethroid insecticides. The chimeric P450 *CYP337B3* enzyme is one of the main mechanisms of pyrethroid resistance in Brazilian *H. armigera* populations.

Keywords: Cotton bollworm; Insect resistance management; Metabolic detoxification; Pyrethroid resistance

*This chapter has been already published at Pesticide Biochemistry and Physiology. Reference: **Durigan, M.R.**; Corrêa, A.S.; Pereira, R.M.; Leite, N.A.; Amado, D.; Sousa, D.R.; Omoto, C. High frequency of *CYP337B3* gene associated with control failures of *Helicoverpa armigera* with pyrethroid insecticides in Brazil. Pesticide Biochemistry and Physiology, 2017.

2.1. Introduction

The cotton bollworm *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) was officially detected for the first time in the Americas in 2013, when larvae were collected under soybean and cotton plants in central Brazil (Czepak et al., 2013; Tay et al., 2013). *H. armigera* has become widely distributed across South America and was recently detected in Puerto Rico and in Florida, USA (Leite et al., 2014; Mastrangelo et al., 2014; Murúa et al., 2014; Hayden, J.E.; Bambila, 2015; Kriticos et al., 2015; Arnemann et al., 2016). Even before *H. armigera* invaded the Americas, it was frequently reported as a serious agricultural pest with a wide host range in Oceania and other Old World countries (Fitt, 1989; Tay et al., 2017). *H. armigera* individuals from Brazil share their genetic material with individuals from Asia, Europe, and Africa, suggesting multiple invasive events in Brazil (Leite et al., 2014; Anderson

et al., 2016; Tay et al., 2017).

H. armigera has become a primary pest of soybean and cotton crops, and its larvae have also caused damage in bean, maize, sorghum and vegetable crops in Brazil (Czepak et al., 2013; Specht et al., 2013; Murúa et al., 2016). This species is more widely polyphagous and tolerant to insecticides than the native lepidopteran pests (Carvalho et al., 2013; Stanley et al., 2009). Therefore, since *H. armigera* was detected, the frequency and dosages of insecticide sprayings and the adoption of Bt genetically modified crops have increased in Brazil.

Pesticide resistance has been reported in *H. armigera* populations around the world (Sparks and Nauen, 2015). The Arthropod Pesticide Resistance Database (2017) (Arthropod Pesticide Resistance Database, 2017) records 763 cases of resistance to 49 different active ingredients, especially pyrethroid insecticides. The multiple selection events of resistance to different insecticides in *H. armigera* populations are associated with intrinsic characteristics of this species, such as its polyphagous feeding habit, short development cycle, high dispersal capacity, and high genetic variability (Fitt, 1989; McCaffery, 1998; Feng et al., 2005).

Resistance to pyrethroid insecticides has been frequently reported in *H. armigera* populations since the 1970s (Forrester, 1990; Pittendrigh, 1997; McCaffery, 1998; Grubor & Heckel, 2007; Djihinto et al., 2009; Brun-barale et al., 2010; Joußen et al., 2012; Rasool et al., 2014; Bird, 2015; Qayyum et al., 2015; Anderson et al., 2016). The main mechanisms of pyrethroid resistance in insects are linked to reduced target-site sensitivity and enzymatic detoxification of insecticide molecules (Gunning et al., 1984; Gunning et al., 1991; Yang et al., 2004; Joußen et al., 2012). Specifically in *H. armigera*, pyrethroid resistance has been associated with the increase of insecticide detoxification by cytochrome P450 monooxygenase enzymes in different regions of the world (Pittendrigh, 1997; Grubor & Heckel, 2007; Feyereisen, 2012; Joußen et al., 2012; Heckel, 2012; David et al., 2013; Liu et al., 2014; Rasool et al., 2014; Han et al., 2015; Xu et al., 2016).

The high selection pressure imposed by the frequent insecticide applications in the field, and the resistance history of *H. armigera* populations in their native geographic areas presage a rapid evolution of pyrethroid resistance in Brazilian agricultural crop systems. In fact, growers and companies have frequently reported insecticide control failures for *H. armigera* in central and northeastern Brazil. It is crucial to understand *H. armigera* pyrethroid susceptibility in order to contribute to insect resistance management (IRM) programs.

For these reasons, we evaluated the susceptibility of *H. armigera* populations from Brazil to two pyrethroid insecticides, deltamethrin and fenvalerate. Then, we monitored the

susceptibility to these insecticides in *H. armigera* populations collected in central and northeastern Brazil from 2013 to 2016. We also investigated the mechanisms involved in *H. armigera* pyrethroid survival. We carried out bioassays with insecticide synergists, and estimated the frequency of the chimeric P450 enzyme *CYP337B3* gene, previously reported as the main genetic mechanism of pyrethroid resistance in *H. armigera* populations in the Old World and Australia (Joußen et al., 2012; Rasool et al., 2014; Daly & Fisk, 1992).

2.2 Material and Methods

2.2.1. Insect sampling

H. armigera larvae were sampled in the main growing regions from 2013 to early 2016, in cotton, soybean, bean, millet and maize. Each population consisted of ≈ 500 to 1000 larvae. For each field-collected sample, we conducted a molecular identification to distinguish *H. armigera* from the native *H. zea*, through the PCR-RFLP technique described by Behere et al. (2008). Field-collected larvae were reared for at least one generation under controlled laboratory conditions of 25 ± 1 °C, 70 ± 10% RH, and 14 h of photophase. Pupae were pretreated with a copper sulfate solution (10%) and were maintained in cylindrical cages made out of PVC tubes (30 cm high × 25 cm diameter) covered with a tulle type material until the emergence of adults. Afterwards, moths were also maintained in the same type of cages with 40 couples each. Eggs were collected from the tulle material, where the females laid their eggs. Neonate larvae were placed in plastic containers containing an artificial diet adapted from Greene et al. (1976). Larvae were kept in the plastic containers containing artificial diet until they reached the third instar, when they were used in the toxicity bioassays.

2.2.2 Dose-mortality response bioassays with pyrethroids

Dose-mortality response bioassays were performed using a topical bioassay with third-instar larvae. For each *H. armigera* population (Table 1: BA33, BA43, BA44, BA45, BA49, GO02, GO03, MS05 and MT11), six to eight doses (1 to 10 μg a.i./larva) of deltamethrin (purity: 98.5%) and fenvalerate (purity: 98.6%) dissolved in 1 μL of acetone were tested. After insecticide application, larvae were individually placed in a 12-well acrylic plate (Costar®) containing artificial diet. Forty-eight larvae were tested for each insecticide concentration. The plates were kept under controlled conditions in a climate-controlled chamber at 25 ± 1 °C, 60 ± 10% RH, and a photoperiod of 14:10 h. Mortality was assessed after 48 h and data

were subjected to a Probit analysis (Finney, 1971) using SAS Software (SAS, 2000) in order to determine the regression lines and slopes, and also to estimate the median and 90th percentile lethal doses (LD_{50} and LD_{90}).

Table 1. Population code, year of collection, crop and location of *H. armigera* populations from major Brazilian producer regions.

Population code	Collection year	Crop	City, State[a]	Latitude (S)	Longitude (W)
BA 27	Feb-2013	Maize	Barreiras - BA	12º09'10"	44º59'24"
[b]BA 33	Jun-2013	Bean	Luis Eduardo Magalhães - BA	12°05'58"	45°47'54"
BA 34	Jan-2014	Soybean	São Desidério - BA	12º21'48"	44º58'24"
[b]BA 43	Jan-2014	Soybean	Luis Eduardo Magalhães - BA	12°05'58"	45°47'54"
[b]BA 44	Feb-2014	Cotton	Luis Eduardo Magalhães - BA	12°05'58"	45°47'54"
[b]BA 49	May-2014	Maize	Correntina - BA	13º20'36"	44º38'12"
BA 52	Nov-2014	Soybean	São Desidério - BA	12º21'48"	44º58'24"
BA 61	Apr-2015	Millet	São Desidério - BA	12º21'48"	44º58'24"
BA 64	Jun-2015	Cotton	Luis Eduardo Magalhães - BA	12°05'58"	45°47'54"
BA 66	Oct-2015	Bean	Luis Eduardo Magalhães - BA	12°05'58"	45°47'54"
BA 69	Jan-2016	Soybean	Luis Eduardo Magalhães - BA	12°05'58"	45°47'54"
BA 70	Mar-2016	Soybean	Luis Eduardo Magalhães - BA	12°05'58"	45°47'54"
GO 02	Mar-2014	Soybean	Mineiros - GO	17º34'10"	52º33'04"
[b]GO 03	Mar-2014	Soybean	Cristalina - GO	16º46'07"	47º36'49"
GO 04	Jan-2015	Soybean	Montividiu - GO	17º26'39"	51º10'29"
GO 05	Feb-2015	Soybean	Santo Antonio do Rio Verde - GO	18º09'57"	47º56'47"
GO 06	Mar-2016	Soybean	Mineiros - GO	17º34'10"	52º33'04"
MA 04	Jan-2013	Soybean	Balsas - MA	07º31'57"	46º02'08"
MS 03	May-2013	Cotton	Chapadão do Sul - MS	18º47'39"	52º37'22"
[b]MS 05	Oct-2013	Cotton	Costa Rica - MS	18º32'38"	53º07'45"
MS 08	Dec-2016	Soybean	Chapadão do Sul - MS	18º47'39"	52º37'22"
MT 06	Nov-2012	Soybean	Rondonópolis - MT	16º28'15"	54º38'08"
[b]MT 11	Oct-2014	Soybean	Primavera do Leste - MT	15º33'32"	54º17'46"
MT 15	May-2015	Cotton	Campo Verde - MT	15º32'48"	55º10'08"
MT 16	May-2015	Cotton	Sapezal -MT	12º59'22"	58º45'51"
MT 17	Jan-2016	Soybean	Sapezal -MT	12º59'22"	58º45'51"
MT 19	Mar-2016	Soybean	Primavera do Leste - MT	15º33'32"	54º17'46"
MT 23	May-2016	Millet	Primavera do Leste - MT	15º33'32"	54º17'46"
MT 27	Oct-2016	Soybean	Nova Mutum - MT	13º49'44"	56º04'56"
PR 05	Feb-2015	Soybean	Londrina - PR	23º18'37"	51º09'46"
RS 02	Mar-2014	Soybean	Itaara - RS	29°36'31"	53°45'55"
SP 15	Jan-2015	Soybean	Viradouro - SP	20º52'23"	48º17'49"
SP 19	Apr-2016	Bean	Limeira - SP	22º33'53"	47º24'06"

[a] State abbreviations: BA, Bahia; GO, Goiás; MA, Maranhão; MS, Mato Grosso do Sul; MT, Mato Grosso; PR, Paraná; RS, Rio Grande do Sul; SP, São Paulo. [b] Populations used in the Baseline Susceptibility bioassays.

2.2.3. Monitoring the susceptibility of *H. armigera* to pyrethroids over growing seasons

The dose of 10 µg a.i./larva of each insecticide was used as a diagnostic dose for monitoring the susceptibility to pyrethroids in *H. armigera* populations collected from different Brazilian agricultural regions. This dose is five times higher than the LC_{50} of 1.864 µg a.i./larva found by Joußen et al. (2012) in a fenvalerate-resistant strain of *H. armigera* from Australia. In each of 15 populations, 240 larvae were monitored through the growing seasons from 2013 to 2016 (Table 1). Larval survival obtained from monitoring was subjected to a multiple comparison analysis, using the many-to-one comparison procedure of Dunnett (1955) using SAS Software (SAS, 2000). All the populations tested were compared with the same control in order to evaluate the evolution of the susceptibility since the detection of *H. armigera* in Brazil. Population BA33, the population collected in Bahia State at the time of the official report of *H. armigera* in Brazil in 2013, was used as the control. This BA33 population is our reference for population susceptibility to insecticides.

2.2.4. Synergist bioassays

Third-instar larvae were treated with the synergists piperonyl butoxide (PBO, Sigma Aldrich, 90%), diethyl maleate (DEM, Sigma Aldrich, 97%) and S,S,S-tributyl phosphorotrithiotate (DEF, Chem Service, 97.2%). The synergists were diluted in pure acetone at a dose of 1 µg a.i./larva each and were applied topically (1 µL on the dorsum of the larva) 2 h before the insecticide application. We tested four treatments for each synergist: (a) Control: acetone; (b) synergists (PBO, DEM and DEF at 1 µg a.i./larva); (d) diagnostic dose of insecticide (10 µg a.i./larva) (c) synergists + diagnostic dose of insecticide (10 µg a.i./larva). Thirty-six larvae each of three different populations (BA33, BA43, and BA52) were tested in each treatment, and mortality was assessed 48 h after application. The results were subjected to the analysis of variance (ANOVA) and means were compared using a Tukey test ($p = 0.05$; SAS, 2000).

2.2.5. Screening of the P450 *CYP337B* gene subfamily

2.2.5.1. Insect sampling and DNA extraction

Screening for the different members of the P450 *CYP337B* gene subfamily was carried out in 33 *H. armigera* populations collected from 2012 to 2016, resulting in 497 individuals

from different crops, years and regions (Table 2). The insects were frozen at −20 °C and genomic DNA was extracted from thoracic tissue of moths using the CTAB method adapted from Clark et al. (2001). DNA concentrations were estimated and diluted to concentration of 10 ng/μL.

2.2.5.2. PCR for detection of the P450 *CYP337B* gene subfamily

Screening for the *CYP337B1*, *CYP337B2* and *CYP337B3* gene subfamilies frequency followed the methodology and three specific primers described by Joußen et al. (2012). PCR amplification was performed with 20 ng of total DNA, 37.5 mM MgCl2 , 2.5 mM dNTPs, 20 pmol of each primer, 1U Taq DNA Polymerase (Life Technologies, Carlsbad, CA, USA), and 10% 10× Taq Buffer and MiliQ water in a total volume of 25 μL. The PCR cycles followed an initial denaturation of 94 °C during 5 min followed by 30 cycles of 30 s at 94 °C, 30 s with the annealing at 55 °C, elongation at 72 °C for 1.5 min, with a final extension step at 72 °C for 10 min. PCR products (2 μL) were observed in an agarose gel (1.5% w/v) stained with SYBR Safe. Afterwards, the presence or absence of each of the three genes (*CYP337B1*, *CYP337B2* or *CYP337B3*) was used to calculate gene frequencies in each population.

2.2.5.3. Sequence analysis

Positive PCR products of each gene in the *CYP337B* subfamily (*CYP337B1*, *CYP337B2* and *CYP337B3*) were sequenced to confirm the PCR specificity (GenBank accession number MF435019–MF435037). The samples were purified with the ExoSap enzymes (Invitrogen). The reactions of purification were incubated for 30 min at 37 °C and then 15 min at 80 °C to denature the enzymes. The sequencing of the samples was performed by the Animal Biotechnology Laboratory from ESALQ/ USP (Piracicaba, SP, Brazil) by the Sanger Sequencing method. The sequencing data were edited and aligned manually with Sequencher 4.8 software (Genes Code Corporation, Ann Arbor, MI, USA) and compared with subfamilies sequences available from GenBank NCBI using the BLASTn tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Afterwards, Bayesian phylogenetic analysis was carried to compare Brazilian *CYP337B3* subfamily alleles of *H. armigera* with others one originated from Australia (JQ284029) (Joußen et al., 2012), China (KM675664, KM675665 and, KM675666) (Han et al., 2015) and Pakistan (KJ636466) (Rasool et al., 2014). All sequences were aligned and interrupted at 328 bp to phylogenetic analysis. We used the

software PAUP* 4.0b10 (Swofford D., 2002) and modeltest 2 (Nylander, 2004) to selected the substitution model using the Akaike information criteria (Akaike, 1974). The Bayesian phylogenies was estimated using Mrbayes, version 3.2 (Ronquist and Huelsenbeck, 2003) with 10million generations using two independent runs with one cold and three heated chains. The *CYP337B2* subfamily allele JQ284028 was defined as outgroup. The first 25% and the last 25% of tree were discarded as burn-ins; the remaining 50% trees were used to construct a consensus tree with Bayesian posterior probabilities observed in the software Figtree version 1.3.1 (Rambaut, 2009).

2.3. Results

2.3.1. Dose-mortality response bioassays with pyrethroids

For all nine *H. armigera* populations tested, the maximum dose tested (10 μg a.i./larva) resulted in mortality lower than 50% of the individuals. Therefore, it was not possible to estimate the LD_{50} or LD_{90}. The maximum mortality caused at 10 μg a.i./larva was 40% for both insecticides.

2.3.2. Susceptibility monitoring through growing seasons

In the monitoring of *H. armigera* susceptibility to deltamethrin, all 15 populations tested showed high survival, above 40% (Fig. 1). The highest survival was observed in population BA69 (93% ± 0.44%) collected during the 2015–2016 growing season. According to Dunnett's test, populations BA43 (50% ± 1.96%) and BA66 (40.4% ± 2.3%) differed from the control sampled in 2013 (BA33, 75% ± 1.83%), showing lower survival for deltamethrin than the control population.

For fenvalerate, population BA43, sampled in 2014, was the most susceptible (40% ± 1.4%) and differed from the control (BA33, 70% ± 1.37%). Populations MT11 (90% ± 1.83%), GO06 (92% ± 2.47%) and MS08 (92% ± 0.81%) also differed from BA33, but showed higher survival than the control. For both insecticides, a decrease was observed of the susceptibility throughout the cropping years.

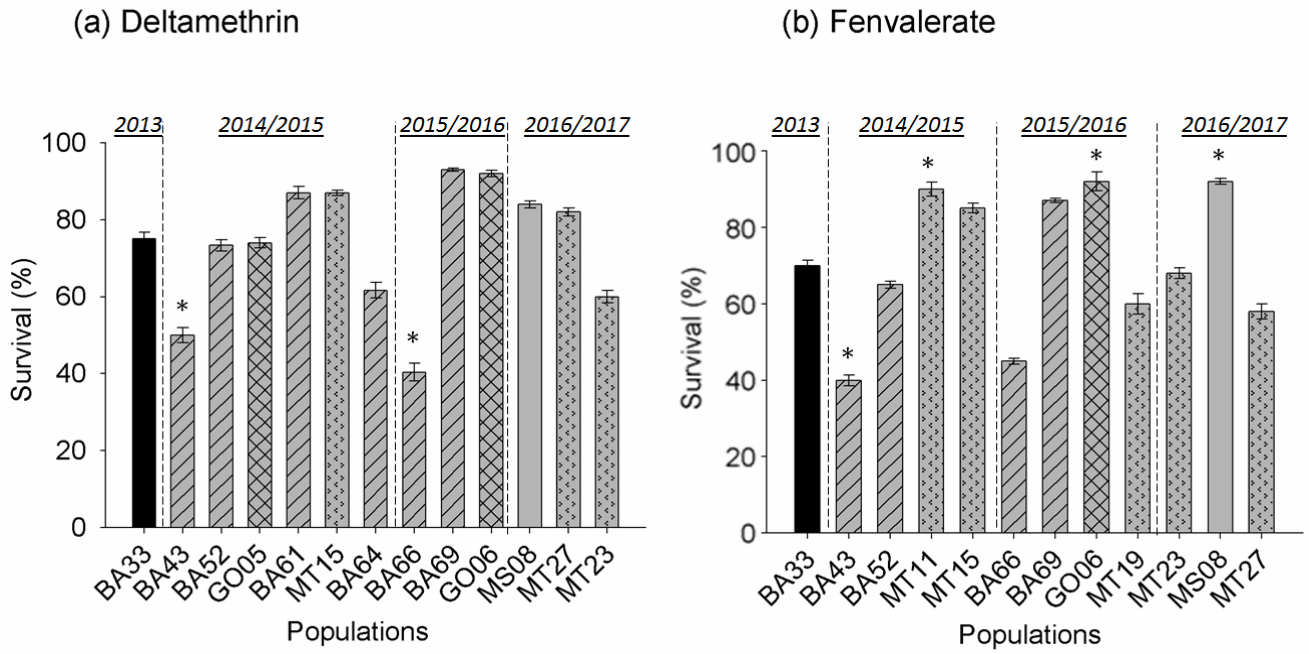

Fig. 1. Survival of *H. armigera* larvae monitored through the crop years with the diagnostic dose of 10 µg a.i./larva of deltamethrin (a) and fenvalerate (b) using a topical bioassay. ■ Population used as a control, collected in 2013; ▨ Populations collected in Bahia State; ▩ Populations collected in Goiás State; ▩ Populations collected in Mato Grosso State; ▭ Populations collected in Mato Grosso do Sul State. * Populations that differ from the control (BA33), by Dunnett's test. *n*=240 larvae.

2.3.3. Synergist bioassays

For the insecticide deltamethrin all the populations had 100% mortality when the larvae were treated with PBO (Fig. 2a, b and c). The treatment with DEF also showed a synergism with deltamethrin presenting mortality values ranging from 79% ± 0.17% to 100%. DEM applied before deltamethrin resulted on an intermediate mortality going from 42.42% ± 0.20% to 65% ± 0.36%. Tukey's test showed that mortality obtained from the treatment with the diagnostic dose + PBO differed from the treatment that used only the diagnostic dose (Fig. 2a, b and c). No significant differences were observed between the treatments with deltamethrin (10 µg a.i./larva) and pretreatment with DEM.

For fenvalerate when only the diagnostic dose (10 µg a.i./larva) was applied the mortality ranged from 36.11% ± 0.63% to 47.22% ± 0.09%. In the treatment with the diagnostic dose of fenvalerate + PBO the populations showed a mortality between 97.22% ± 0.10% and 100%. Mortality at the diagnostic dose of fenvalerate + DEF treatment was between 47% ± 0.09% to 83.33% ± 0.17%, while the treatment with diagnostic dose of fenvalerate + DEM showed a mortality from 37.14% ± 0.26% to 50% ± 0.60% (Fig. 2d, e and f). No mortality was observed when we applied only the synergists. Based on Tukey's test, for all populations the treatment with the diagnostic dose of fenvalerate + PBO showed higher

mortality than only the diagnostic dose of fenvalerate treatment, however the application with DEM had no effect on the insects mortality. Therefore, for both insecticides the toxicity increased when PBO synergist was used as pretreatment followed by the DEF pretreatment.

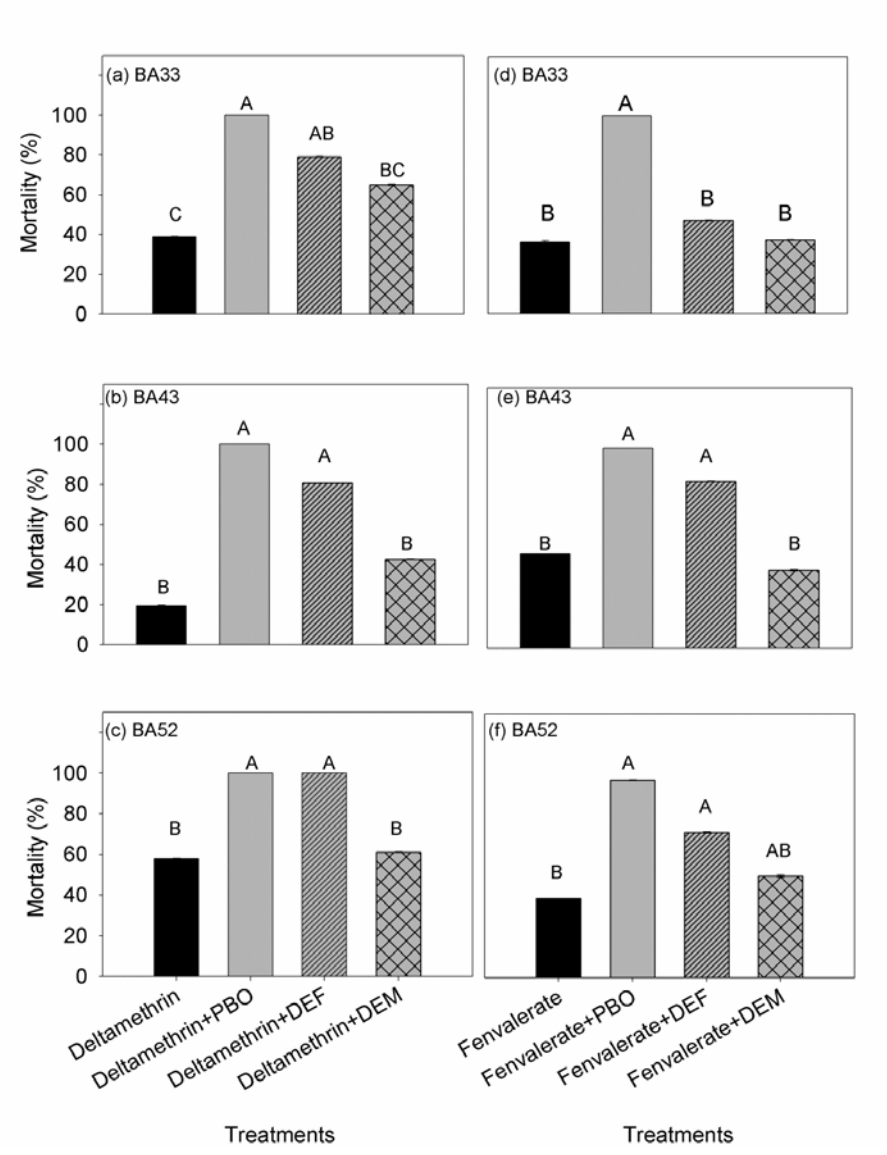

Fig. 2. Effect of synergist (PBO, DEF and DEM) treatment on *H. armigera* (third-instar larvae) mortality to deltamethrin (a, b and c) and fenvalerate (d, e and f). *Bars followed by the same letter do not differ from each other according to Tukey's test ($p< 0.05$).

3.4. *CYP337B* gene subfamily frequencies in Brazilian populations of *H. armigera*

All insects screened were positive for the chimeric P450 *CYP337B3* gene subfamily. Of the total populations screened (33), 21 showed a *CYP337B3* frequency equal to 1.0 (Fig. 3). Of the 994 alleles screened, 980 were positive for *CYP337B3*, eight positive for

CYP337B2 and six positive for *CYP337B1*. The *CYP337B2* gene was found in the populations collected in Bahia state (BA33, BA 44, BA66, BA69 and BA70: f = 0.025) and in Mato Grosso state (MT11: f = 0.025; MT23: f = 0.042; MT27: f = 0.071) which were heterozygous with *CYP337B3*. The *CYP337B1* was positive in populations collected in Bahia state (BA27: f = 0.028; BA34: f = 0.056 and BA44: f = 0.025), Mato Grosso state (MT27: f = 0.071) and Mato Grosso do Sul state (MS03: f = 0.038; MS05: f = 0.029). The individuals who were positive for *CYP337B1* and *CYP337B2* were also positive for *CYP337B3*, i.e. heterozygous. No frequency differences were observed through the years sampled, indicating that the *CYP337B3* frequency has always been high since the introduction of this species into Brazil.

The phylogenetic analysis indicates that Brazilian *CYP337B3* subfamily alleles of *H. armigera* are closer to Chinese and Pakistan when compared to the Australian allele (Fig. 4). The Brazilian *CYP337B3* alleles MF435033, MF435034, MF435035, and MF435036 have 100% of identity to Chinese allele KM675664 and 99% identity to Pakistan and Australia allele when compared for the BLASTn tool. The Brazilian *CYP337B3* allele MF435037 has 99% of identity with Chinese (KM675664 and KM675665) and Pakistan (KJ636466) alleles and 98% of identity with the Australian allele.

Table 2. Population code, year of collection, crop, location, total of insects screened and *CYP337B1*, *CYP337B2* and *CYP337B3* gene frequencies in *H. armigera* populations in Brazil.

Population code	Collection year	Crop	Colletion site	Total insects screened	*CYP337B1* frequency	*CYP337B2* frequency	*CYP337B3* frequency
BA 27	Feb-2013	Maize	Barreiras - BA	18	0.028	0.000	0.972
BA 33	Jun-2013	Bean	Luis Eduardo Magalhães - BA	20	0.000	0.025	0.975
BA 34	Jan-2014	Soybean	São Desidério - BA	09	0.056	0.000	0.944
BA 43	Jan-2014	Soybean	Luis Eduardo Magalhães - BA	20	0.000	0.000	1.000
BA 44	Feb-2014	Cotton	Luis Eduardo Magalhães - BA	20	0.025	0.025	0.950
BA 49	May-2014	Maize	Correntina - BA	19	0.000	0.000	1.000
BA 52	Nov-2014	Soybean	São Desidério - BA	20	0.000	0.000	1.000
BA 61	Apr-2015	Millet	São Desidério - BA	04	0.000	0.000	1.000
BA 64	Jun-2015	Cotton	Luis Eduardo Magalhães - BA	15	0.000	0.000	1.000
BA 66	Oct-2015	Bean	Luis Eduardo Magalhães - BA	20	0.000	0.025	0.975
BA 69	Jan-2016	Soybean	Luis Eduardo Magalhães - BA	20	0.000	0.025	0.975
BA 70	Mar-2016	Soybean	Luis Eduardo Magalhães - BA	20	0.000	0.025	0.975
GO 02	Mar-2014	Soybean	Mineiros - GO	03	0.000	0.000	1.000

GO 03	Mar-2014	Soybean	Cristalina - GO	03	0.000	0.000	1.000
GO 04	Jan-2015	Soybean	Montividiu - GO	20	0.000	0.000	1.000
GO 05	Feb-2015	Soybean	Santo Antonio do Rio Verde - GO	08	0.000	0.000	1.000
GO 06	Mar-2016	Soybean	Mineiros - GO	20	0.000	0.000	1.000
MA 04	Jan-2013	Soybean	Balsas - MA	05	0.000	0.000	1.000
MS 03	May-2013	Cotton	Chapadão do Sul - MS	13	0.038	0.000	0.962
MS 05	Oct-2013	Cotton	Costa Rica - MS	17	0.029	0.000	0.971
MS 08	Dec-2016	Soybean	Chapadão do Sul - MS	14	0.000	0.000	1.000
MT 06	Nov-2012	Soybean	Rondonópolis - MT	07	0.000	0.000	1.000
MT 11	Oct-2014	Soybean	Primavera do Leste - MT	20	0.000	0.025	0.975
MT 15	May-2015	Cotton	Campo Verde - MT	20	0.000	0.000	1.000
MT 16	May-2015	Cotton	Sapezal -MT	20	0.000	0.000	1.000
MT 17	Jan-2016	Soybean	Sapezal -MT	20	0.000	0.000	1.000
MT 19	Mar-2016	Soybean	Primavera do Leste - MT	18	0.000	0.000	1.000
MT 23	May-2016	Millet	Primavera do Leste - MT	12	0.000	0.042	0.958
MT 27	Oct-2016	Soybean	Nova Mutum - MT	07	0.071	0.071	0.857
PR 05	Feb-15	Soybean	Londrina - PR	20	0.000	0.000	1.000

RS 02	Mar-14	Soybean	Itaara - RS	12	0.000	0.000	1.000
SP 15	Jan-15	Soybean	Viradouro - SP	15	0.000	0.000	1.000
SP 19	Apr-16	Bean	Limeira - SP	20	0.000	0.000	1.000

State abbreviations: BA, Bahia; GO, Goiás; MA, Maranhão; MS, Mato Grosso do Sul; MT, Mato Grosso; PR, Paraná; RS, Rio Grande do Sul; SP, São Paulo.

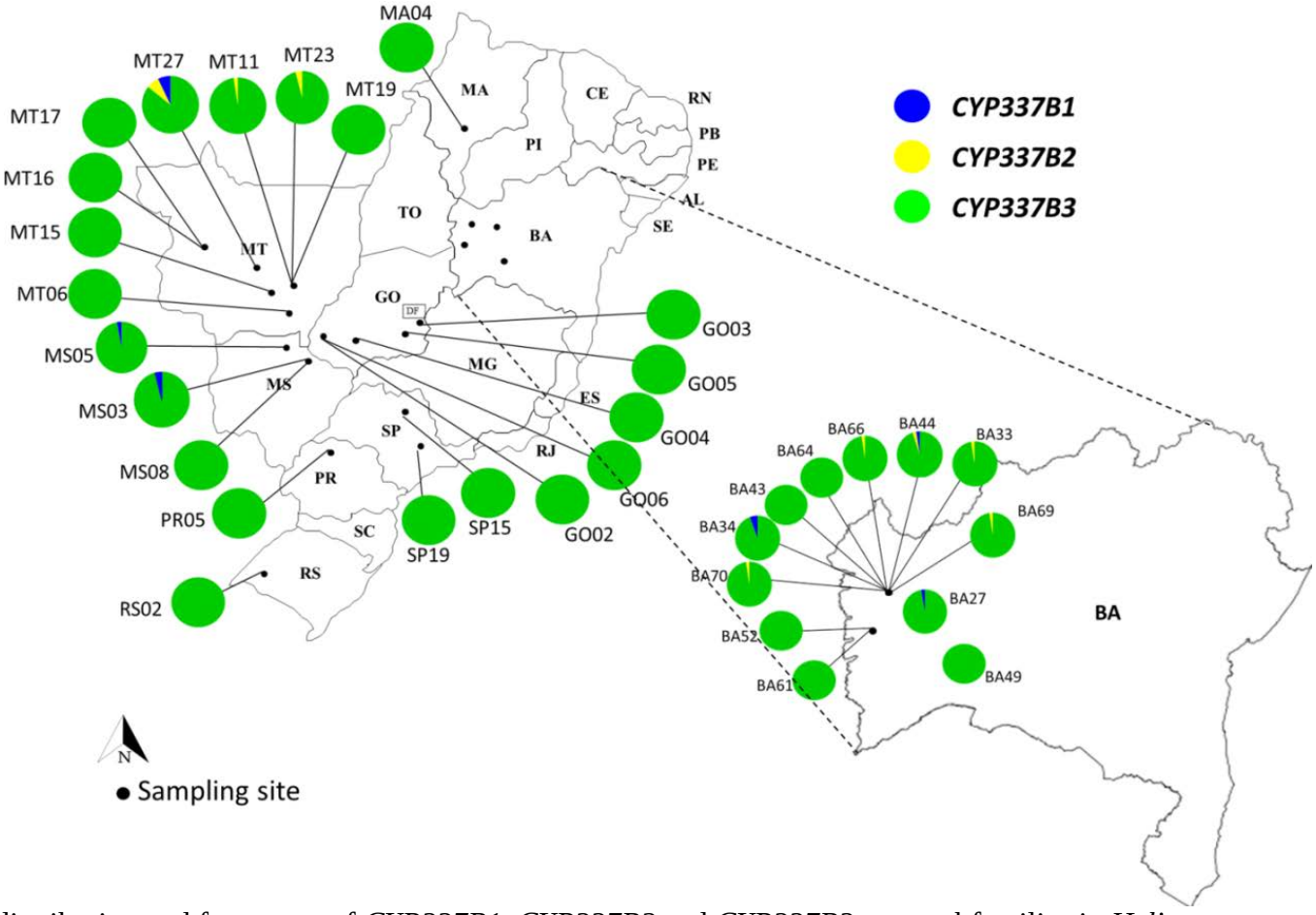

Fig. 3. Geographic distribution and frequency of *CYP337B1*, *CYP337B2* and *CYP337B3* gene subfamilies in *Helicoverpa armigera* (Lepidoptera: Noctuidae) populations in Brazil. State abbreviations: BA, Bahia; GO, Goiás; MA, Maranhão; MS, Mato Grosso do Sul; MT, Mato Grosso; PR, Paraná; RS, Rio Grande do Sul; SP, São Paulo.

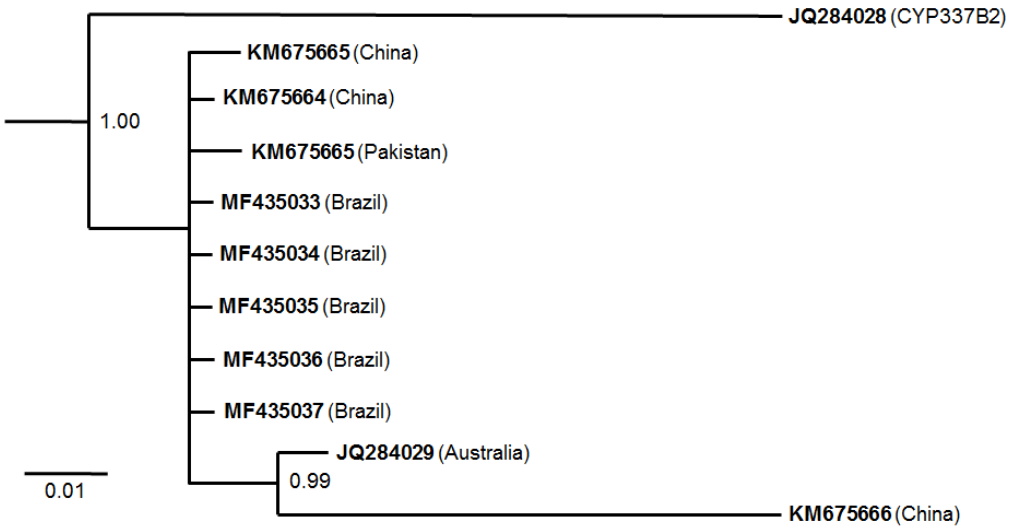

Fig. 4. Bayesian phylogeny of *CYP337B3* subfamily alleles (328 bp) of *H. armigera*. Nodes number shows Bayesian posterior probabilities supports values. Nodes below 50% (bootstrap) or 0.50 (posteriori probability) were not recorded in the tree.

2.4 Discussion

Pesticide resistance and tolerance are commonly reported traits in invasive agricultural pests (Haddi et al., 2012; Wang et al., 2013). Tolerance is considered as the natural ability of a population to resist the toxic effect of an insecticide. It can be developed within a single generation, because of physiological adaptation, as a detoxification process by enzyme induction. The resistance level may decrease when the insects cease to be exposed to the insecticide. Resistance is a genetic change in response to selection pressure by the toxic compound, where a structural genetic change occurs and is also heritable (Yu, 2008). Here, we confirmed the resistance of Brazilian *H. armigera* populations to pyrethroid insecticides. This result was expected based on the many cases of pyrethroid resistance reported in Old World countries that are possible origins of the Brazilian *H. armigera* populations (Martin et al., 2002; Mironidis et al., 2013; Yang et al., 2013; Rasool et al., 2014; Han et al., 2015; Qayyum et al., 2015; Anderson et al., 2016; Xu et al., 2016; Tay et al., 2017).

Brazilian growers have frequently reported control failures for pyrethroid insecticides in the field. This was confirmed by the high larval survival of *H. armigera* populations in our susceptibility-monitoring bioassays (40 to 90% survival for both deltamethrin and fenvalerate). Pyrethroids are also used in Brazil to control other pest species that occur in the same crop field as *H. armigera*, and most of the pyrethroids are sprayed in mixtures with other compounds such as diamide insecticides, contributing to the high selection pressure in the field and the increase in the survival of this species during the growing season.

The high pyrethroid resistance and survival in monitoring tests of *H. armigera* populations in Brazil are higher than reported in its native regions (Rasool et al., 2014; Han et al., 2015; Rossiter, 2008). This could be explained by the high frequency of resistance alleles in *H. armigera* founder individuals and the high selection pressure applied in Brazil. As soon as *H. armigera* was detected, the government agencies recommended pyrethroids as emergency insecticides to control this pest (Embrapa, 2013).

The susceptibility of the *H. armigera* populations assessed here changed over time. Populations collected in summer 2013–2014 (BA33 and BA43) were more susceptible than populations tested in later years (BA69 and GO06). Currently, pyrethroid insecticides have been replaced by diamide, spinosyn or avermectin.

The screening for P450 enzyme suppression with PBO and PCR suggested that the chimeric *CYP337B3* P450 enzyme is an important mechanism of pyrethroid resistance in Brazilian *H. armigera* populations (Joußen et al., 2012; Rasool et al., 2014). The enzyme-suppression bioassays indicate suppression of P450 and esterase enzymes in the three populations tested, which showed almost 100% mortality to both pyrethroid insecticides when exposed to PBO. Furthermore, PCR screening of the chimeric P450 enzyme *CYP337B3* gene showed a frequency higher than 95% in all 33 tested populations from different Brazilian regions. The chimeric P450 enzyme *CYP337B3* had been described as promoting cross-resistance to fenvalerate and cypermethrin, and our results suggest cross-resistance to deltamethrin (Rasool et al., 2014).

The chimeric *CYP337B3* is derived from an unequal crossover from two other P450 gene subfamilies, *CYP337B1* and *CYP337B2*, resulting in an enzyme with higher substrate specificity and capacity to detoxify pyrethroid molecules (Joußen et al., 2012). Brazilian *CYP337B3* alleles are more similar to the Chinese alleles and Pakistan allele when compared to the Australian allele. This fact reinforces the hypothesis of Asiatic origin of Brazilian *H. armigera* individuals (Leite et al., 2014; Anderson et al., 2016; Tay et al., 2017).

We detected only 14 susceptible genes (*CYP337B1* or *CYP337B2*), and found no homozygous susceptible individuals. Synergist bioassays indicate esterase enzymes as a secondary pyrethroid resistance mechanism in Brazilian *H. armigera* populations, since treatment with the synergist DEF, an inhibitor of esterase enzymes, increased the mortality of *H. armigera* individuals when applied together with deltamethrin and fenvalerate. The mortality varied among populations and insecticides, with higher mortality when DEF was applied before deltamethrin. Thus, we suggest that a secondary pyrethroid resistance mechanism confers differing pyrethroid susceptibilities among *H. armigera* populations in

Brazil. Enhanced esterase enzyme production was also reported to cause resistance in Australian and Old World *H. armigera* populations (Gunning, 1996; Gunning et al., 1999; Kranthi et al., 2001; Young et al., 2005; Achaleke et al., 2009). However, the esterase enzymes have been associated with a high fitness cost, while the chimeric P450 *CYP337B3* enzyme has not so far been shown to be associated with a fitness cost (Gunning, 1996; Gunning et al., 1999; Gunning et al., 2005; Gunning et al., 2007; Gunning & Moores, 2010).

In summary, Brazilian *H. armigera* populations showed high tolerance to pyrethroids, and our results suggest that the chimeric P450 enzyme *CYP337B3* is an important mechanism of pyrethroid resistance. However, since our diagnostic dose of 10 μg a.i./larva resulted in a lower mortality than the LC_{50} of 2 μg a.i./larva in the Australian population homozygous for *CYP337B3*, it is likely that additional P450s and perhaps esterases also contribute to pyrethroid resistance in Brazilian populations of *H. armigera*.

These important results may stimulate Brazilian growers, companies and government agencies to implement a resistance-management program using the successful example of the Australian Insecticide Resistance Management strategy for pyrethroids. Through this program, since the application of pyrethroids was strictly limited to once a year, *CYP337B3*-mediated pyrethroid resistance increased only slowly over a 10-year period (Forrester, 1990; Forrester et al., 1993). This demonstrates the importance and effectiveness of insecticide resistance-management strategies.

The high frequency of the chimeric P450 enzyme CYP337B3 gene in *H. armigera* populations indicates that pyrethroid insecticides should not be recommended in Brazil or in the rest of the Americas, due to the high dispersal capacity and gene flow among *H. armigera* populations (Feng et al., 2005; Jones et al., 2015; Anderson et al., 2016; Leite et al., 2016). Resistance-management programs for chemical insecticides and *Bt* are crucial and must be implemented with urgency to avoid the loss of commercial products that will make it more difficult to manage this invasive species in the field.

3. Conclusions

- ✓ *H. armigera* populations in Brazil are highly resistant to pyrethroids;
- ✓ *CYP337B3* alleles found in Brazil are the same found in China and Pakistan confirming its pest origin;
- ✓ Pyrethroids should not be recommended to control this pest.

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3. INHERITANCE OF PYRETHROID RESISTANCE IN *Helicoverpa armigera* (LEPIDOPTERA: NOCTUIDAE) IN BRAZIL

Abstract

Knowledge of inheritance of insecticide resistance can contribute to understand the rate of evolution of resistance in the field. Resistance of *Helicoverpa armigera* (Hübner) to pyrethroids has reported from several continents and more recently in Brazil. Here, we explored the genetic basis of this resistance. Reciprocal crosses and backcrosses with susceptible (TWB3) and resistant (BA43) strains of *H. armigera* to pyrethroids were conducted to estimate the degree of dominance and evaluate whether resistance is autosomal or not and the number of genes involved in this resistance. The LC_{50} values for the pyrethroid fenvalerate were 0.161 μg a.i./larva for the susceptible strain (TWB3) and 125.53 μg a.i./larva for the resistant strain (BA43), and the resistance ratio was 780-fold. LC_{50} values for crosses ♂TWB3 × ♀BA43 and ♀TWB3 × ♂BA43 were 40.72 μg a.i./larva and 72.16 μg a.i./larva, respectively. Larvae from the backcrosses (♂H1_F × ♀TWB3 and ♂H1_M5 × ♀TWB3) exposed to the diagnostic dose of 10 μg a.i./larva showed a mean survival of 53%. Dominance degree ranged from 0.66 to 0.92, i.e. suggesting incomplete to complete dominance. Resistance of *H. armigera* to pyrethroids in Brazil was autosomal and polygenic. These results explain field failures with the use of pyrethroids to control *H. armigera* in Brazil.

Keywords: Cotton bollworm; Resistance inheritance; Fenvalerate; Insect resistance management

3.1 Introduction

The cotton bollworm *Helicoverpa armigera* (Hübner) rapidly evolved resistance to pyrethroids, as reported in several countries (Gunning et al., 1984; Forrester, 1990; Forrester et al., 1993; Djihinto et al., 2009; Martin et al., 2010; Joußen et al., 2012; Rasool et al., 2014; Bird, 2015; Qayyum et al., 2015; Durigan et al., 2017). The rate of evolution of resistance are associated with genetic variability and high selection pressure (Roush and McKenzie, 1987). Biological, operational and genetic factors also contribute to the rates of resistance evolution (Georghiou, 1972; Georghiou & Taylor, 1977a; Georghiou & Taylor, 1977b; Roush & Daly, 1990). Only the operational factor can be manipulated by implementing resistance-management strategies to prevent or reverse resistance. Biological factors involve intrinsic characteristics such as life-cycle duration, mating success and egg fertility of a species. Genetic factors are associated with inheritance patterns involving the degree of dominance, whether resistance is autosomal or not, and the number of genes involved in resistance (Georghiou & Taylor, 1977a; Georghiou & Taylor, 1977b).

Knowledge of the genetics of resistance is essential to understand how the rate of evolution of resistance will be affected and if resistance will develop over a longer or shorter

period (Georghiou, 1972; Taylor and Georghiou, 1979). If the degree of dominance is fully dominant, autosomal and monogenic resistance will evolve more rapidly, since the heterozygotes (RS) will show a resistant phenotype (Tabashnik et al., 2003). Heterozygote individuals are the main carriers of the resistance allele, so if RS individuals are equally resistant, it will be necessary to use high pesticide doses to control the heterozygotes and reduce the frequency of the R allele in the field (Roush and McKenzie, 1987; Tabashnik et al., 2003). This type of information is crucial for the implementation of Insect Resistance Management (IRM) programs, and can also help to estimate the correct dose for field use and to prevent resistance evolution.

The insecticide fenvalerate came into use in Australian cotton fields in 1977, with a high application frequency that resulted in high selection pressure. Five years later, in 1983, the first report of *H. armigera* resistance to fenvalerate in Australia appeared (Gunning et al., 1984), and the frequency of the resistant allele was very high in populations of this pest all over the country. As fenvalerate had become inefficient in controlling *H. armigera*, programs to manage and reverse this resistance situation were needed (Forrester, 1990; Forrester et al., 1993). A few years later, by conducting crosses and backcrosses between pyrethroid-resistant and susceptible strains, Gunning et al. (1996) found that pyrethroid resistance in Australia was incompletely dominant and autosomal. Since this discovery, several studies have been conducted in order to understand how the genetics of *H. armigera* resistance to pyrethroids can contribute to the rapid evolution of resistance in the field (Daly and Fisk, 1992; Ru et al., 1998; Achaleke and Brévault, 2009).

In Brazil, resistance of *H. armigera* to pyrethroids was reported, associated with a frequency of the chimeric P450 gene *CYP337B3* higher than 95% (Durigan et al., 2017). Most *H. armigera* populations in Brazil showed LC_{50} values for fenvalerate higher than 10 µg a.i./larva, a very high concentration compared to other LC_{50} values reported in China, Australia and Pakistan (Wu et al., 2006; Joußen et al., 2012; Rasool et al., 2014; Qayyum et al., 2015). The high tolerance of Brazilian populations to pyrethroids suggests that not only the chimeric *CYP337B3* gene is contributing to *H. armigera* resistance in Brazil, but also additional mechanisms of resistance are involved.

The genetics of pyrethroid resistance of *H. armigera* populations in Brazil must be elucidated in order to generate data for the implementation of an Insect Resistance Management (IRM) program in this and other New World countries recently invaded by this pest. We used a highly resistant strain to characterize the genetic basis of pyrethroid resistance in *H. armigera* in Brazil, where the inheritance pattern was assessed after crossing and

backcrossing with a susceptible strain.

3.2 Material and Methods

3.2.1 *H. armigera* strains

The Brazilian pyrethroid-resistant strain (BA43) was collected in January 2014 from a soybean field in Luís Eduardo Magalhães, Bahia State, and has been reared in the Arthropod Resistance Laboratory at the Luiz de Queiroz College of Agriculture (ESALQ-USP) in Piracicaba, São Paulo. In each generation, third-instar larvae from the BA43 strain were treated with fenvalerate at 10 µg a.i./larva to maintain the selection pressure. As most of the populations in Brazil are resistant to pyrethroids, and the greatest challenge is to find a strain that is more susceptible to this group of insecticides (Durigan et al., 2017), the BA43 strain was sent to the Department of Entomology at the Max Planck Institute for Chemical Ecology in Jena, Germany (Export permit: 17BR023456/DF) to investigate the differences in resistance patterns between the Brazilian pyrethroid-resistant and the Australian strain, which has been reared in the German laboratory since 2003 without selection pressure. The Australian strain (TWB3) was collected in Towoomba, Queensland in January 2003. This strain also possess the chimeric P450 gene *CYP337B3* (Joußen et al., 2012), which is also reported in all Brazilian populations (Durigan et al., 2017). However, as it has been maintained for more than 10 years without selections pressure, and at the diagnostic dose of 10 µg a.i./larva TWB3 resistance is functionally recessive. Therefore, the TWB3 strain was considered as susceptible reference strain in our studies. The BA43 strain was reared in a mass cross in acrylic boxes covered with voile, while the TWB3 strain was reared as single-pair matings in 450 mL paper cups, also covered with voile, and all adults were treated with a 10% honey solution. The voile cloths containing the eggs from both strains were collected three times per week and were transferred to a Petri dish containing the Bio-Serv diet. Larvae were kept in the Petri dishes until they reached the third instar, when they were used in the topical bioassay. Both strains were maintained in a walk-in climate chamber at 26°C and 55% relative humidity with a photoperiod of 16:8 h (light:dark).

3.2.2 Crosses and backcrosses

Crosses between strains BA43 and TWB3 were conducted as single-pair matings and also as mass matings, in order to search for new resistance mechanisms in the resistant

Brazilian strain, besides the chimeric P450 gene reported previously. TWB3 and BA43 strains were crossed and backcrossed to increase the possibility of finding other resistance genes in BA43, and also for further investigation of genes that are possibly linked to *CYP337B3*. Initially, crosses were established using females-informative and males-informative from strain BA43. The female-informative cross used females from BA43 and males from TWB3 (♀BA43 × ♂TWB3) as single-pair matings, while the male-informative cross used males from BA43 and females from TWB3 (♂BA43 × ♀TWB3) as mass crosses. The difference in the mating systems between the two crosses is due to the low mating success of males from BA43 when they are in single pairs. The heterozygotes (H1) provided from the two informative crosses were backcrossed with the most-distant parental strain, in this case TWB3. The backcrosses consisted of single-pair matings between a male H1 provided from the crosses described previously and a female from strain TWB3. Rearing conditions were as described in section 3.1.

3.2.3 Mortality responses to fenvalerate

Dose-response curves were prepared for strains BA43, TWB3 and H1 provided from both informative crosses, using the insecticide fenvalerate (Sigma-Aldrich, PESTANAL®, 99.6% purity). Six to eight concentrations (1 to 100 µg a.i./larva) and a control (acetone) were used in order to estimate the LC_{50} for each strain and a diagnostic dose. Doses were diluted in acetone and 1 µL of solution was applied topically on the dorsum of each third-instar larva, using a Hamilton syringe held in a Hamilton PB600-1 Repeating Dispenser (Hamilton Messtechnik). The insects were maintained under the same rearing conditions, in a climate chamber at 26°C and 55% relative humidity with a photoperiod of 16:8 h (light: dark). Mortality was assessed after 48 h; larvae that were unable to move when touched with a tweezer were considered dead. Larvae from the backcrosses (BCK) were exposed to the diagnostic dose of 10 µg a.i./larva, and mortality assessment and criteria were as described previously. Mortality data were submitted to a Probit analysis (Finney, 1971) performed in SAS Software (SAS Institute 2000).

3.2.4 Inheritance and dominance of resistance

Mortality data were also used to calculate the degree of dominance of resistance at the LC_{50} (D_{LC}) by means of the method described by Bourguet et al. (2000), using the equation:

$$D_{LC} = \frac{(log\ LC_{RS} - logLC_{SS})}{(log\ LC_{RR} - logLC_{SS})}$$

where: LC_{RR} is the lethal concentration for the resistant strain (BA43), LC_{RS} is the lethal concentration for the hybrid (H1: ♀BA43 × ♂TWB3 and ♂BA43 × ♀TWB3), and LC_{SS} is the lethal concentration for the susceptible strain (in this case, TWB3). If the D_{LC} value is close to zero, resistance is considered completely recessive; while if the D_{LC} value is close to 1, resistance is considered completely dominant.

Mortality data were also used to calculate the effective dominance (D_{ML}) in each insecticide concentration, using the formula described by Bourguet et al. (2000):

$$D_{ML} = \frac{ML_{RS} - ML_{SS}}{ML_{RR} - ML_{SS}}$$

where ML_{RS}, ML_{SS} and ML_{RR} are the percentage mortality at a particular concentration for the heterozygous (H1: ♀BA43 × ♂TWB3 and ♂BA43 × ♀TWB3), susceptible (TWB3), and resistant (BA43) strains, respectively. As described in the previous method, D_{ML} values can range from 0 to 1, where 0 means completely recessive and 1 means completely dominant (Bourguet et al., 2000). We also performed a dominance calculation, using the equation proposed by Stone (1968):

$$D = \frac{2X_F - X_R - X_S}{X_R - X_S}$$

X_F, X_R and X_S are the $\log_{10}$ of LC_{50} for the hybrid (♀BA43 × ♂TWB3 and ♂BA43 × ♀TWB3), resistant (BA43), and susceptible (TWB3) strains, respectively. In this analysis D values can range from –1, completely recessive, to 1, completely dominant.

The number of loci was estimated in order to predict the number of genes involved in effecting resistance in the Brazilian resistant strain. To perform this estimate we used the χ^2 test based on the goodness-of-fit between the mortalities for the BKC and TWB3 larvae at the diagnostic dose of 10 µg a.i./larva. First, the expected mortality (p) was estimated for the BKC larvae using the Mendelian model, as proposed by Georghiou (1969):

$$p = \frac{a+b}{2}$$

where *a* is the mortality for the parental strain used in the backcross (TWB3) and *b* is the mortality for the BKC larvae at 10 µg a.i./larva.

Next, χ^2 values were estimated using the method described by Sokal and Rohlf (1981), by means of the following equation:

$$\chi^2 = \frac{(Ni - pni)^2}{pqni}$$

where *Ni* is the mortality observed for the BKC larvae at the diagnostic dose, *p* is the expected mortality estimated by the Mendelian model, *ni* is the number of individuals tested at the diagnostic dose, and $q = 1 - p$.

3.3. Results

3.3.1 Mortality response curves and test with diagnostic dose

LC_{50} values for strains TWB3 and BA43 were 0.161 (0.07–0.37) µg a.i./larva and 125.53 (81.37–270.62) µg a.i./larva respectively, resulting in a resistance ratio of 780 times for strain BA43: strain TWB3 (Table 3.1). For the female informative cross (♂TWB3 × ♀BA43) the heterozygotes showed an LC_{50} value of 40.72 (24.02–93.91) µg a.i./larva, whereas for the male informative cross (♀TWB3 × ♂BA43) the heterozygotes showed an LC_{50} value equal to 72.16 (52.39–112.93) µg a.i./larva. The highest slope was found for strain TWB3 (2.03 ± 0.30) while BA43 showed the lowest slope value (1.35 ± 0.25), followed by the heterozygotes ♀TWB3 × ♂BA43 (1.41 ± 0.17) and ♂TWB3 × ♀BA43 (1.65 ± 0.23), indicating that the resistant BA43 and both heterozygotes had a low response to the increased dose of fenvalerate (Figure 3.1).

Table 3.1. Mortality response to fenvalerate of *H. armigera* larvae (third instar) from TWB3, BA43 and heterozygotes (♀TWB3 × ♂BA43, ♂TWB3 × ♀BA43) with topical bioassay.

Strain	*n*[1]	Slope ± SE[2]	LC_{50} (95% CI)[3]	χ^2 [4]	d.f.[5]	RR[6]
TWB3	216	2.03 ± 0.30	0.161 (0.07–0.37)	7.88	4	–
BA43	336	1.35 ± 0.25	125.53 (81.37–270.62)	7.61	5	780
♀TWB3 × ♂BA43	360	1.41 ± 0.17	72.16 (52.39–112.93)	4.40	6	448
♂TWB3 × ♀BA43	280	1.65 ± 0.23	40.72 (24.02–93.91)	9.40	5	253

[1]number of larvae tested; [2]slope and standard error; [3]lethal concentration that kills 50% of larvae (µg a.i./cm^2) and 95% confidence interval; [4]chi-square; [5]degrees of freedom; [6]Resistance ratio based on LC_{50}.

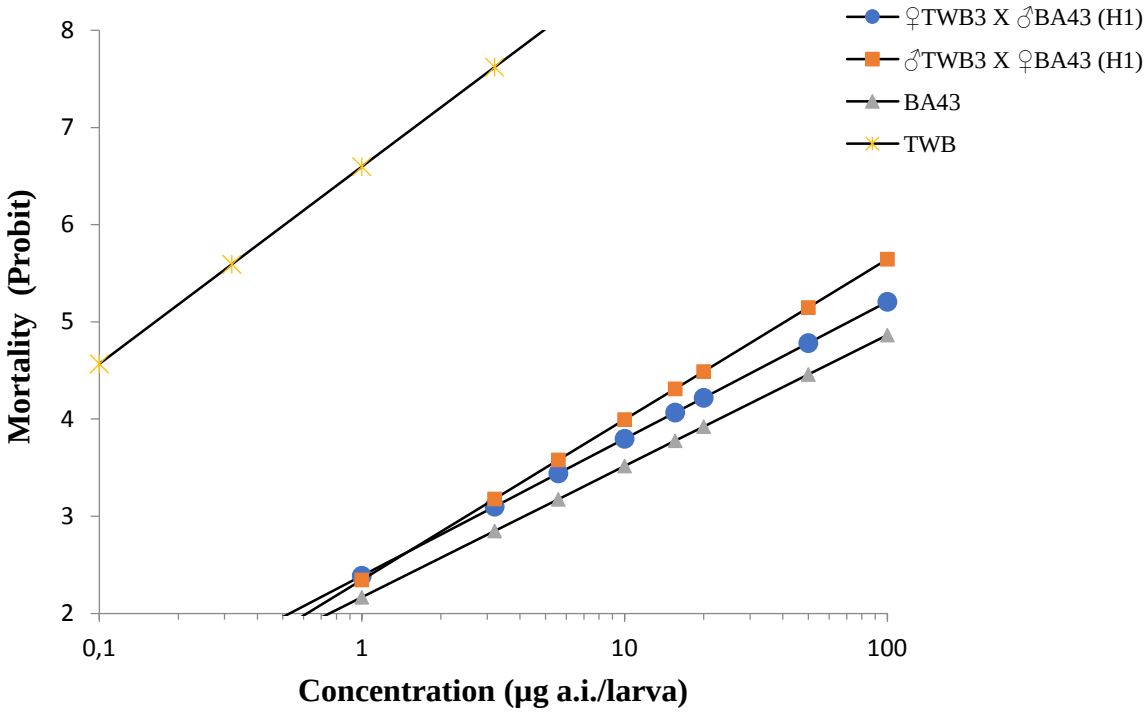

Figure 3.1. Mortality response curves of third-instar larvae from the heterozygotes (♀TWB3 × ♂BA43 and ♂TWB3 × ♀BA43), BA43 and TWB strains treated with different concentrations of fenvalerate.

When third-instar larvae from the two parental strains (BA43 and TWB3) and the backcrosses (♂H1_F × ♀TWB3 and ♂H1_M5 × ♀TWB3) were treated with the diagnostic dose of 10 µg a.i./larva of fenvalerate, the Brazilian pyrethroid-resistant strain BA43 showed 85±1.2% survival, while the Australian pyrethroid-resistant strain showed 100% mortality. Third-instar larvae from the backcrosses ♂H1_F × ♀TWB3 and ♂H1_M5 × ♀TWB3 had 47.4±1.4% and 45.5±1.7% survival, respectively (Figure 3.2).

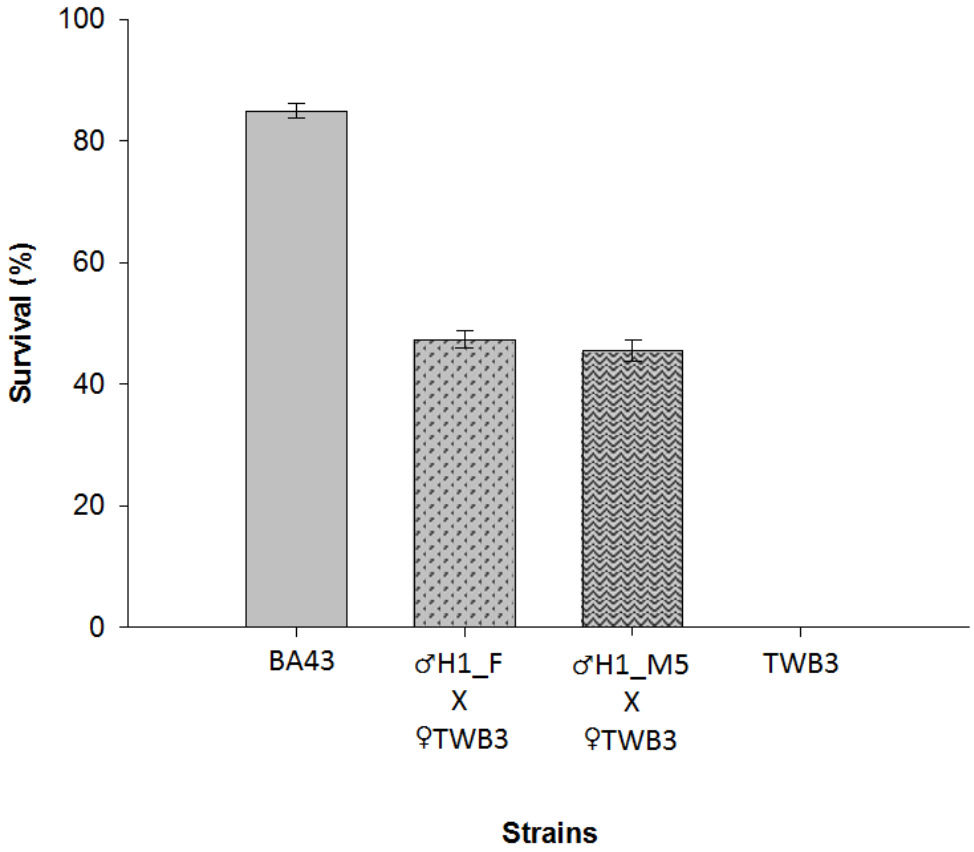

Figure 3.2. Survival (% ± SE) of third-instar larvae from strain BA43, backcrosses (♂H1_F × ♀TWB3 and ♂H1_M5 × ♀TWB3) and TWB3 treated with 10 µg a.i./larva of fenvalerate.

3.3.2 Inheritance of *H. armigera* resistance to fenvalerate

The degrees of dominance (D) estimated based on LC_{50} values for the heterozygotes provided from the male informative cross (♂BA43 × ♀TWB3) were 0.92 and 0.83 according to the methods of Bourguet and Stone, respectively; whereas for the female informative cross (♀BA43 × ♂TWB3) the D values were 0.83 (Bourguet method) and 0.66 (Stone method). As all D values were between 0.5 and 1, fenvalerate resistance in the Brazilian strain can be considered incompletely dominant. In addition, as both heterozygotes showed similar LC_{50} values with overlap of the confidence interval, the resistance is autosomal and not sex-linked or with maternal effect (Table 3.2).

Table 3.2. Degree of dominance estimated based on LC_{50} values using Bourguet and Stone methods.

Strain	LC_{50} µg a.i./larva	DLC_{50}*	D (LC_{50})**
BA43	125.53	–	–
TWB3	0.16	–	–
♂BA43 × ♀TWB3	72.16	**0.92**	**0.83**
♀BA43 × ♂TWB3	40.72	**0.83**	**0.66**

* Bourguet et al. (2000); ** Stone (1968)

The effective dominance (DML) was estimated for each individual dose of fenvalerate used in the mortality-response curves, which showed that resistance to fenvalerate was completely dominant at most of the doses tested, from 0.01 to 3.2 µg a.i./larva and 15.6 to 50 µg a.i./larva. For the male informative cross (♂BA43 × ♀TWB3) at the doses of 5.6, 10 and 100 µg a.i./larva, resistance was incompletely dominant; while for the female informative cross (♀BA43 × ♂TWB3) at doses of 5.6, 10 and 50 µg a.i./larva, resistance was also incompletely dominant (Table 3.3). Incompletely recessive resistance was found at 100 µg a.i./larva for the female informative cross (♀BA43 × ♂TWB3).

Table 3.3. Effective dominance in each concentration estimated with the Bourguet method.

Dose	**Mortality (%)**				**Dominance (DML)**	
µg a.i./larva	**BA43**	**TWB3**	**♂BA43 × ♀TWB3**	**♀BA43 × ♂TWB3**	**♂BA43 × ♀TWB3**	**♀BA43 × ♂TWB3**
0.01	0.0	4.2	0.0	0.0	1.0	1.0
0.032	0.0	8.3	0.0	0.0	1.0	1.0
0.1	0.0	20.8	0.0	0.0	1.0	1.0
0.32	0.0	70.8	0.0	0.0	1.0	1.0
1	0.0	100.0	0.0	3.3	1.0	1.0
3.2	0.0	100.0	4.8	3.3	1.0	1.0
5.6	0.0	100.0	6.7	6.4	0.9	0.9
10	0.0	100.0	11.7	10.0	0.9	0.9
15.6	16.7	100.0	13.3	16.7	1.0	1.0
20	20.0	100.0	20.0	36.0	1.0	1.0
50	28.3	100.0	26.7	53.3	1.0	0.7
100	40.0	100.0	63.1	80.0	0.6	0.3

Observed and expected mortalities for third-instar larvae from the backcrosses with TWB3 treated with 10 µg a.i./larva of fenvalerate were 52.6 and 76.3 for ♂H1_F × ♀TWB3 respectively, while for ♂H1_M5 × ♀TWB3 the observed and expected mortalities were 54.5 and 77.3 (Table 3.4). Chi-square values for both backcrosses were significant at $P < 0.001$, indicating that pyrethroid resistance in the Brazilian resistant strain is polygenic.

Table 3.4. Observed and expected mortalities estimated based on Mendelian inheritance and χ^2 of the backcrosses between heterozygotes and strain TWB3 at the diagnostic concentration.

Concentration	♂ H1_F × ♀TWB3			♂ H1_M5 × ♀TWB3		
	Observed	Expected	χ^2(d.f.=1)	Observed	Expected	χ^2(d.f.=1)
10 µg i.a./larva	52.6	76.3	317.5*	54.5	77.3	335.7*

* Significant difference ($P < 0.001$, d.f. = 1).

3.4 Discussion

In this study we showed that the resistance of Brazilian *H. armigera* to pyrethroids is much higher than that of the Australian resistant strain (780-fold). As both strains have the chimeric P450 *CYP337B3* gene, our results indicated that this is not the only resistance mechanism in the Brazilian populations, and that probably additional mechanisms are involved in insecticide detoxification. In addition, the chi-square values found here were higher than the tabulated value ($P < 0.001$, d.f. = 1) and so the observed and expected mortality were significantly different, confirming that more than one gene is responsible for this high resistance.

The heterozygotes showed LC_{50} values that were more similar to the Brazilian resistant strain, and there was no overlap in the confidence intervals (95%) of the LC_{50} values estimated for the two crosses (♀TWB3 × ♂BA43 and ♂TWB3 × ♀BA43). This suggests that pyrethroid resistance in the Brazilian populations does not have a maternal origin nor is it sex-linked, but rather is autosomal and therefore present in both the male and female chromosomes. Autosomal resistance has been commonly reported and contributes significantly to the rapid evolution of resistance in sexually reproducing species (Heather, 1986; Daly and Fisk, 1998; Achaleke and Brévault, 2009).

The results for the degree of dominance showed that *H. armigera* resistance to pyrethroids in Brazil extends from incomplete dominance to complete dominance at most of

the concentrations tested. The results also indicated that dominance only started to become incompletely recessive at a very high dose (100 µg a.i./larva), which is impractical for field use. Knowledge of the dominance degree is essential for the implementation of resistance-management measures, and allows us to determine the best dose to use in the field, in order to kill the heterozygotes and prevent the evolution of resistance (Roush and McKenzie, 1987; Roush & Daly, 1990; Lenormand and Raymond, 1998; Tabashnik et al., 2003).

The individuals of *H. armigera* that originated the populations in Brazil carried the pyrethroid-resistance gene *CYP337B3* (Durigan et al., 2017). Our results suggest that they may have carried other genes for resistance, and also, the high selection pressure in Brazilian fields may be contributing to the emergence of new mechanisms of resistance. The array of genetic characteristics found in this study explains why resistance to pyrethroids in Brazil evolved so rapidly and is now well fixed in all *H. armigera* populations.

In Africa, resistance of *H. armigera* to cypermethrin was also characterized as completely dominant and autosomal; the chi-square values suggested that resistance is monogenic, controlled by a single locus (Achaleke and Brévault, 2009). In Australia, Daly and Fisk (Daly and Fisk, 1992; Daly and Fisk, 1993) found that resistance of *H. armigera* to fenvalerate was also governed by a single, but incompletely dominant gene. Later, Joußen et al. (2012) characterized the resistance of *H. armigera* to fenvalerate as dominant. Inheritance of *H. armigera* resistance to fenvalerate was assessed in Chinese and Pakistani strains as autosomal, monogenic and incompletely recessive (Ru et al., 1998; Tan and McCaffery, 1999). However, a few years later in Pakistan, resistance to fenvalerate was reported as monogenic, autosomal and completely dominant (Rasool et al., 2014).

The inheritance of resistance has been investigated for other species of Heliothinae, *Heliothis virescens* and *Helicoverpa zea*. For *H. virescens*, some studies have reported resistance to lambda-cyhalothrin as incompletely recessive (Payne et al., 1988) or incompletely dominant (Elzen et al., 1994). In the United States, resistance of *H. zea* to lambda-cyhalothrin was reported as incompletely dominant (Brown et al., 1998).

Resistance is usually associated with a fitness cost, and in the absence of selection pressure, susceptibility can be recovered (Tabashnik et al., 2003). In Australia, the susceptibility of *H. armigera* to fenvalerate was recovered after 2 years of a resistance-management program, where cotton growers had to maintain a window with no pyrethroid spraying (Forrester et al., 1993). Also in Australia and in China, the susceptibility of *H. armigera* to fenvalerate was restored after the introduction of *Bt* cotton, which contributed to a decrease in insecticide spraying (Wu et al., 2005).

Our results explain field failures with the use of pyrethroids to control *H. armigera* in Brazil. Therefore, pyrethroids should not be used to control this pest even in a mixture strategy with other group of insecticides such as diamides and insect growth regulators.

3.4. Conclusions

- ✓ Resistance of *H. armigera* to pyrethroids in Brazil is autosomal, incomplete to complete dominance and polygenic;
- ✓ The resistance ratio of *H. armigera* to fenvarelate was very high (780-fold) indicating that the Brazilian pyrethroid-resistant strain possesses additional mechanisms of resistance besides the chimeric P450 *CYP337B3.*

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4. INVESTIGATING RESISTANCE MECHANISMS OF *Helicoverpa armigera* (LEPIDOPTERA: NOCTUIDAE) TO PYRETHROIDS IN BRAZIL

Abstract

Insecticide resistance occurs through specific mechanisms, which may involve a behavioral change, reduction in cuticle penetration, mutation at the target site, or the metabolization of the insecticide. Resistance of *H. armigera* to pyrethroids was first reported in the 1980s and since then many studies have been conducted to elucidate resistance mechanisms. A Brazilian pyrethroid-resistant strain (BA43) was used in this study, where initially the total RNA and cDNA were extracted and synthesized, respectively, from fourth-instar larvae. Specific primers for the voltage-gated sodium channel were designed and samples were submitted to a PCR. After sequencing the sequences were aligned and compared with others available on GenBank. The same cDNA samples were submitted to a RT-qPCR where the regulation of more than 50 P450 genes was assessed. Two mutations were found in BA43 strain, V937G and Q960H. The P450 genes *CYP6AB10, CYP301A, CYP4S13 and CYP321A5* were found to be up-regulated in BA43. These findings suggest that the resistance of *H. armigera* to pyrethroids in Brazil is due to multiple mechanisms. However, additional studies are needed to confirm if these findings are linked to the high level of pyrethroid resistance in *H. armigera* populations.

Keywords: Cotton bollworm; Sodium channel; Target-site mutations; Detoxification; P450 enzymes

4.1 Introduction

Helicoverpa armigera is among the pest species with the most cases of resistance to insecticides reported worldwide (Sparks and Nauen, 2015; Joußen and Heckel, 2016). This polyphagous, highly destructive pest has developed resistance to more than 45 active ingredients, with 856 cases of resistance currently known (Arthropod Pesticide Resistance Database, 2018). Among these cases, 403 are associated with pyrethroids (Group 3A). Pyrethroids act on the voltage-gated sodium channels, affecting the nervous and muscle systems. Pyrethroids are some of the most important insecticides marketed worldwide, comprising of 16% sales, behind only neonicotinoids (Sparks and Nauen, 2015).

The wide use of this group of insecticides has resulted in a high selection pressure in crops worldwide, and has contributed to the rapid evolution of resistance in many pest species, including *Bemisia tabaci* (Gnankiné et al., 2018), *Spodoptera litura* (Wang et al., 2018), *Tuta absoluta* (Silva et al., 2015), *Spodoptera frugiperda* (Carvalho et al., 2013), *Plutella xylostella* (Cheng, 1988), *Helicoverpa zea* (Hopkins and Pietrantonio, 2010) and *Heliothis virescens* (Park and Taylor, 1997). For *H. armigera* many researchers have reported resistance of this pest to different molecules including fenvalerate, deltamethrin, bifenthrin

and lambda-cyhalothrin (Gunning et al., 1984; Djihinto et al., 2009; Brun-Barale et al., 2010; Joußen et al., 2012; Rasool et al., 2014; Bird, 2015; Qayyum et al., 2015; Durigan et al., 2017).

Resistance is a genetic and inheritable characteristic, conferred by one or more mechanisms (Roush and McKenzie, 1987). Four major mechanisms can confer resistance to insecticides, i.e., behavioral, morphological, target-site or metabolic (Pittendrig et al., 2008). Most cases of resistance of *H. armigera* to insecticides and *Bt* toxins were reported to be associated with target-site mutations and metabolic mechanisms (Gunning, 1996; Yang et al., 2008; Xiao et al., 2015; Bretschneider et al., 2016; Joußen and Heckel, 2016; Durigan et al., 2017).

Brazilian populations of *H. armigera* have a high level of resistance to pyrethroids, where the chimeric P450 *CYP337B3* plays a highly important role (Durigan et al., 2017). The high level of resistance in the Brazilian populations compared to others in Australia and Pakistan (Joußen et al., 2012; Rasool et al., 2014) indicates that the metabolic resistance conferred by the *CYP337B3* gene is likely not the only mechanism involved in this high resistance.

As *H. armigera* was recently introduced in Brazil (Czepak et al., 2013; Specht et al., 2013), it is crucial to explore the mechanisms of resistance involved in pyrethroid resistance, to better understand resistance evolution and determine the best strategies for implementing resistance-management programs and preventing the emergence of cross- or multiple resistance to other insecticides. Here, we evaluated the involvement of other P540 genes, using quantitative real-time PCR to assess levels of gene expression. We also sequenced and analyzed the voltage-gated sodium channel in order to search for possible target-site mutations.

4.2 Material and Methods

4.2.1 *H. armigera* strains and insect preparation

The *H. armigera* strains used in this study were the same described in chapter 3 section 3.1. Three biological replicates were initially established for each strain (BA43 and TWB3), each replicate containing 5 fourth-instar larvae. Each larva was placed in a dish and covered with nuclease-free water (Anbion) to preserve RNA in the cells, and the gut was dissected out from the rest of the body. All the biological replicates were maintained in a freezer at –80°C.

4.2.2 RNA extraction and cDNA synthesis

Total RNA was isolated with the RNeasy Mini Kit (Qiagen) following the manufacture's instructions. Afterwards, cDNA was synthesized using the SuperScript III First-Strand kit (Invitrogen), where 5 µL of RNA was used in a total volume of 20 µL. cDNA synthesis was carried out in three steps. The first step used 3 µL of RNAse-free water, 1 µL of 10 mM dNTP mix and 1 µL of RT primer (200 pmol/µL). Samples were incubated at 65°C for 5 min and then chilled on ice for 5 min. In the second step, a Mater Mix was prepared using 2 µL 10× RT Buffer, 4 µL 25 mM $MgCl_2$, 2 µL 0.1 M DTT, 1 µL RNase Out (40 u/µL) and 1 µL SuperScript RTase (200 u/µL). Samples were maintained in a thermocycler at 50°C for 60 min, followed by 85°C for 5 min, and then chilled on ice. In the third step, 1 µL *E. coli* RNase (2 u/µL) was added to each sample and the samples were incubated for 20 min at 37°C. RNA and cDNA concentrations were estimated using a NanoDrop (Thermo Fischer Scientific).

4.2.3 Primers and PCR conditions for the sodium channel amplification

The primers used to amplify the sodium channel in strain BA43 were designed based on the sodium-channel sequence in other *H. armigera* strains from Australia and South Korea (Tables 4.1 and 4.1, unpublished: provided by in-house data from the Max Planck Institute for Chemical Ecology). 400 ng of cDNA from the non-gut body of the 3 biological replicates was submitted to a PCR (20 µL) with the following conditions: milliQ water (12.25 µL), dNTP (1.6 µL), forward and reverse primers (1.0 µL each), 10× Buffer (2.0 µL), Taq – Takara (0.15 µL) and cDNA (2.0 µL). The thermocycle conditions for the PCR were: initial denaturation temperature at 95°C for 5 min (1×); 3 cycles of 20 s at 94°C, 20 s at 63°C, 1:30 min at 72°C; 3 cycles of 20 s at 94°C, 20 s at 61°C, 1:30 min at 72°C; 3 cycles of 20 s at 94°C, 20 s at 59°C, 1:30 min at 72°C; 30 cycles of 20 s at 94°C, 20 s at 57°C, 1:30 min at 72°C, and a final extension at 72°C for 2 min. PCR results were visualized in 1% agarose gel stained with SYBR Safe, using 8 µL of PCR solution after running for 1 h at 120 volts.

Table 4. 1. Primers used on the *H. armigera* sodium channel amplification.

Primers	***Sequence (5'>3')***	***Position***	***Expected size (pb)***
Ha_para-F2	TGGCTGTCCCTATCAGCGAT		1843
para-MS-5F4-2R	CGGCGATCCAGGCAGTGATA	1843 - 1824	
para-MS-5F4-2	CCTGGATCGCCGTTCAATTTG	1832 - 1852	
m_VSSC-7R	CTACGAAGTTTATAAGCGAGACCA	4126 - 4149	2317
moth_VSSC-R1	GAGAATGACGGCGATGTACATG	5579 - 5558	3747
para-MS3-R	CACGGCGTCCAGCAGCAC	6214-6197	4382
para-MS3-F2	CACCCGAGCGAGTCGACAA	3446 - 3465	2961
para-MS3-F3	TCCTTGTGGGTGATGACGAATC	3971 - 3993	2414
Moth_VSSC-F7	TGGTCTCGCTTATAAACTTCGTAG	4278-4301	2129
Ha_para_3'UTR-F	CGTGCTGGACGGCATCATCAA	5569 - 5589	838
Ha_para_3UTR-R1	GCGCTACCACGCTATATGTACA		

Table 4.2. Primer combinations used to amplify specific fragments from *H. armigera* sodium channel.

F primer	*R primer*	*Size (pb)*
Ha_para-F2	para-MS-5F4-2R	> 2000
para-MS-5F4-2	m_VSSC-7R	2317
	moth_VSSC-R1	3747
	para-MS3-R	4382
para-MS3-F2	Ha_para_3UTR-R1	2961
para-MS3-F3		2414
Moth_VSSC-F7		2129
Ha_para_3'UTR-F		838

4.2.4 Sequence analysis and alignment

Positive gel fragments revealed in the PCR were cut and the samples were purified with QIAquick Gel Extraction Kit (Qiagen), following the instructions provided by the manufacturer. Purified samples were sent for sequencing in South Korea (Macrogen). Results were analyzed in Sequencher and aligned with the software DNASTAR (SeqMan NGen® version 12.0, DNASTAR, Madison, WI). The alignment was done between the sequences from strains BA43 and TWB3, resistant and susceptible strains.

4.2.5 RT-qPCR to assess the expression of new P450 genes

A Real-Time Quantitative Reverse Transcription PCR (RT-qPCR) was used to assess the expression level of different P450 genes in BA43 compared with strain TWB3. Primers for 54 different P450 genes were designed based on the in-house database of the Department of Entomology in the Max Planck Institute for Chemical Ecology, and 2 reference genes were used in the analysis, *eIF4A* (eukaryotic Initiation Factor 4A) and *RpS18* (ribosomal protein S18) (Table 4.3). For the RT-qPCR reaction, the Absolute Blue qPCR SYBR Green Kit (Thermo Scientific) was used in a total volume of 20 µL containing 10 µL of 2× Absolute SYBR Green, 1.4 µL of each primer (forward and reverse), 5.2 µL of Nuclease-free water and 2 µL cDNA diluted to 250 µg μL^{-1}. The analysis was performed using four 96-well plates containing the biological replicates from BA43 and TWB3. Each experimental plate included at least one reference gene. The RT-qPCR analyses were conducted in a CFX Connect Real-Time PCR Detection System (Bio-Rad) with an initial temperature for enzyme activation at 95°C for 15 min, followed by 40 cycles at 95°C for 15 s, 55°C for 30 s and 72°C for 30 s. The

dissociation curve was set at 95°C for 30 s, 55°C for 30 s and 95°C for 10 s, this last step repeated 80 times. Results for the mean Cq values and gene regulation were calculated and analyzed in CFX Manager 3.1 Software (Bio-Rad). BA43 results were compared against TWB3 in a *t* test in R.

Table 4.3. Forward and reverse primers list by each P450 and reference genes used on RT-qPCR.

Gene	Forward primer (5'-3')	Reverse Primer (5'-3')
CYP4G8	TCTGACCTGAAGGAGGAGGA	TAGCAGGCTTTGGTTGGTTT
CYP4G9	CCACCCTCAGCACTACAAGG	GGTCCAGCACTGAATGGAAT
CYP4G26	TGACCTCCGATGTGACTGAG	TCTGCCTGGGTTCAATCTTC
CYP4L5	GTGCTGGTCCGAGAAATTGT	GTTCAGGCTCTTGAGGTGCT
CYP4L11	GCATTCTTGGACCTGTTGCT	TCGTGACCCTCAAACATGAA
CYP4M6v2	AATAGCCGTAGCGGAGGTTT	CAGGTCCTTCGTTCCTGAAG
CYP4M7v2	AGCGCTGGTCCTAGAAATTG	ATGGTTTCGTCACTGGCTTC
CYP4M10v2	ATTGCGGAACTTCGTATTG	TGAAATTGACGACCACTGGA
CYP4S12	TGCGTAACTTCAAGCTGGTG	AGATAGGGTCAACGGGTCTG
CYP4S13	CTATGCATCCGTACGCCTTT	CGACAAATTCCACTCAGCAC
CYP4AU1	TGCAGTAGTGCCAATGGTGT	TCGGTCCACACTGTTCTCTG
CYP6B2v3	ATTGGACCGAAAGGAGGAAT	TTCATGGTCCGTTGTCGTTA
CYP6B6v2	ATTGGACCGAAAGGAGGAAT	CCGTTACTTGTACCGTCATTATAGC
CYP6B43	TTGTAGGGAGGTTATTGAAGCAG	TGCCTTCTCGTCTCAAGTCC
CYP6AB9	CATAGGTGAGCGGCTAGGTC	TGCACTATGCTGGACTGAGG
CYP6AB10	TGTGTTGGTGCCAGATTAGG	CTACGAAACCTTCGGAAACG
CYP6AE11	TCTCACATTGCCTTTCACTCC	ACTCCCAAAGAACCCACTCC
CYP6AE12	AGATGAGGCTCTGCGTCTGT	CAGATGCACCCTCAGACCTT
CYP6AE14	GGATCCGGAGGAGTATAGGC	TACAAAGTCTCGGGCCTTCA
CYP6AE15v2	CCCTTACTGAGCCGATTTGA	ATCTGCGTGTCGGGAGATAG
CYP6AE17	GTTCCTGCCTGAGAACAAGC	TAATTATTCCCGCCGTCATC
CYP6AE19	GCATATGTTCTTGCGCCTTC	TTTGATTGCCCATGAATCTG
CYP6AE24	CCAAATTGAGAACCTCCTCCT	CCACGCACTTAGGAACACAA
CYP6AN1	ATCGAAGAGTTGGGCATCAC	TATCCGGCATGAACTTCTCC
CYP9A3	GTGCTTATTCGGGAGATGGA	CCCTGTAGCTGCATGTTGAA
CYP9A14	ATGGAAGTCAGCGAGCAGAT	TATCACATCGTTGGCGTAGC
CYP9A15	GGATCGAGGTTTGCTCTCTG	ATTGTTAGCCGCAAGTTTGG
CYP9A23v3	CAGATCTTACGGCACATGGA	AAGCCAGTGTCCTCCTTTCA
CYP9A34	TGTGCGAGATGAAAGCGATA	AGGTTGAACTGATCGGGAGA
CYP9G5	TTCCCGAACCAGACAAGTTT	ATCATCGCGAACCTCATACC
CYP304F1	CCTGCCGTACAGTTTGTCCT	GACGACGCGGTCTATCTCTT
CYP305B1	AGTACTCATGTCGCTGGGAGA	CGCTTTCGTCAATGAACCTT
CYP321A5	TGGAATGGGAAACAGGACAT	CTGCTGGCTTTCTGGGATAG
CYP321B1	CGAGATACGCACGAATTCAA	TTGTACTGGATGCCTCCTTTG
CYP324A1	TATGCCGTTTGGAGAAGGAC	TGTTTGGCAGCACTCTCACT
CYP332A1	CCCGAGGTTCTGCATAGGTA	CCACGATCTTAGGTGCATCA
CYP333A1	CGGCTTCGGAATAAGGAGTT	CCCTCCCACGTAACTTTGAA
CYP340G1	GCAGATGTCACCAAGATGGA	CCTCATCCTCATCACCAACA
CYP340H1	CCGGACAAGGATCAGTTCAT	CCGATACAGTTGCGTCTGC

CYP340H2	TATGCACACGCAACAGATGA	GCTTGCCGAAGTCCATTTAT
CYP340J1	TCAATATGGCGGATGAACAA	TGGATTTGACCCAAGAAGGA
CYP340K1	ACGGCAAACAAGACTTTGCT	CGCCAACATCACCTTTCATA
CYP341B2	GGCTACCAATACGCGATGAT	GCCATTACTTCTGGCCATGT
CYP354A3	GGTCTGAGATTCGCCATGTT	CTGAATGGGATCAACCGTCT
CYP367A8	TGAACCAGAAAGGGCCACTA	TTTGCTGTCTTCCGTTGGTT
CYP367B2	AGAGCGGTTCAATCCAGAAA	TGCCGAAGTATCTTCCCAAA
CYP428A1	CCATCTGGCAAACTCATTAGC	GGCCTTTGTCCATTGAAGTG
eIF4A	AGCAAATCCAAAAGGTGGTG	AGAGCACGGCGAGTTATCAT
RpS18	ACTGCCATCAAGGGTGTTG	TGTATTGCCTGGGGTTAGACA

4.3 Results

4.3.1 Sodium channel sequence

The sodium channel sequenced from cDNA for the resistant strain BA43 resulted in a sequence with 6,162 bp (6 kb) (GenBank MG674159), generated based on the overlapping of 8 fragments generated with the primer combinations provided in Tables 4.1 and 4.2. After translation of the bases into amino acids and alignment with the Australian TWB3 strain sodium-channel sequences, 2 non-synonymous mutations were detected in the Brazilian pyrethroid-resistant strain BA43 (Figure 4.1). Mutations were found at points V937G, resulting in glycine instead of valine and Q960H, resulting in histidine instead of glutamine. The mutation point V937G is located in between regions IIS4 and IIS5, whereas the Q960H mutation is located in IIS5 region.

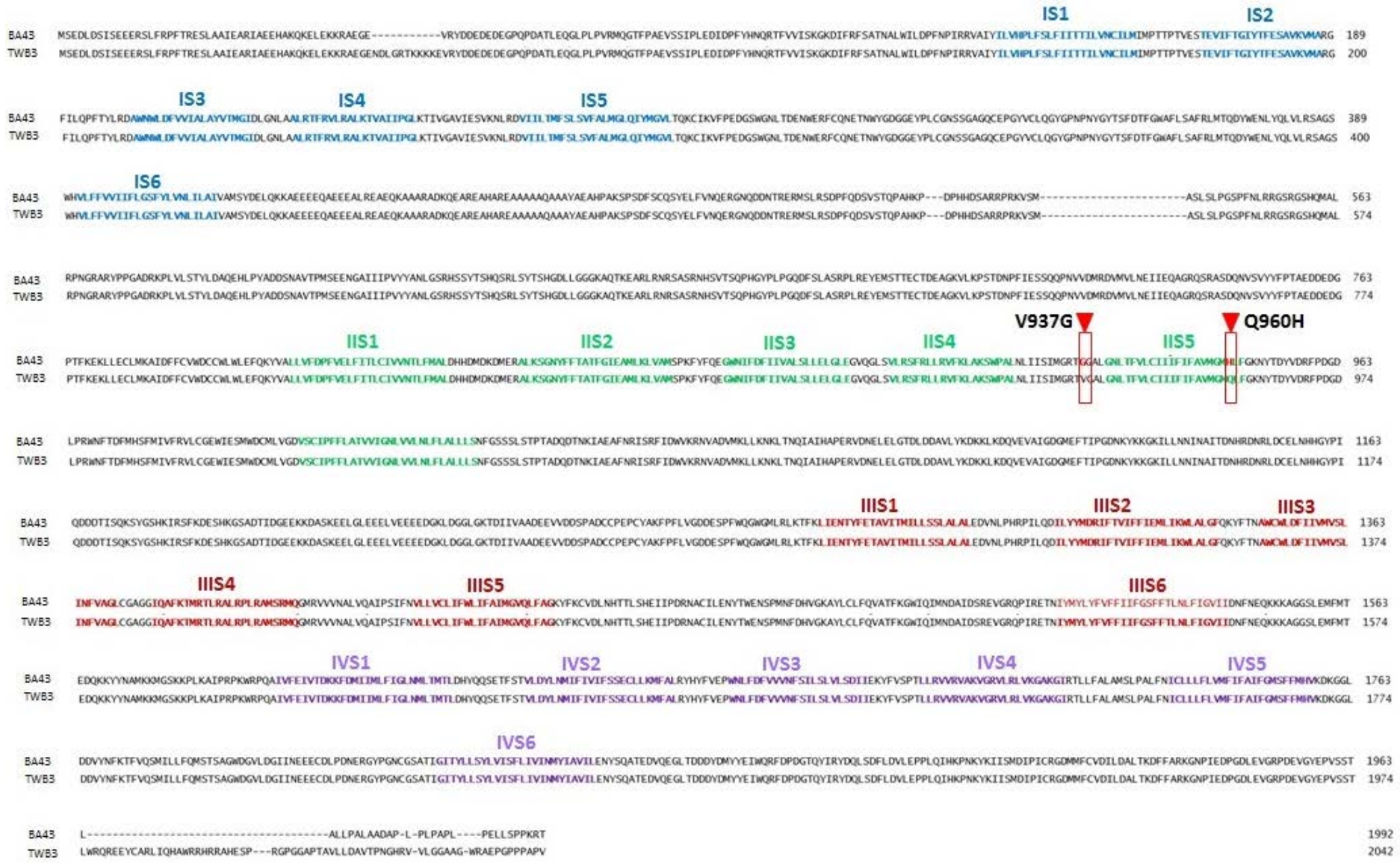

Figure 4.1. Alignment of sodium channel aminoacids sequences of BA43 and TWB3 (TWB3-provided by the inhouse database from the Max Planck Institute for Chemical Ecology). Mutations V937G, Q960H are marked with red arrows. Domains I, II, III and IV are represented in blue, green, red and purple letters, respecvelly.

4.3.2 Expression of other P450 genes in RT-Qpcr

The gene expression of BA43 was compared against TWB3, where the regulation threshold provided by the CFX Manager software was equal to 2. According to this threshold, genes that showed a regulation higher than 2 were considered up-regulated. In the non-gut body tissue, 25 genes were classified as up-regulated in BA43: *CYP6AB10, CYP301A, CYP4S13, CYP321A5, CYP4M6v2, CYP6B43, CYP4S14, CYP6B6v2, CYP4L5, CYP6AB9, CYP4M7v2, CYP340H1, CYP332A1, CYP6AE24, CYP4AU1, CYP4L11, CYP340K1, CYP6AE17, CYP6AE11, CYP9A3, CYP333A1, CYP4M10v2 CYP4M10v2, CYP9A34* and *CYP340J1* (Table 4.4). Of these, 4 genes proved to be highly up-regulated *CY6AB10* (92.71), *CYP301A* (43.93), *CYP4S13* (25.94) and *CYP321A5* (22.46). In contrast, 10 genes appeared to be down-regulated: *CYP324A1, CYP36AN1, CYP4G26, CYP4S12, CYP9AJ3, CYP4G8, CYP4G9, CYB321B1, CYP304F* and *CYP340G1*.

In the gut tissue, 19 genes were considered up-regulated in BA43 (Table 4.5): *CYP4S13, CYP6AB10, CYP321A5, CYP4L5, CYP4M7V2, CYP4L11, CYP4G8, CYP333A1, CYP367B2, CYP6AE24,* NADPH reductase, *CYP6AE15, CYP354A4, CYP6AE12, CYP6B43, CYP324A1, CYP6AB9, CYP4G9* and *CYP4G26*, and of these, the genes *CYP4S13, CYP6AB10* and *CYP321A5* were up-regulated at a higher level (92.39, 35.71 and 24.03 respectively). In this same tissue, 14 genes were down-regulated in BA43 compared to TBW3: *CYP340H2, CYP4AU1, CYP305B1, CYP337B1, CYP6AE11, CYP9G5, CYP4M6, CYP9A15, CYP36AN1, CYP6AE14, CYP304F1, CYP321B1, CYP9AJ3* and *CYP9A23.*

P values were significant for genes *CYP301A, CYP6B6v2, CYP4L5, CYP4L11* and *CYP340F1* ($P < 0.001$) in the non-gut body tissue. In the gut tissue, P values were significant for the genes *CYP4S13, CYP333A1,* NADPH reductase and *CYP9A23* ($P < 0.001$).

Table 4.4. Mean Cq ± SE and gene regulation in BA43 compared to TWB3 strain for the rest of the body tissue by RT-qPCR. Statistical significance of t-test: * P < 0.05, ** P < 0.01, *** P < 0.001.

Gene	Regulation	Compared to Regulation Threshold	P-Value	Mean Cq ± SE	
	BA43 X TWB3			*BA43*	*TWB3*
CYP6AB10	92.71	Up regulated	0.2337	25.27±1.02	31.93±0.22
CYP301A	43.93	Up regulated	<0.001***	33.55±0.94	39.06±0.16
CYP4S13	25.94	Up regulated	0.1338	26.63±1.13	31.38±0.06
CYP321A5	22.46	Up regulated	0.1094	21.16±0.96	26.22±0.20
CYP4M6V2	13.06	Up regulated	0.3578	27.47±1.98	31.3±0.07
CYP6B43	11.12	Up regulated	0.3391	26.35±1.59	29.88±0.10
CYP4S14	6.33	Up regulated	0.3538	28.69±1.77	31.43±0.29
CYP6B6v2	6.25	Up regulated	<0.001***	22.42±0.09	25.64±0.40
CYP4L5	6.01	Up regulated	<0.001***	21.38±0.11	24.09±0.07
CYP6AB9	5.99	Up regulated	0.2846	24.18±1.15	26.88±0.17
CYP4M7V2	5.50	Up regulated	0.3431	24.96±1.48	27.55±0.02
CYP340H1	5.21	Up regulated	0.2024	24.96±1.34	27.42±0.50
CYP332A1	5.17	Up regulated	0.3465	26.19±1.52	29.14±0.09
CYP6AE24	3.75	Up regulated	0.1137	27.26±0.78	29.74±0.14
CYP4AU1	3.63	Up regulated	0.0199*	25.97±0.27	27.91±0.38
CYP4L11	3.32	Up regulated	<0.001***	22.77±0.08	24.63±0.07
CYP340K1	2.85	Up regulated	0.1229	26.48±0.67	28.11±0.05
CYP6AE17	2.75	Up regulated	0.1574	24.09±1.16	26.13±0.14
CYP6AE11	2.70	Up regulated	0.2020	24.23±0.72	25.75±0.23
CYP9A3	2.66	Up regulated	0.0519	27.28±0.39	28.74±0.09
CYP333A1	2.52	Up regulated	0.1523	24.71±0.48	26.1±0.13
CYP4M10V2	2.44	Up regulated	0.1593	28.93±0.62	30.34±0.07
CYP9A34	2.06	Up regulated	0.006**	26.49±0.14	27.59±0.06
CYP340J1	2.06	Up regulated	0.4021	26.42±1.54	27.54±0.97
CYP9G5	1.96	No change	0.1628	27.3±0.67	28.33±0.04
CYB354A4	1.89	No change	0.008**	25.79±0.12	26.84±0.11
CYP9A14	1.87	No change	0.1441	26.77±0.42	27.73±0.28
CYP9A15	1.84	No change	0.3646	27.45±0.81	28.91±0.14
NADPH reductase	1.83	No change	0.0846	21.38±0.30	22.31±0.05
CYP6AE19	1.77	No change	0.5083	25.52±0.23	26.43±0.70
CYP341B2	1.77	No change	0.2291	27.27±1.12	28.18±0.02
CYP305B1	1.54	No change	0.3523	26.19±0.59	27.39±0.13
CYP9A23v3	1.50	No change	0.3274	28.29±0.48	28.94±0.04
CYP337B1	1.13	No change	0.5731	21.73±0.65	21.99±0.04
CYB367B2	1.11	No change	0.5577	29.56±0.78	29.84±0.20
CYP6B7	1.09	No change	0.5774	25.69±0.80	25.9±0.24
CYP367A2	1.05	No change	0.5326	32.28±1.01	32.41±0.15
CYP340H2	-1.06	No change	0.6154	28.32±0.92	28.31±0.02

CYP6AE14	-1.09	No change	0.6947	25.99±0.24	25.93±0.35
CYP6AE12v1	-1.13	No change	0.4585	25.5±1.98	25.9±0.09
CYP6B2v3	-1.17	No change	0.8114	25.17±0.43	25.52±0.18
CYP341D1	-1.29	No change	0.5954	28.89±1.57	28.58±0.53
CYP6AE15v2	-1.41	No change	0.9931	28.93±0.81	29.02±0.04
CYP324A1	-2.16	Down regulated	0.9974	25.86±1.36	25.33±0.05
CYP36AN1	-2.44	Down regulated	0.005**	23.67±0.14	22.97±0.13
CYP4G26	-2.60	Down regulated	0.3527	22.95±3.16	21.64±0.30
CYP4S12	-3.12	Down regulated	0.6296	30.51±2.18	28.99±0.43
CYP9AJ3	-4.17	Down regulated	0.1467	30.66±1.38	29.18±0.07
CYP4G8	-5.28	Down regulated	0.6481	20.67±2.66	18.4±0.17
CYP4G9	-5.79	Down regulated	0.6391	23.2±2.79	20.79±0.08
CYB321B1	-6.82	Down regulated	0.018*	30.55±0.77	27.9±0.34
CYP304F1	-8.43	Down regulated	<0.001***	32.83±0.28	30.33±0.08
CYP340G1	-9.00	Down regulated	0.034*	29.53±1.26	26.48±0.23

Table 4.5 Mean Cq ± SE and gene regulation in BA43 compared to TWB3 strain for the rest of the gut tissue by RT-qPCR. Statistical significance of t-test: * P < 0.05, ** P < 0.01, *** P < 0.001.

Gene	**Regulation**	**Compared to Regulation Threshold**	**P-Value**	**Mean Cq ± SE**	
	BA43 X TWB3			***BA43***	***TWB3***
CYP4S13	92.39	Up regulated	<0.001***	25.58±0.91	33.41±0.67
CYP6AB10	35.71	Up regulated	0.093	24.19±1.25	29.93±0.33
CYP321A5	24.03	Up regulated	0.137	20.01±1.20	25.91±0.49
CYP4L5	8.72	Up regulated	0.047*	20.54±0.58	24.25±0.40
CYP4M7V2	8.33	Up regulated	0.121	23.6±1.40	27.24±0.23
CYP4L11	8.28	Up regulated	0.064	22.13±0.68	25.76±0.27
CYP4G8	6.68	Up regulated	0.374	24.34±2.95	27.66±0.19
CYP333A1	4.85	Up regulated	<0.001***	22.36±0.01	25.94±0.13
CYP367B2	4.04	Up regulated	0.312	29.95±1.16	32.55±0.59
CYP6AE24	3.84	Up regulated	0.014*	26.13±0.28	29.37±0.17
NADPH reductase	3.31	Up regulated	<0.001***	20.37±0.01	23.39±0.24
CYP6AE15	2.57	Up regulated	0.151	26.05±0.77	28.72±0.08
CYP354A4	2.52	Up regulated	0.009**	25.55±0.17	27.47±0.21
CYP6AE12	2.47	Up regulated	0.316	26.06±1.07	28.66±0.25
CYP6B43	2.38	Up regulated	0.030*	23.3±0.28	25.85±0.12
CYP324A1	2.12	Up regulated	0.346	26.01±1.09	28.4±0.18
CYP6AB9	2.10	Up regulated	0.359	22.67±1.60	24.32±0.11
CYP4G9	2.09	Up regulated	0.383	26.82±2.75	28.47±0.09
CYP4G26	2.05	Up regulated	0.378	27.35±3.44	29.94±0.14
CYP341B2	1.84	No change	0.374	28±1.01	30.43±0.13
CYP4S14	1.46	No change	0.366	30.21±1.32	32.31±0.38
CYP9A34	1.41	No change	0.309	25.78±0.62	27.57±0.17

CYP6B2	1.31	No change	0.284	23.83±1.88	25.52±0.37
CYP4M10V2	1.23	No change	0.417	29.7±0.38	30.58±0.19
CYP6AE19	1.23	No change	0.863	24.88±0.32	26.73±0.87
CYP301A	1.15	No change	0.700	37.54±0.87	39.04±0.71
CYP6AE17	1.08	No change	0.504	22.31±1.39	23.73±0.42
CYP9A14	1.06	No change	0.844	26.34±0.09	27.73±0.24
CYP6B7	-1.03	No change	0.328	25.35±2.37	26.87±0.37
CYP9A3	-1.09	No change	0.582	28.26±0.08	29.44±0.22
CYP340J1	-1.36	No change	0.590	28.77±1.48	29.89±0.22
CYP4S12	-1.37	No change	0.218	28.66±0.25	28.79±0.14
CYP340G1	-1.46	No change	0.537	30.87±1.97	30.91±0.70
CYP340H1	-1.53	No change	0.628	27.3±1.49	28.25±0.19
CYP341D1	-1.55	No change	0.974	31.42±1.45	32.08±0.63
CYP340K1	-1.55	No change	0.955	27.51±0.98	27.45±0.17
CYP367A2	-1.59	No change	0.174	29.92±0.35	30.56±0.21
CYP332A1	-1.59	No change	0.905	24.48±1.31	25.11±0.18
CYP6B6v2	-1.90	No change	0.909	21.66±1.73	22.04±0.48
CYP340H2	-3.09	Down regulated	0.632	29.87±1.25	29.8±0.14
CYP4AU1	-3.45	Down regulated	0.037*	25.84±0.40	25.61±0.29
CYP305B1	-3.61	Down regulated	0.038*	27.05±0.42	26.5±0.32
CYP337B1	-4.05	Down regulated	0.173	22±1.11	21.54±0.32
CYP6AE11	-4.40	Down regulated	0.088	21.88±1.36	21.3±0.26
CYP9G5	-4.56	Down regulated	0.036*	29.36±0.35	28.46±0.43
CYP4M6	-6.30	Down regulated	0.185	25.67±2.36	23.6±0.08
CYP9A15	-6.51	Down regulated	0.023*	24.9±0.98	23.5±0.24
CYP36AN1	-7.18	Down regulated	0.003**	23.25±0.23	21.71±0.20
CYP6AE14	-7.69	Down regulated	0.050	25.86±1.00	24.48±0.38
CYP304F1	-8.01	Down regulated	0.001**	31.93±0.44	30.23±0.14
CYP321B1	-9.55	Down regulated	0.002**	32.24±0.94	29.57±0.05
CYP9AJ3	-11.54	Down regulated	0.013*	31.1±1.55	28.88±0.17
CYP9A23	-12.86	Down regulated	<0.001***	28.41±0.56	26.03±0.11

4.4 Discussion

In this study we confirmed the existence of 2 non-synonymous mutations on the sodium channel of the Brazilian pyrethroid-resistant strain BA43. Mutations V937G and Q960H have not been reported previously in this species. Some of the pyrethroid-resistance mechanisms found previously in *H. armigera* were associated with a condition of nerve insensitivity, termed *kdr* and *super-kdr* mutations (Dong, 2007). This type of *kdr* and *super-kdr* resistance was reported in pyrethroid-resistant strains of *H. armigera* in Australia (Gunning, 1996; Gunning et al., 1996), Thailand (Ahmad, 2007), China and India (McCaffery, 1998).

In 1998 Head et al. (1998) investigated the *para*-homologous sodium-channel gene

from a North American strain of *H. virescens* and a Chinese strain of *H. armigera*, both reported with nerve-insensitivity resistance to pyrethroids (McCaffery, 1998). After analyzing and comparing the sequences, the authors found two point-mutations, GAC to GTC and GAA to GGA, termed D1561V and E1565G mutations, which are located between regions III and IV (Head et al., 1998). In the Brazilian resistant strain, we found different point-mutations located in different regions from those reported by Head and coworkers. In our case, the mutations were found in regions IIS5 and between IIS4 and IIS5.

H. zea is considered a complex of cryptic species, because of the morphological similarity and evolution process (Leite et al., 2014; Pearce et al., 2017), and is the closest to *H. armigera*. Although in Brazil *H. zea* is extremely susceptible to pyrethroids, in the United States this pest is resistant to this group of insecticides (Hopkins and Pietrantonio, 2010). The voltage-gated sodium channel of a pyrethroid-resistant strain of *H. zea* was sequenced and analyzed in comparison to a susceptible line. The authors found the mutations V421M, V421 residue, V421A, V421G, I951V and L1029H, all located in domains I and II, subunits 5 and 6 (IS5, IS6 and IIS6) (Hopkins and Pietrantonio, 2010). A similar mutation, L1029H, has been found previously in *H. virescens* (Park and Taylor, 1997).

Here we report 2 mutations (V937G and Q960H) in the *H. armigera* voltage-gated sodium channel. Further studies are needed to confirm how these mutations are linked to pyrethroid resistance in Brazil or if they are SNPs present among Brazilian strains. As previous results with the synergists PBO and DEF demonstrated a suppression of P450s and esterase activity against pyrethroids in Brazilian populations (Durigan et al., 2017), there is a strong indication that the high resistance in Brazil is associated with metabolic enzymes.

The chimeric P450 *CYP337B3* is a resistance mechanism developed in *H. armigera* worldwide, by recombination of two genes (*CYP337B1* and *CYP337B2*). It is associated with metabolization of fenvalerate, cypermethrin and deltamethrin (Joußen et al., 2012; Joußen and Heckel, 2016; Durigan et al., 2017). However, this is not the only mechanism associated with *H. armigera* pyrethroid resistance in China and Pakistan (Rasool et al., 2014) (Han et al., 2015). In the Brazilian pyrethroid-resistant strain BA43 we showed through RT-qPCR that some of the P450 genes tested were up-regulated in comparison with the Australian TWB3 strain.

The up-regulation of other P450 genes found in this study may help to better understand why the Brazilian strain possesses this high level of resistance. In both tissues analyzed, the gut and the remainder of the body, we found three main up-regulated genes, *CYP6AB10*, *CYP4S13* and *CYP321A5*. Among these genes, *CYP6AB10* was previously

reported as overexpressed in the body tissue of *H. armigera* and is related to detoxification of gossypol secondary metabolites (Celorio-Mancera et al., 2011). In the same study, the expressions of *CYP4S13* and *CYP321A5* were characterized as unchanged and down-regulated in the non-gut body tissue (Celorio-Mancera et al., 2011).

In a pyrethroid-resistant strain from Pakistan Rasool et al. (2014) found the gene *CYP340G1* up-regulated in 146-fold and associated with *H. armigera* resistance to cypermethrin. For this same CYP cluster we found two other genes overexpressed in BA43, *CYP340K1* and *CYP340J1*. In a fenvalerate-resistant strain of *H. armigera* from Australia, a 2.2-fold up-regulation was reported for the gene *CYP4G8* (Pittendrigh, 1997), while in this study we found that this same gene was overexpressed 6.68-fold in the gut tissue of strain BA43.

In China, resistance mechanisms of *H. armigera* to pyrethroids were explored by Wee et al. (2008) through molecular techniques. They found an overexpression of not only P450s but also one esterase and one glutathione, termed *CYP4S1, CYP337B1, ESTX18 and GSTX01*, but only the P450s and esterase were involved with resistance. Another important cluster of P450 genes is the *CYP6B* cluster (Grubor and Heckel, 2007); some genes that belong to this group are associated with *H. armigera* resistance to pyrethroids and also with detoxification of other metabolites. The gene *CYP6B7* was reported to be linked with fenvalerate resistance in two *H. armigera* strains, AN02 and HDFR, from Australia and China respectively (Grubor and Heckel, 2007; Zhang et al., 2010). Other P450s were reported to promote very high levels of resistance to pyrethroids in China, where the genes *CYP9A12*, *CYP9A14* and *CYP9A17(v2)* showed a high affinity with and ability to metabolize esfenvalerate, deltamethrin, bifenthrin and cyhalothrin (Yang et al., 2008; Zhang S., 2008).

Resistance of *H. armigera* to pyrethroids emerged more than 30 years ago. However, many aspects of the characteristics of this resistance in many countries, including in recently invaded Brazil, remain unclear. Until now, only a few P450 genes were recognized as involved in pyrethroid resistance; some carboxylesterases (Gunning et al., 1996) (Gunning et al., 1999) (Wee et al., 2008) were also reported, and until now no GST was found to be associated with resistance (Joußen and Heckel, 2016).

In the current study, we report the presence of two mutations in the *H. armigera* voltage-gated sodium channel in a Brazilian pyrethroid-resistant strain, and also up-regulation of some P450 genes that may be involved in the high resistance level found in Brazil. Additional research is needed to prove the association between these findings and pyrethroid resistance, in order to help to manage insect resistance and implement integrated pest-

management programs in Brazil.

4.5 Conclusions

- ✓ Two mutations, V937G and Q960H were found in the Brazilian pyrethroid-resistant strain;
- ✓ 25 P450 genes were up-regulated in most body tissues, and 19 genes up-regulated in gut tissue in strain BA43;
- ✓ Further studies are needed to confirm if these mutations and also up-regulation of P450s are linked to pyrethroid resistance in Brazil.

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5. BASELINE SUSCEPTIBILITY TO INDOXACARB IN *Helicoverpa armigera* (LEPIDOPTERA: NOCTUIDAE) POPULATIONS IN BRAZIL

Abstract

The cotton bollworm *Helicoverpa armigera* was officially detected in Brazil in 2013. This species has evolved resistance to many insecticides including indoxacarb, an important molecule used for pest control. Understanding the susceptibility of *H. armigera* populations to insecticides is crucial for the implementation of Insect Resistance Management Programs and delaying the development of resistance in the field. In this study we characterized the mortality-response curves and monitored the susceptibility of *H. armigera* to indoxacarb in Brazil from 2013 to 2017. All the bioassays were conducted with third-instar larvae, using the diet-overlay method, and mortality data were assessed after 48 h. LC_{50} values ranged from 0.22 (0.16–0.28) μg a.i./cm^2 to 0.57 (0.41–0.82) μg a.i./cm^2, varying 2.60 times. Populations monitored with the diagnostic dose of 6.1 μg a.i./ cm^2 reached the highest survival in 2017 (53.51% in Mato Grosso do Sul State). An indoxacarb-resistant strain was selected in the laboratory, and the resistance ratio, estimated against a susceptible strain, showed a 297.5-fold difference. The susceptibility of *H. armigera* to indoxacarb has been decreasing in Brazil over the crop years analyzed. These results indicate that growers, industry and government must implement resistance management strategies to delay the evolution of indoxacarb resistance in Brazil.

Keywords: Cotton bollworm; Resistance management; Dose-response curves; Oxadiazine; Susceptibility monitoring

5.1 Introduction

Since 2013 when it was first detected in Brazil, the cotton bollworm *Helicoverpa armigera* (Hübner) has caused severe damage to many important crops (Czepak et al., 2013; Specht et al., 2013; Tay et al., 2013; Leite et al., 2014; Durigan et al., 2017). *H. armigera* is a polyphagous and highly destructive pest, responsible for economic losses of U$5 billion per year in the Old World, i.e. Europe, Asia, Africa and Australia alone (Pearce et al., 2017; Tay et al., 2017). In Brazil the loss was U$0.8 to U$2 billion, mainly in the state of Bahia in 2012–2013, when the occurrence of this pest was confirmed in soybean and cotton fields (Sosa-Gómez et al., 2016).

Most of the harm from *H. armigera* results from the cost to control this species when it reaches the level of economic damage to crops (Tay et al., 2017). As a consequence of *H. armigera* damage in Brazil, growers began to use high doses of insecticides and increased the number of sprayings in their crops, inevitably increasing the selection pressure in the field.

One of the insecticides recommended for control of this species in Brazil and worldwide is the oxadiazine indoxacarb (Group 22A), a sodium-channel blocker (Wing et al., 2000; Silver et al., 2017). Similarly to pyrethroids, indoxacarb acts on the sodium channel; however, while pyrethroids keep the channels open, resulting in insect overexcitation, the

prodrug indoxacarb locks the channels and prevents membrane depolarization and nerve-impulse transmission, and the insect becomes paralyzed, stops feeding, and dies (Wing et al., 2000; Lapied et al., 2001). Indoxacarb is the most recently discovered molecule that targets sodium channels. It is commonly called a prodrug, because once ingested by the insect it must be metabolized by an esterase or amidase to cleave the carbomethoxy group from the urea bond and then release the metabolite that will actually bind in the sodium channels, the free urea N-decarbomethoxylated DPX-MP062, DCMP or DCJW (Silver et al., 2017).

In most countries where *H. armigera* originated, indoxacarb is still considered an efficient control. However, as a consequence of the increase of sprayings and doses in the field, the Arthropod Pesticide Resistance Database (Arthropod Pesticide Resistance Database, 2018) indicates that 18 cases of resistance to indoxacarb have been reported in Australia, China and Pakistan (Aheer et al., 2009; Qayyum et al., 2015; Bird, 2015; Bird, 2017; Wang et al., 2017). The evolution of insecticide resistance in the field is a consequence of the selection pressure combined with the high genetic variability among individuals (Georghiou & Taylor, 1977). Nevertheless, resistance can be prevented or reversed with an Insect Resistance Management (IRM) program (Roush and McKenzie, 1987).

Considering that cases of resistance to indoxacarb have been reported in the Old World, that indoxacarb and pyrethroids share the same target site, that pyrethroid resistance has been reported for *H. armigera* in Brazil (Durigan et al., 2017), and that the selection pressure in Brazilian fields is very high, chances are high that this species will evolve resistance to this oxadiazine. The objectives of this study were to characterize and understand the susceptibility of *H. armigera* populations to indoxacarb in Brazil; to monitor the susceptibility of populations over several crop years, based on a diagnostic dose; and to develop an indoxacarb-resistant strain under laboratory conditions.

5.2 Material and Methods

5.2.1 Insect sampling and rearing

H. armigera populations in the field were sampled from 2013 to 2018. We chose the most representative regions in Brazil, in the states of Bahia, Goiás, Mato Grosso, Mato Grosso do Sul and São Paulo, based on their economic importance and soybean, cotton and bean production (Table 5.1). Larvae sampled in the field were brought to the Arthropod Resistance Laboratory at the University of São Paulo in Piracicaba, São Paulo, where the rearing and bioassays were conducted. Once the larvae pupated they were treated with a 1%

copper solution for decontamination. They were maintained in cylindrical cages made of PVC tubes (30 cm high × 25 cm diameter) covered with tulle, until the adults emerged; as the adults emerged they were provided a 10% honey solution. The populations were reared in a mass cross with 30 to 40 pairs in each cage. Three times a week the eggs laid on the tulle were collected and transferred to another container and maintained on an artificial diet adapted from Greene et al. (1976). Third-instar larvae were used to perform the dose-response bioassays with the insecticide. Larvae and adults were reared in the laboratory under controlled conditions, with 25 ± 1°C, 70 ± 10% RH and 14:10 (light: dark) photoperiod. All populations were identified by a molecular method using genomic DNA extracted from the moths, by the PCR-RFLP technique described by Behere et al. (2008), in order to distinguish *H. armigera* from the morphologically similar *H. zea* and separate the two species.

5.2.2 Susceptible strain

The susceptible strain TWB was collected in Towoomba, Queensland, Australia in January 2003, and since that time has been kept under laboratory conditions without selection pressure. This strain was donated to the Arthropod Resistance Laboratory of USP/ESALQ by Dr. David Heckel, researcher at the Max Planck Institute for Chemical Ecology, Jena, Germany (Import permit: 17BR025050/DF).

Table 5.1. Population codes used in the laboratory, municipality, crop where *Helicoverpa armigera* were sampled, and date of sampling.

Population code	Collection year	Crop	Municipality, State[a]	Latitude (S)	Longitude (W)
[b]BA 33	Jun 2013	Bean	Luís Eduardo Magalhães, BA	12°05'58"	45°47'54"
[b]BA 43	Jan 2014	Soybean	Luís Eduardo Magalhães, BA	12°05'58"	45°47'54"
[b]BA 45	Feb 2014	Cotton	Luís Eduardo Magalhães, BA	12°05'58"	45°47'54"
BA 52	Nov 2014	Soybean	São Desidério, BA	12°21'48"	44°58'24"
BA 64	Jun 2015	Cotton	Luís Eduardo Magalhães, BA	12°05'58"	45°47'54"
BA 66	Oct 2015	Bean	Luís Eduardo Magalhães, BA	12°05'58"	45°47'54"
BA 68	Dec 2015	Soybean	Luís Eduardo Magalhães, BA	12°05'58"	45°47'54"
BA 69	Jan 2016	Soybean	Luís Eduardo Magalhães, BA	12°05'58"	45°47'54"
BA 74	Oct 2016	Millet	Barreiras, BA	12°09'10"	44°59'24"
BA 76	Apr 2017	Cotton	Barreiras, BA	12°09'10"	44°59'24"
[b]GO 02	Mar 2014	Soybean	Mineiros, GO	17°34'10"	52°33'04"
GO 05	Feb 2015	Soybean	Santo Antônio do Rio Verde, GO	18°09'57"	47°56'47"
GO 06	Mar 2016	Soybean	Mineiros, GO	17°34'10"	52°33'04"
GO 09	May 2017	Cotton	Mineiros, GO	17°34'10"	52°33'04"
[b]MS 05	Oct 2013	Cotton	Costa Rica, MS	18°32'38"	53°07'45"
MS 08	Dec 2016	Soybean	Chapadão do Sul, MS	18°47'39"	52°37'22"
MS 09	Apr 2017	Cotton	Chapadão do Sul, MS	18°47'39"	52°37'22"
[b]MT 11	Oct 2014	Soybean	Primavera do Leste, MT	15°33'32"	54°17'46"
MT 15	May 2015	Cotton	Campo Verde, MT	15°32'48"	55°10'08"
MT 19	Mar 2016	Soybean	Primavera do Leste, MT	15°33'32"	54°17'46"
MT 23	May 2016	Millet	Primavera do Leste, MT	15°33'32"	54°17'46"
MT 25	Jun 2016	Millet	Campo Verde, MT	15°32'48"	55°10'08"
MT 26	Oct 2016	Soybean	Campo Novo do Parecis, MT	13°40'31"	57°53'31"
MT 27	Oct 2016	Soybean	Nova Mutum, MT	13°49'44"	56°04'56"
MT 28	May 2017	Cotton	Juscimeira, MT	16°03'03"	54°52'50"
SP 19	Apr 2016	Bean	Limeira, SP	22°33'53"	47°24'06"
BA77	Jan 2018	Soybean	Luís Eduardo Magalhães, BA	12°05'58"	45°47'54"

[a] State abbreviations: BA, Bahia; GO, Goiás; MA, Maranhão; MS, Mato Grosso do Sul; MT, Mato Grosso; PR, Paraná; RS, Rio Grande do Sul; SP, São Paulo.
[b] Populations used in the baseline susceptibility bioassays.

5.2.3 Baseline susceptibility bioassays

Diet-overlay bioassays were carried out for the formulated insecticide using 24-well acrylic plates (Costar®) containing 1.3 ml of artificial diet per well. For each *H. armigera*

population, six to eight concentrations of indoxacarb 150 g L^{-1} (Avaunt/Avatar, FMC®) were tested. The insecticide was diluted at different concentrations in distilled water, and 0.1% Triton X-100 was added to obtain a uniform spread of the solution over the diet surface. Each well received 30 µl of the insecticide-water solutions, applied on the diet surface. The control solution consisted of distilled water and 0.1% Triton X-100. After a drying period, one third-instar larva was added in each well. The plates were kept in a climate-controlled chamber with 25 ± 1°C, 60 ± 10% RH and 14 h photophase. Mortality was assessed after 48 h; larvae that did not move when touched with a tweezer were considered dead.

5.2.4 Data analysis

For the baseline susceptibility, mortality data were submitted to a Probit analysis (Finney, 1971) to estimate the LC_{50} and LC_{90} concentrations. Natural mortality was corrected using Abbott's formula (Abbott, 1925). The diagnostic dose for each insecticide was also estimated based on a LC_{99} where the same data were submitted to a joint analysis with a binomial model, using the log-log complement connection function. Data for larval survival obtained from the diagnostic-dose assays were transformed to $\arcsin\sqrt{x}/100$ and then subjected to an analysis of variance and also to a multiple-comparison analysis with the many-to-one comparison procedure of Dunnett (1955). All statistical analyses were performed in SAS Software (SAS Institute, 2000).

5.2.5 Selection of a resistant strain in the laboratory

The indoxacarb-resistant strain (AVA-100) was selected from a strain collected in Chapadão do Sul, MS, in a soybean field in December 2016 (MS08). Initially, third-instar larvae from the MS08 population were exposed to the diagnostic dose for susceptibility monitoring. Larvae that survived after treatment with this dose were transferred from the bioassay plate containing the insecticide to a plastic cup containing artificial diet without insecticide. They were reared until the next generation (F2), and the resulting third-instar larvae were treated with the same diagnostic dose. Only 30% of F2-generation larvae survived after treatment with the diagnostic dose. For the next generations, selection pressure was maintained with a concentration of 100 ppm until all the treated larvae survived, and this resistant strain was then termed AVA-100. This selection pressure was maintained for 5 generations in order to maintain the selection of the most resistant individuals and increase the

resistance ratio.

5.3 Results

5.3.1 Baseline susceptibility of *H. armigera* to indoxacarb

LC_{50} values for indoxacarb ranged from 0.22 (0.16–0.28) µg a.i./cm^2 in the BA45 population to 0.57 (0.41–0.82) µg a.i./cm^2 in the MT11 population, i.e., a 2.60-fold variation (Table 5.2). Based on the overlap of confidence intervals (95% CI), population BA45 differed from populations BA43, MS05 and MT11; while MT11 differed only from BA33. Population MT11 also showed the highest LC_{90} value (3.29 (1.95–8.10) µg a.i./cm^2); however, population BA33 showed the lowest LC_{90} (1.53 (1.16–2.23) µg a.i./cm^2). Slope values varied from 1.17±0.11 to 1.87±0.17 (Table 5.2), indicating a good mortality response of the *H. armigera* populations with increases in the insecticide dose.

Table 5.2. Mortality response of *Helicoverpa armigera* larvae (third instars) to indoxacarb, with diet-overlay bioassay.

Population code	*n*[1]	Slope ± SE[2]	LC_{50} (95% CI)[3]	LC_{90} (95% CI)[4]	$\chi^{2(5)}$	*P*-value[6]	d.f.[7]
BA33	434	1.87 ± 0.17	0.32 (0.26 – 0.38)	1.53 (1.16 – 2.23)	3.83	0.70	6
BA43	430	1.67 ± 0.16	0.42 (0.34 – 0.52)	2.45 (1.75 – 3.87)	8.39	0.21	6
BA45	405	1.35 ± 0.13	0.22 (0.16 – 0.28)	1.92 (1.30– 3.29)	1.71	0.88	5
GO02	758	1.17 ± 0.11	0.24 (0.18 – 0.30)	3.03 (2.08 – 5.13)	7.63	0.11	4
MS05	430	1.81 ± 0.19	0.46 (0.37 – 0.57)	2.33 (1.6 – 3.89)	8.50	0.13	5
MT11	425	1.69 ± 0.17	0.57 (0.41 – 0.82)	3.29 (1.95 – 8.10)	9.58	0.14	6

[1]number of larvae tested; [2]slope and standard error; [3]lethal concentration that kills 50% of larvae (µg a.i./cm^2) and 95% confidence interval; [4]lethal concentration that kills 90% of larvae (µg a.i./cm^2) and 95% confidence interval; [5]chi-square; [6] P-value (> 0.05) and [7]degrees of freedom.

5.3.2 Monitoring of *H. armigera* susceptibility to indoxacarb

The LC_{99} estimated with the joint analysis was 6.1 µg a.i./cm^2 (95% CI: 2.8–10.8 µg a.i./cm^2) and was used as the diagnostic dose for monitoring the susceptibility of the *H. armigera* populations over the crop years (Figure 5.1). BA33 was the first population sampled in 2013 and also the first used to validate the diagnostic dose, showing a survival of 30.42% (±0.58% SE). The lowest survival was observed in population BA43, sampled in early 2014, with only 1.25% (±0.13% SE) survival; while the highest survival was found in MS08, with 53.51% (±0.66% SE). Monitoring in Bahia revealed wide variation in population survival, ranging from 1.25% (BA43) to 31.25% (BA74). In Mato Grosso susceptibility was signifi-

cantly lower, ranging from population MT15 with 9.58% (±0.40% SE) survival in 2014, to 47.08% (±1.32% SE) in population MT26 in 2017. In general, over the crop years the susceptibility of *H. armigera* populations to indoxacarb decreased; this was most evident in the 2016/2017 season when survival rates reached an average 34%.

To conduct the many-to-one multiple comparisons by Dunnett's method (Dunnett, 1955), initially population BA33, sampled in early 2013, the year when *H. armigera* was detected in Brazil, was set as the susceptibility reference. Subsequently all the monitored populations were compared against BA33. Based on the results of Dunnett's test, the survival found in populations BA43, BA52, BA66, BA68, MT15, MT19, MT23, SP19 and BA77 was significantly lower than in BA33, while the survival in MS8 was significantly higher ($P < 0.0001$) than in the reference population BA33 (Figure 5.1).

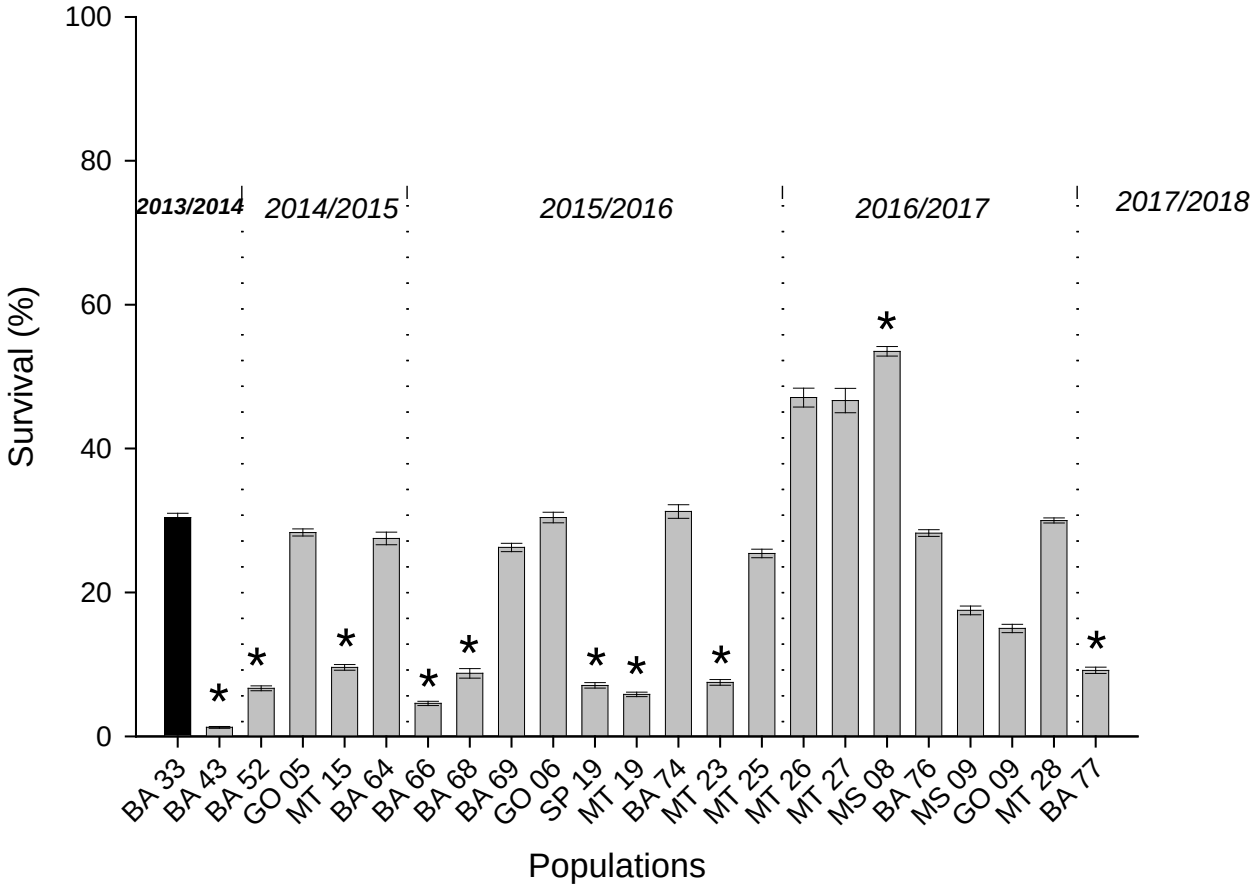

Figure 5.1. Survival of *Helicoverpa armigera* larvae (third instars) monitored over the crop years 2013–2017, with the diagnostic dose of 6.1 µg a.i./cm^2 of indoxacarb, using the diet-overlay bioassay.

* Populations that differ from the control (BA33), by Dunnett's test. *n*=240 larvae.

** State abbreviations: BA, Bahia; GO, Goiás; MS, Mato Grosso do Sul; MT, Mato Grosso; SP, São Paulo.

5.3.3 Resistance ratio for the laboratory-selected indoxacarb-resistant strain

The LC_{50} value estimated for the selected indoxacarb-resistant strain (AVA-100) was 3.57 μg a.i./cm^2 (95% CI: 2.89–4.40 μg a.i./cm^2), while the LC_{50} for the susceptible strain (TWB) was 0.012 μg a.i./cm^2 (95% CI: 0.009–0.01 μg a.i./cm^2). The resistance ratio (RR) was estimated by dividing the LC_{50} for AVA-100 by the value for TWB, resulting in a RR of 297.5 times (Table 5.3). LC_{90} ranged from 0.05 μg a.i./cm^2 (0.04–0.07 μg a.i./cm^2) in TWB to 17.62 μg a.i./cm^2 (12.92–31.36 μg a.i./cm^2) in AVA-100, showing a resistance ratio equal to 352 times. The susceptible TBW strain showed a higher slope value (2.02 ± 0.19), indicating that this strain had a higher mortality response as the indoxacarb dose increased.

Table 5.3. Mortality response of *Helicoverpa armigera* larvae (third instars) from AVA-100 and TWB strains to indoxacarb, with diet-overlay bioassay.

Strain	n[1]	Slope ± SE[2]	LC_{50} (95%CI)[3]	LC_{90} (95%CI)[4]	$\chi^{2(5)}$	d.f.[6]	RR[7]
AVA-100	336	1.8 ± 0.21	3.57 (2.89–4.40)	17.62 (12.92–31.36)	3.1	4	297.5
TWB	384	2.02 ± 0.19	0.012 (0.009–0.02)	0.05 (0.04–0.07)	2.67	5	–

[1]number of larvae tested; [2]slope and standard error; [3]lethal concentration that kills 50% of larvae (μg a.i./cm^2) and 95% confidence interval; [4]lethal concentration that kills 90% of larvae (μg a.i./cm^2) and 95% confidence interval; [5]chi-square; [6]degrees of freedom; [7]resistance ratio based on LC_{50}.

5.4 Discussion

The determination of the mortality-response curves is the first step in studies to elucidate insect resistance (Roush and McKenzie, 1987). The results of the bioassays indicate how the mortality response to a certain insecticide varies among different populations. The low variation between LC_{50} values for indoxacarb found here for *H. armigera* populations suggests that when this species invaded Brazil in 2013 and 2014, most individuals showed the same response to this insecticide, mainly because the 3-fold variation among them is considered a natural level of variation among wild individuals.

A similar variation in LC_{50} values (3.52 times) was observed among 22 *H. armigera* populations in Australia (Bird, 2015), and even after more than 10 years of indoxacarb use in the field, the author did not find large differences in that country. A low variation (5.6 times) was also observed among Pakistani populations of *H. armigera* in 2008 (Sayyed & Ahmad, 2008). However, a few years later in Pakistan, the difference in LC_{50} values estimated for 20 populations varied from 1 to 20 times, suggesting that in some regions the selection pressure

increased and is contributing to a higher tolerance and evolution of resistance to indoxacarb in that country (Qayyum et al., 2015).

The second step in studies of resistance is to monitor the susceptibility of populations over several years in order to generate enough data to prevent the evolution of resistance in the field (Roush and Miller, 1986). To perform this monitoring, it is first necessary to estimate a diagnostic dose, which is usually based on the LC_{99} concentration. Both baseline susceptibility and monitoring should be done by commercial companies before the release and marketing of a new product, in order to prevent the development of resistance and maintain the effectiveness of the insecticide for a long period in the field (Sparks and Nauen, 2015). Nevertheless, most of the time the pesticide companies only begin to monitor susceptibility after a long period, when control failures occur.

Results found in this study during 4 years of monitoring *H. armigera* susceptibility to indoxacarb in Brazil indicate that probably the founding individuals of *H. armigera* populations in this country arrived already carrying resistance genes or with intrinsic characteristics that confer a high tolerance to this insecticide. When BA33 was exposed to the diagnostic dose of 6.1 µg a.i./cm^2 in 2013, survival was considered relatively high (31.25%), and although some other populations showed lower survival in 2014 and 2015, ranging from 1.25% to 9.58% at this same dose, most of the populations monitored showed moderate susceptibility to indoxacarb. The most worrisome scenario was observed in the 2016/2017 crop year, due to the increase in *H. armigera* survival at the diagnostic dose. The recommended doses of indoxacarb to control *H. armigera* in cotton and soybean are 76 ppm and 38 ppm respectively (Ministério da Agricultura Pecuária e Abastecimento, 2017), 7.4 and 14.7 times lower than the diagnostic dose used to monitor its susceptibility in this study (560 ppm).

Some of the reasons for the large increase in *H. armigera* survival have been questioned, especially because it is known that resistance evolves mainly with an increase in selection pressure from a specific insecticide (Georghiou, 1977; Roush and McKenzie, 1987), and indoxacarb is not widely used by Brazilian growers. Farmers prefer to use insecticides of the diamide and growth-regulator groups, as well as emamectin benzoate. Considering this point, the main hypothesis is that the decrease in susceptibility of *H. armigera* to indoxacarb in Brazil is occurring as a consequence of the high selection pressure exercised not only by indoxacarb but, mainly, by other insecticides.

Pearce et al. (2017) compared the genomes of *H. armigera* and *H. zea* and found that *H. armigera* has a higher expression and 70 more genes associated with its defense against

xenobiotics, and that the expression of detoxification genes is induced and increases when it is exposed to a toxic compound such as an insecticide. Therefore, what may be occurring in *H. armigera* is an increase in detoxification due to this natural defense, conferring a high tolerance to most insecticides including indoxacarb.

The resistance ratio of 357 times between the laboratory-selected resistant strain AVA-100 and the Australian susceptible TWB found in this study is higher than the resistance ratio of 198 times found by Bird et al. (2017) for an indoxacarb-resistant strain from Australia. These authors also discovered that the inheritance of indoxacarb resistance in Australia is autosomal, incompletely dominant, and monogenic, characteristics that contribute to the rate of resistance evolution in the field by conferring a resistant phenotype on the heterozygotes. In Pakistan a resistance ratio of 379 times was found for a population of *H. armigera* sampled in the field, whereas the ratio of resistance to indoxacarb found for a Cry1Ac-resistant strain was 3088-fold, suggesting that selection for Cry1Ac also increased the selection for indoxacarb (Alvi et al., 2012).

Some aspects of indoxacarb resistance in *H. armigera* remain unknown, mainly regarding its mechanisms of resistance. Some researchers from Australia, Pakistan, India and China, after conducting tests using synergists such as PBO, have suggested that resistance of *H. armigera* to indoxacarb is associated with P450 enzymes, and more-recent investigations have indicated that this resistance is linked to the *CYP6AE* gene cluster.

Whereas the mechanisms of resistance to indoxacarb in *H. armigera* are still being explored, for other pest species such as *Plutella xylostella, Tuta absoluta, Spodoptera litura* and *Sitophilus zeamais*, such studies have advanced further (Sayyed et al., 2008; Haddi et al., 2015; Wang et al., 2016; Zhang et al., 2017; Roditakis et al., 2017). In China and India, *P. xylostella* resistance to indoxacarb is conferred by both metabolic and target-site mutations, where cross-resistance with mataflumizone, beta-cypertmethrin and chlorfenapyr was also reported (Wang et al., 2016; Zhang et al., 2017; Marak et al., 2017). An indication of cross-resistance between indoxacarb and both spinosad and emamectin was reported for *S. litura* in Pakistan, where resistance was associated with detoxification by P450 and esterases (Sayyed et al., 2008). Indoxacarb resistance in *T. absoluta* was also associated with metabolic changes and mutations in the sodium channel that rendered the molecule incompatible with the target site (Roditakis et al., 2017).

The susceptibility to indoxacarb of the invasive *H. armigera* in Brazil is a matter of concern. The moderate susceptibility levels found in this study suggest that within a short period of time, indoxacarb could become inefficient for controlling this pest in the field. The

successful development of a resistant strain under laboratory conditions indicates that several mechanisms are involved in indoxacarb detoxification, or there may be mutations at the DCJW target site which confer resistance, and can also be present in low frequencies in wild populations. If Brazilian growers continue to increase the selection pressure, resistance will evolve rapidly in the field. The present results can be used to help companies and farmers in their pest-management decisions, and will alert stakeholders to the importance of implementing an insect resistance-management (IRM) program in Brazil.

5.5 Conclusions

- ✓ Susceptibility of Brazilian *H. armigera* populations to indoxacarb decreased over crop years (2013/14 to 2016/17);
- ✓ The potential for Brazilian populations of *H. armigera* to evolve resistance to indoxacarb is high.

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6. FINAL CONSIDERATIONS

The results found in the current study confirm the hypothesis that *H. armigera* individuals who invaded Brazil were already resistant to pyrethroids. Here we prove that one of the main mechanisms of pyrethroid resistance, the chimeric P450 *CYP337B3*, is present in every insect of *H. armigera* in Brazil, in a frequency of > 95% in most of the populations sampled from 2013 to 2017. We also prove that the inheritance of this resistance has a dominant characteristc beeing also autosome, two factors that contribute for the rapid spread of the resistant allele increasing resistance evolution rate in the field. Furthermore our findings suggest that pyrethroid resistance in *H. armigera* populations in Brazil is due not by a unique gene but there are probably others mechanisms confering the high resistance found. The results show that the susceptibility of *H. armigera* to indoxacarb decreased trhoughou the cropping years and if it may become a problem in the future and as the laboratory selected strain also posses the *CYP337B3* gene, it is possible the existence of a multiple resistance case in Brazil. The two non-synonimous mutations found in the sodium channel and also the high expression of some others P450s must be more explored to confirm their linkage with pyrethroid or indoxacarb resistance.

Based on these findings we highlight the importance to know and monitor the pest in the field in order to choose the best control methods based in the concept of integraded pest management (IPM), integrating different tactics such as biological, chemical, cultural, biotechnology control. In the case of an invasive pest, it is very important to know and understand the resistance background in its countries of oringin and recommend a program for emergencial control based on this background. In the case of *H. armigera* in Brazil, the authorities recommended pyrethroids in emergencial use however the resistance frequency in the countries of its pest origin was extremely high. Finally our results suggest that the implementation of a resistance management program for *H. armigera* in Brazil is urgent and essential if we want to keep our fields productivity and sustainability. The implementation of an IRM program in Brazil is crucial to preserve the insecticides molecules and also Bt toxins available and effective for a longer period of time in the field.