CHARACTERIZATION OF RESISTANCE MECHANISMS AND INHERITANCE OF THE FALL ARMYWORM *SPODOPTERA FRUGIPERDA* (J.E. SMITH) (LEPIDOPTERA: NOCTUIDAE) FROM PUERTO RICO TO SYNTHETIC AND BIOLOGICAL INSECTICIDES.

By

Omar Alejandro Posos-Parra

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ABSTRACT

The fall armyworm (FAW) (*Spodoptera frugiperda* J.E. Smith) poses a considerable challenge to global corn production owing to its remarkable ability to develop resistance to synthetic insecticides and insecticides derived from microorganisms, thereby undermining conventional pest management strategies. A remarkable FAW adaptation to pesticides was detected in Puerto Rico (PR). This region has one of the most strategic nurseries in the world for seed augmentation and research. However, a combination of factors including more than ten FAW generations per year, a high pest infestation, isolated geographical conditions, and 29 insecticide sprays per season prompt FAW to develop quick resistance. This doctoral thesis comprises three detailed studies that address the FAW inheritance and metabolic mechanism of resistance to synthetic insecticides and derived insecticides from microorganisms *Saccharopolyspora spinosa.* Diet overlay bioassays were conducted in all experiments, employing third early instar larvae.

In the initial study, I characterized the resistance of the PR strain of FAW to the diamide insecticides chlorantraniliprole and flubendiamide, uncovering high resistance ratios (RR_{50} = 96-fold for chlorantraniliprole and RR_{50} = 2,762-fold for flubendiamide). This resistance exhibits distinct inheritance patterns, with chlorantraniliprole showing autosomal inheritance and flubendiamide demonstrating an X-linked inheritance mode. Additionally, the resistant strains exhibited cross-resistance to cyantraniliprole and cyclaniliprole. Synergist bioassays using DEF, DEM, PBO and verapamil reveal crucial role of detoxification enzymes as glutathione S-transferases, ABC transporters and esterases in resistance to flubendiamide and reduced metabolism for chlorantraniliprole. High levels of resistance from the PR strain to pyrethroid insecticides esfenvalerate (62-fold) and deltamethrin (15-fold) were detected. The inheritance of

resistance exhibited an incompletely dominant resistance trait. Cytochrome P450 monooxygenases, esterases, glutathione S-transferases, and ABC transporters had an important role in the detoxification of the esfenvalerate. For deltamethrin, significant resistance was attributed to glutathione S-transferases, with lesser roles for ABC transporters and minor contributions from cytochrome P450 monooxygenases and esterases. This sheds light on the complex interplay of detoxification enzymes in pyrethroid resistance.

The final study investigates resistance mechanisms in the PR strain to spinosyn insecticides. Very high levels of resistance were found for spinosad (214-fold) and spinetoram (63-fold), revealing an autosomal inheritance of resistance for spinosad and an X-linked for spinetoram. A higher role of esterases and glutathione S-transferases, followed by ABC transporters, and cytochrome P450 monooxygenases was observed in the Puerto Rican strain. Conversely, resistance to spinetoram was primarily mediated through by glutathione S-transferases, followed by ABC transporters, and lesser contributions from cytochrome P450 monooxygenases and esterases. These findings underscore the critical roles of detoxification enzymes, both individually and collectively, in conferring resistance. The synergist bioassays, employing both diet-overlay and topical applications, validated the hypothesis regarding the involvement of detoxification enzymes in resistance to spinosyns in the PR strain.

These studies analyze the genetic and biochemical factors behind insecticide resistance in FAW, highlighting the complexity and cross-resistance. They emphasize the need for new, holistic pest management methods and insecticide rotation. This research enhances understanding and provides crucial knowledge to manage resistance, potentially mitigating FAW's impact on agriculture worldwide.

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LIST OF ABBREVIATIONS

IRM	Integrated Resistance Management
IPM	Integrated Pest Management
FAW	Fall armyworm
PRABIA	Puerto Rico Agricultural Biotechnology
Bt	*Bacillus thuringiensis*
IRAC	Insecticide Resistance Action Committee

CHAPTER 1:

INTRODUCTION.

Evolution of agriculture and food security

The concept of agriculture today encompasses a profound understanding of its influence on the nature of social institutions, moral virtues, and the evolution of cultural forms (Barnhill et al., 2018). Archaeological evidence points to the origins of agriculture around 23,000 years ago, with early human civilizations initiating trial cultivations near the Sea of Galilee, Israel, marking the first known human impact on the environment for agricultural purposes (Price and Bar-Yosef, 2011; Snir et al., 2015). Over millennia, agriculture has undergone significant transformations, evolving independently across various parts of the world. From the pioneering shifts of the Neolithic Revolution to today's emphasis on Sustainable Agriculture, there has been a continuous evolution towards practices that protect the environment, enhance the natural resource base, and maintain soil fertility. This evolution reflects a commitment to ensuring sustainable and efficient agricultural practices for future generations (Rasmussen, 2020; USDA NIFA, 2020).

The concept of food security, as defined by the World Food Summit in 1992, emphasizes the assurance that all individuals have both physical and economic access to sufficient, safe, and nutritious food to meet their dietary needs for an active and healthy life (Chakraborty and Bird, 2020). The "2019 Revision of World Population Prospects" by the United Nations forecasts a global population increase of 2 billion over the next 30 years from the current 7.7 billion (UN, 2019; Wells, 2019). This surge in population is pinpointed as a key driver behind food shortages and climate change—issues that magnify the difficulties in achieving optimal crop cycles, further aggravated by pests and diseases (FAO, 2017; *Global Report on Food Crises*, 2019; Premanandh, 2011). To meet the anticipated crop demands by 2050, it is projected that food production must increase by 25% to 70% (Hunter et al., 2017). Therefore, addressing critical challenges in crop protection and devising innovative strategies to enhance food production are crucial steps toward securing a sustainable future for both the present and future populations.

Moreover, the implications of climate change add another layer of urgency to this issue. As temperatures rise and weather patterns become more unpredictable, the viability of traditional crop-growing regions is threatened, potentially reducing yields and making food production less predictable (Skendzic et al., 2021). This unpredictability, coupled with the increased incidence of pests and diseases, poses a formidable challenge to maintaining and improving food security. Adapting agricultural practices to cope with these changes, including the development of more resilient crop varieties and sustainable pest management strategies, becomes imperative (Kambrekar et al., 2015).

Advances and challenges in crop protection

Agriculture and, specifically, crop protection, has experienced continuous evolution in response to diseases and pests. Initially, ancient humans coexisted with pests without the means or necessity for control. However, as competition for food intensified, they began to develop methods to protect their crops, employing cultural techniques and physical and biological controls (Shankar and Abrol, 2012). The earliest recorded pest control technique, dating back to 2500 B.C., involved the use of sulfur compounds to combat mites and insects. A millennium later, around 1500 B.C., botanical insecticides and cultural control methods were applied for seed treatment in China, marking the advent of more sophisticated pest management strategies. The period from 1750 to 1880 was marked by the discovery of natural pyrethrins extracted from pyrethrum flowers and rotenone, heralding the advent of technified agriculture (Taylor et al., 2007; Matsuo, 2019). During this time, the first commercial spraying machine was introduced, which laid the groundwork for future advancements. The introduction of arsenicals and lime sulfur as pesticides was a significant milestone in the control of agricultural pests (Costa, 1987). These compounds were widely adopted due to their efficacy in combating a variety of insect and plant pathogens, thereby expanding the range of pest management strategies available to farmers. This period of innovation was crucial in evolving agricultural practices towards more sophisticated and targeted approaches to pest control. However, the phenomenon of insect resistance was first documented in 1914 (Melander, 1914), and by 1929, the first area-wide eradication effort targeted the Mediterranean fruit fly in Florida, USA (Papadopoulos, 2014). It was not until 1959 that the concepts of economic thresholds, economic injury levels, and integrated pest management were introduced by Stern, laying the foundation for modern crop protection strategies (Stern, 1959).

In the recent decades, the Green Revolution, spearheaded by Norman Borlaug, addressed the pressing hunger challenges of the time by developing the necessary institutional capacity for efficient plant breeding tools. These innovations aimed to significantly boost global agricultural production. Approximately a decade later, the concept of "integrated pest management" (IPM) alongside "life systems" was introduced by L.R. Clark, P.W. Geier, R.D. Hughes, and R.F. Morris (Waterhouse, 1967). This period also saw a pivotal shift in the perception of pesticides, largely influenced by Rachel Carson's seminal work, "Silent Spring," during the 1960s and 1970s. The book's publication led to the banning of DDT and paved the way for the advent of *Bacillus thuringiensis* (Bt)-based insecticide technologies, an early forerunner of genetically modified organism (GMO) technology, heralding what was perceived as a comprehensive solution to agricultural and food production challenges at the time (Ibrahim et al., 2010: Abdul Aziz et al., 2022).

However, this breakthrough also introduced significant challenges. The widespread and often indiscriminate application of pesticides, coupled with the adaptation of arthropods to GMO technologies—particularly those involving Cry toxin genes—has introduced new complexities in agricultural pest management. Despite these hurdles, pesticides remain indispensable to produce vegetables. Acknowledging the adverse impacts on beneficial insects, extensive efforts are being made to mitigate these effects through controlled and sustainable practices (Bonny, 2016; Duan et al., 2008; Duke and Powles, 2008; Losey et al., 1999; Malone and Burgess, 2009; Tabashnik et al., 2023).

Pesticide resistance and the FAW

Arthropod resistance to pesticides has reached a critical stage, and this trend is expected to continue to worsen on an annual basis. To date, over 18,871 cases of resistance to nearly 364 distinct insecticides and acaricides are documented. Moreover, an estimated 632 species of mites and insects exhibit resistance to one or more active ingredients, highlighting the gravity and intricacy of the issue (Dhaliwal and Ramesh, 1996; Mota-Sanchez and Wise, 2024; Nauen et al., 2019; Pingali, 2012; Shankar and Abrol, 2012; Whalon et al., 2008). Since 2008, the top 20 most resistant arthropods have included two-spotted spider mite (*Tetranychus urticae*), diamondback moth (*Plutella xylostella*), green peach aphid (*Myzus persicae*), Colorado potato beetle (*Leptinotarsa decemlineata*), and housefly (*Musca domestica*), all of which continue to pose significant challenges (Whalon et al., 2008). Recently, the fall armyworm *Spodoptera frugiperda* has been included with a group of species (Lepidoptera: Noctuidae), that has emerged as significant contributor to resistance, including the beet armyworm (*Spodoptera exigua*), Mediterranean climbing cutworm (*Spodoptera littoralis*), cotton earworm (*Helicoverpa armigera*) (Mota-Sanchez and Wise, 2024).

Over the past decade, FAW has garnered global attention not only for its potential to devastate up to 60% of a crop's yield, particularly in corn (Nagaratna et al. 2023), but also for its unique migratory capabilities, and escalating resistance to a variety of pesticides (Richardson et al., 2020; Kenis et al., 2023). This pest's adaptability is further underscored by its host range of more than 350 plant species in 76 botanical families (Cruz, 1995; Montezano et al., 2018; Wyckhuys and O'Neil, 2006), and perhaps one reasons for its pest adaption to pesticides (Alyokhin and Chen, 2017), underlining the threat it poses to a diverse array of crops. The FAW's ability to continually infest areas, aided by its non-dormant migratory nature, sets it apart from other migratory pests, enabling its relentless spread across regions. Originating in the Americas, FAW is one of the oldest pests documented by the Tzeltal tribes. A tribe descendent from the Maya civilization, employed historical management practices dating back to 1200 B.C. The Tzeltal's traditional method of controlling FAW (Chanul ixim) involved the manual removal of pests with a stick and crushing them underfoot (Nigh and Nations, 1980). This historical significance underscores the historical presence of FAW and the importance of integrating modern scientific strategies to combat this pervasive agricultural threat effectively (Gómez-Muñoz, 2004; Sparks, 1979; Westbrook et al. 2016).

The FAW's lifecycle can vary from an average of 30 days to up to 90 days in cooler conditions (Capinera, 2000; Kenis et al., 2023). Its reproductive cycle involves nocturnal activities where females, after mating, lay clusters of eggs on the undersides of leaves, typically producing around 1,500 eggs in their lifetime (Sparks, 1979; Capinera, 2000; Kenis et al., 2023). In general, the FAW goes through six to seven larval instars (Santos et al., 2003). Initially, larvae feed on their eggshells before moving on to the host plant's foliage. Due to its cannibalistic nature, it is mentioned that is rare but not impossible to find multiple larvae on a single plant

(Sparks, 1979; Capinera, 2000; Pannuti et al., 2015). In corn, second to six instars are often found in the whorl. Before pupation, larvae disengage from the plant and pupate in the soil, completing their development away from the host. This complex lifecycle, combined with its broad host range, genetic variability, and resistance to pest control methods, underscores the FAW's resilience and the multifaceted challenge it presents in agricultural management and crop protection strategies.

Until 2016, the FAW was primarily a concern for the American continent, notably affecting American countries and some Caribbean islands (Smith, 1797; Biondi et al., 2018). This scenario dramatically changed when FAW invaded the African continent presenting a new economic threat to those countries (Goergen et al., 2016). The situation escalated as the following years witnessed the spread of FAW reaching Asia, Australia, Europe, and more countries in Africa by 2024, confirming the accuracy of expert prediction models made in prior years (Early et al., 2018; Kalleshwaraswamy, 2018; Kim and Babcock, 2019; Li et al., 2019; Nagoshi et al., 2017; Richardson et al., 2020). Presently, the threat posed by FAW remains significant, with predictions indicating its continued adaptation and expansion across various territories and seasons.

Integrated Pest Management (IPM) is conceived as an ecosystem-based strategy aimed at uniting a variety of tools and techniques—ranging from cultural practices and the use of resistant crop varieties to biological and chemical controls, alongside habitat manipulation. The goal is to prevent and mitigate the long-term impacts of pests and diseases (UC IPM, 2020). Over the past decades, the effectiveness of IPM has largely hinged on comprehensive educational programs supported by growers, academic researchers, extension agents, and agricultural companies (EPA, 2023). In the United States, many agricultural colleges and research universities have established robust IPM programs at universities. The Environmental Protection Agency (EPA) and the

United States Department of Agriculture (USDA) annually offer grant opportunities, allocating funds ranging from $250,000 to $2.1 million per proposal, aimed at fostering innovative strategies to address pressing agricultural challenges (Finn, 2015; IPM Institute of North America, 2024). Furthermore, international bodies such as the Insecticide Resistance Action Committee (IRAC), along with the European and Mediterranean Plant Protection Organization (EPPO), the Arthropod Pesticide Resistance Database (APRD), Scientist Animations Without Borders (SAWBO), and CropLife International, collaborate as a global network. These organizations work together to ensure the flow of communication and education regarding insecticides, acaricides, nematicides, and fungicides, promoting the development of resistance management strategies. Collectively, they strive to advance and harmonize cutting-edge agricultural techniques worldwide, safeguarding agricultural sustainability for current and future generations (FRAC, 2020; IRAC, 2020; *IPM IMPACT*, 2024).

In recent years, a concerted effort by associations and committees has been dedicated to enhancing IPM programs, specifically targeting the rapid global spread of the FAW (FAO, 2022). This focus arises from the urgent need to address the pest's ability to migrate expansively, putting countries previously unexposed to FAW at significant risk due to their lack of experience and tools for managing such invasions.

Corn and rice strains of FAW

Research has unveiled two genetically distinct strains of FAW, each with preferences for specific hosts, notably corn and rice, showcasing the pest's genetic diversity and adaptation to different environments (Nagoshi et al., 2019; Nagoshi and Meagher, 2022). The application of genetic markers, such as the Cytochrome Oxidase Subunit I (COI) and Triosephosphate isomerase (TPi), has unveiled significant insights into the pest's population structure. Despite their morphological similarities and shared physiological and reproductive behaviors, including resistance development and pheromone activity, these strains exhibit distinct ecological niches (Feldmann et al., 2019; Nagoshi et al., 2017). Molecular analyses have been pivotal in tracing the origins of new infestations, revealing their potential provenance in the Eastern Hemisphere (Gutierrez-Moreno et al., 2019; Nagoshi et al., 2019; Souza et al., 2019; Mahalle et al., 2024a). The challenge is particularly acute for developing countries, where establishing robust Insect Resistance Management (IRM) programs and accessing new active ingredients are limited by economic constraints. Intriguingly, much of the African FAW population has been identified as a distinct haplotype, presenting unique behavioral traits and open the question if this strain, distinct to the corn and rice known strains, could have different adaptation strategies (Nagoshi et al., 2020; Acharya et al., 2021).

The unique case of FAW adaptation to pesticides in island type conditions

Puerto Rico is a strategic location for agricultural research, and seed increase, worldwide, particularly in plant breeding, due to its year-round favorable climate, and biotechnology-friendly environment (Belay et al., 2012; PRABIA, 2020; Storer et al., 2010). According to the Puerto Rico Agricultural Biotechnology Industry Association (PRABIA), over 85% of seeds used in biotechnological agriculture globally are developed under Puerto Rico's optimal conditions at some point in the process (PRABIA, 2020). However, this competitive advantage comes at significant cost. For instance, the climate in Puerto Rico is optimal for the development of more than 10 generations of FAW per year, and a very high larval infestation during crop production. To manage FAW, up to 30 pesticide applications are made in three to four consecutive growing cycles, totaling 90 to 120 sprays per year. This practice has led to pest resistance. As a result, FAW has developed high levels of resistance to synthetic pesticides and Bt toxins, which threatens the sustainability of the seed industry (Belay et al., 2012; Gutierrez-Moreno et al., 2019, Gutierrez-Moreno 2020).

To date, there are 252 cases reported of FAW resistance described involving 47 different active ingredients of which six are Bt Cry proteins and 41 are synthetic pesticides (Table 1.1.). It is noteworthy that nearly 10% of these resistance cases were documented in Puerto Rico over the past decade, emphasizing the island's pivotal role in the global struggle against FAW resistance (Mota-Sanchez and Wise, 2024). The agricultural fields of Puerto Rico face a persistent phytosanitary challenge, with pesticide resistance shortening the effective lifespan of these chemicals due to inadequate control measures and cross-resistance issues (Gutierrez-Moreno et al., 2019; Zhu et al., 2015).

Table 1.1. Number of FAW resistance cases (Mota-Sanchez and Wise, 2024).

Mode of action	**Cases of resistance**	**Number of countries**	**Countries**
Acetylcholine esterase inhibitors	53	6	Brazil, China, Mexico, USA and Venezuela
Chloride channel activators	21	2	Brazil and China
GABA-gated chloride channel antagonists	4	2	Bolivia and Paraguay
Inhibitors of chitin biosynthesis, type 0	3	2	Brazil and USA
Microbial disruptors of insect midgut membranes (includes transgenic crops)	75	5	Argentina, Brazil, China, and USA
Nicotinic Acetylcholine receptor agonists (allosteric)	11	3	Brazil, China and USA
Ryanodine receptor modulators	23	5	Brazil, China, Mexico, and USA
Sodium channel modulators	50	8	Bolivia, Brazil, China, Colombia, Mexico, USA and Venezuela
Voltage-dependent sodium channel blocker	12	1	China

In response, the seed industry has promoted collaboration among companies, entomologists, and agronomists to develop and implement strategies for the continuous monitoring and management of FAW resistance. In 2010, research on FAW resistance in Puerto Rico initiated by determining the efficacy of various active ingredients against FAW infestations (Storer et al., 2010). Initial findings revealed a growing resistance to Bt proteins, particularly elevated resistance levels to Cry1F and moderate resistance to Cry1Ac (Storer et al., 2010; Blanco et al., 2010). Subsequent studies conducted in 2013 and 2014 delved deeper into the genetic and enzymatic underpinnings of this resistance. In 2013, research demonstrated that the resistance to Cry1F in Puerto Rican FAW populations was recessive and autosomal, potentially linked to a single genetic locus, without evidence of cross-resistance to other Bt proteins (Vélez et al., 2013). One year later, a novel enzymatic profile was identified in the midgut of Puerto Rican FAW, revealing a complex interplay of esterases, glutathione S-transferases (GST), trypsin, and chymotrypsin in the resistance mechanism, along with evidence of shared binding sites contributing to cross-resistance between Cry1F and the insecticide organophosphate, acephate (Camargo et al., 2017; Jakka et al., 2014; Zhu et al., 2015; Gutierrez-Moreno et al., 2020).

Recent research has examined FAW populations in Mexico and Puerto Rico, revealing significant resistance and cross-resistance among synthetic insecticides, including considerable resistance to the diamide class. This indicates persistent selection pressure from the overuse of pesticides, highlighting the complexity of resistance mechanisms and inheritance patterns that remain to be fully understood (Gutierrez-Moreno et al., 2019). Addressing these gaps is crucial for extending the effective lifespan of active ingredients in Puerto Rico and enhancing IRM and

IPM strategies, especially in regions recently confronted with FAW threats. These advancements are essential for addressing future food security challenges.

Dissertation overview

This dissertation aims to elucidate the field-evolved resistance of FAW in Puerto Rico, to synthetic and biological insecticides from both the genetic and physiological perspectives. By analyzing a well-documented field resistant FAW population, this study diverges from conventional resistance research by focusing on naturally resistant strains rather than those derived from F_2 screening or genetic engineering. This approach contrasts with the methods previously reported in the literature (Carvalho et al., 2013; Lira et al., 2020), with the aim of providing a more comprehensive understanding of resistance mechanisms. Despite resistance development to synthetic compounds and Bt toxins, numerous questions about resistance behaviors across pesticide classes remain unanswered, with new cases of cross-resistance emerging. Some resistance levels have intensified over the past five years (Mota-Sanchez and Wise, 2024; Sparks et al., 2020). This project aimed to reveal the genetic and physiological profiles underlying the resistance of Puerto Rico's FAW population to various old and novel insecticides, including pyrethroids, diamides, and spinosyns. Understanding these resistance mechanisms is crucial for addressing the challenges faced by plant breeding companies in Puerto Rico and for mitigating the broader impacts on global food security through effective pest management.

Chapter 2 delivers an in-depth examination of the inheritance patterns of resistance to diamides in FAW populations from Puerto Rico. Diamide compounds target ryanodine receptors, triggering an uncontrolled release of calcium ions within the insect's muscle cells, leading to paralysis and death (Cordova et al., 2021; Nauen, 2006). This chapter not only updates the current understanding of FAW populations' susceptibility to diamides but also delves into the intricate relationship among genetic variations including dominance or reccesiveness of genes, and pest management practices that drive the development of resistance. The chapter highlights the consequences of diamide resistance for pest management strategies, emphasizing the need for integrated approaches to sustainably manage pest populations and protect crop health.

Chapter 3 provides a thorough analysis of the inheritance patterns and resistance mechanisms to pyrethroids, which work by altering the function of sodium channels in the nerve cell membranes, in FAW strain from Puerto Rico. By updating the susceptibility status of these populations to pyrethroids, this section highlights adaptive genetic variations and elucidates the involved biochemical pathways. Furthermore, the narrative places these findings within the context of ongoing efforts to manage resistance, considering both the efficacy and the limitations of current pest control methods.

Chapter 4 delves into the rise of field-evolved resistance to spinosyns including Spinosad and the semisynthetic derivative spinetoram. Spinosyns —compounds are produced by the fermentation of the soil bacterium *Saccharopolyspora spinosa*. This resistance is particularly noted among FAW populations in Puerto Rico, serving as a key case study in resistance management challenges. The chapter evaluates the susceptibility to both susceptible and resistant strains following exposure to significant doses of spinosyns, situating these findings within the context of actual practical resistance situation. It captures essential lessons derived from these resistance patterns, providing valuable insights into the future of sustainable pest management strategies and highlighting the severe implications of failing to act.

In Chapter 5 of this dissertation, I presented the conclusions of this research and the future directions. My research reveals the complex resistance mechanisms that the FAW from

Puerto Rico has developed against diamides, pyrethroids and spinosyns, emphasizing detoxification enzymes and specific inheritance patterns. My results open avenues for future research on genetic markers and fitness costs associated with resistance. Recent studies suggest microRNAs (miRNAs) play a key role in detoxification and pesticide resistance (Mahalle et al., 2024a). Understanding the FAW genome and detoxification processes will enhance resistance management strategies. Despite extensive research, more work is needed to address practical resistance in field conditions. Puerto Rico's unique agricultural climate and biotechnology-friendly environment make it a significant player in plant breeding research, but high pest pressures have led to FAW resistance. Integrating field trials, rotation programs, and resistance monitoring is vital for strengthening IPM and IRM strategies. This research provides globally significant insights and methodologies to address pesticide resistance in FAW, contributing to food security and sustainable agriculture.

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CHAPTER 2:

CHARACTERIZATION OF THE INHERITANCE OF FIELD-EVOLVED RESISTANCE TO DIAMIDES IN THE FALL ARMYWORM (*Spodoptera frugiperda*) (LEPIDOPTERA: NOCTUIDAE) POPULATION FROM PUERTO RICO.

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Introduction

The fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), is one of the most destructive pests of corn. It is native to the Americas, mainly Latin America, Caribbean islands, and the extreme southern part of the US, with annual migrations north into the US corn belt and Canada (Biondi et al., 2018; Kasoma et al., 2021). In 2016, infestations were found for the first time in Africa, setting the stage for a dramatic change in its distribution and economic impacts (Goergen et al., 2016; Nagoshi et al., 2017). Its current expanded range includes India, China, Japan, Malaysia, Vietnam, Egypt, the Republic of Korea, and Australia (Deshmukh et al., 2018; Kim and Babcock, 2019; Qi et al., 2021; Richardson et al., 2020; Tepa-Yotto et al., 2021). FAW has several traits that make it one of the most economically important pests of the 21st century including a high reproductive rate, no diapause, and the ability to rapidly adapt to new environments, including the adaptation to novel insecticides (Barros and de Freitas Bueno, 2010; Nagoshi et al., 2015). There are 192 reported cases of FAW resistance to 43 different active ingredients belonging to eight modes of action (Mota-Sanchez and Wise, 2023).

In addition to direct losses to food and feed from FAW, losses in the seed industry are often overlooked. Puerto Rico plays a crucial role in agricultural seed production for both research and bulk seed production. Its tropical climate allows for three to four seasons of corn production per year. It is estimated that 85% of all certified field crop seeds used for food consumption worldwide pass-through Puerto Rico's fields and nurseries at some point in development (PRABIA, 2020). However, tropical conditions are optimal for FAW populations, resulting in high and constant pest pressure in seed corn fields. To manage FAW injury, there is high pesticide usage, with up to 30 applications per season of products in at least nine modes of action (Head, 2016). As a result of such constant pest pressure and insecticide use, FAW populations in Puerto Rico have developed resistance to a wide diversity of synthetic insecticides (Gutierrez-Moreno et al., 2019) and to *Bacillus thuringiensis* (Bt) proteins in GMO corn (Blanco et al., 2010; Gutierrez-Moreno et al., 2020; Storer et al., 2010; Storer et al., 2012; Vélez et al., 2013; Zhu et al., 2015).

With the increase in FAW resistance globally, diamide insecticides (Group 28, IRAC) have become one of the critical tools for integrated pest management (IPM) of this species. The diamide insecticides selectively activate ryanodine receptors (RyR) in the endoplasmic reticulum of insects, a novel mode of action in Lepidoptera pests (Cordova et al., 2006; Richardson et al., 2020). RyR modulate the release of Ca^{2+} cations from intracellular stores, allowing insect muscles to contract. When diamides activate RyRs, insects suffer irreversible muscle contraction and paralysis (Cordova et al., 2021; Cordova et al., 2006; Ebbinghaus-Kintscher et al., 2006; Lahm et al., 2007; Nauen, 2006). They are highly selective against insects and exhibit reduced toxicity in mammals. In general, they are safer and more ecologically friendly than some older insecticides. For instance, the acute oral toxicity of chlorantraniliprole is much lower than that of chlorpyrifos, with LD_{50} values of over 5,000 mg/kg (in rats) and over 60 mg/kg (in rats), respectively (Mahajan et al., 2022; *Pesticide Fact Sheet: Chlorantraniliprole*, 2008). As a result, diamides use to be one of the most common MOAs used today (Richardson et al., 2020; Troczka et al., 2017). Diamide insecticides primarily target Lepidopteran species which is very important since many Lepidopteran species have developed extensive resistance to pesticides. Additionally, diamides are also effective against other order as Coleoptera and Hemiptera (Mota-Sanchez and Wise, 2023; Sparks et al., 2020).

The diamides, chlorantraniliprole and flubendiamide, have both been heavily used to control FAW in seed corn production in Puerto Rico since its registration in 2016 (Lewis, 2016). Flubendiamide is not used anymore due to environmental restrictions (Wise, 2016); however, chlorantraniliprole continues to be used. Resistance to these compounds was recently detected in FAW populations from Puerto Rico (Gutierrez-Moreno et al., 2019). However, the inheritance and mechanism of this resistance remains unknown. Understanding these aspects is essential for managing resistance and prolonging the use of diamide products.

The objective of this study was to determine the inheritance and begin to explore the mechanisms of resistance using synergists to diamide insecticides in Puerto Rican FAW from seed corn production. This work increases our understanding of the inheritance of this resistance and its mechanism, as well as provides insights into FAW management in Puerto Rico and globally.

Materials and Methods

FAW Strains

An (assumed) diamide-resistant strain [PR] originated directly collected from infested plants in a seed corn field in Ponce, Puerto Rico, in January 2019. Larvae were collected by personnel of Corteva under a collaboration Michigan State University-Corteva. Larvae were placed in 60 mL plastic cups with 5 mL of artificial FAW diet (Southland Products Inc., Lake Village, AR) and shipped to our laboratory at Michigan State University, East Lansing, MI to complete development under controlled conditions. Larvae were checked every three days to monitor feeding and development. The known diamide-susceptible population [SUS] was provided by Bayer USA from their rearing facilities in Union City, Tennessee. We have been using this susceptible strain for at least 8 years.

Colony maintenance

Larvae were maintained under controlled conditions (26 ± 2 ºC; 35 ± 2% RH) and a photoperiod of 16:8 hours (L:D), inside an FXC-19 Growth Chamber (BioChamber, Winnipeg, Manitoba, Canada) After pupation, twenty pairs of pupas (even number male and female) were placed in a 5 L paper brown bag to provide space for mating and egg-laying. Bags were placed inside of cylindric mesh cages to avoid accidental escape. Adults were fed lime or orange liquid Gatorade (PepsiCo, Harrison NY). Egg masses were collected every other day by cutting out the sections of the bag containing the egg masses. These were collected every other day and placed in a closed plastic container with moist paper towels. Egg masses were transferred to 60 mL plastic cups with 10 mL of artificial FAW diet to ensure safe emergence and efficient feeding from the beginning of the larval cycle. As eggs hatched, multiple neonates were moved to diet cups using a fine paintbrush. Once reaching third instar stage, larvae were collected either bioassays or for the colony, placing one larva per cup.

Diet Overlay Bioassays – General methods

Diet overlay bioassays were performed using 24-well trays (ProCell, Alkali Scientific Inc., Fort Lauderdale, FL) with 1 mL of artificial FAW diet per well. The diet surface area in each well was 2.0 cm^2. Products to be tested and appropriate controls were applied to the diet and left to air dry for an hour. A total of 30 μL of insecticide or control solution was applied to each well. Then one early third instar was deposited on the treated surface of each well. Mortality was recorded four days after placing the larva over the treated diet. Larvae were considered dead if they did not react after prodding insect with a small paintbrush or showed severe intoxication symptoms (defined as slow movement, interrupted molting, or reduced size). We selected the overlay diet assay over diet (Bolzan et al., 2019; Muraro et al., 2021) incorporated assay because the fast and clean procedure to perform this type of bioassays.

Mortality data was corrected using Abbott's equation (1925). Probit analysis (Finney, 1971) was performed using the PROC PROBIT procedure from SAS version 9.4 (2023) to estimate slope values, median lethal concentration 50% (LC_{50}), and 90% (LC_{90}), fiducial limits (95%), and X^2 for each strain. Resistance ratios at 50% and 90% (RR_{50} and RR_{90}) were calculated by dividing LC_{50} or LC_{90} values of the PR strain by the LC_{50} or LC_{90} values of the SUS strain. Where appropriate, parallelism and equality tests ($P<0.05$) were also calculated to compare the responses of the strains to the diamide compounds using the software PoloJR (PoloJR, 2021). Log concentration responses were plotted using the software OriginLab (OriginLab, 2021)

Evaluating resistance to diamides used in PR seed corn

For both the PR and SUS populations, bioassays were done for two diamide formulations: chlorantraniliprole (Altacor® 35 WG, 35 g a.i./kg, FMC Corporation, Philadelphia, PA) and flubendiamide (Belt® 480 SC, 480 g a.i./L, Bayer CropScience LP, Research Triangle Park, NC). The formulated material was resuspended in distilled water, and non-ionic surfactant (Triton X-100, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was added in a concentration of 0.05% v/v. Each insecticide was tested at concentrations covering a range of mortality from 5% to 95%. Four to five replications per concentration were performed. A single replication consisted of twelve wells with one early third-instar per well. The control treatment consisted of distilled water with only the surfactant. Mortality was assessed at four days, as described in the general methods.

Bioassays were performed using the procedure for both strains to determine if there was cross-resistance among diamides. Diamides tested were cyclaniliprole (Harvanta® 50 SL, Summit Agro USA, Durham, NC) and cyantraniliprole (Exirel® 100 SE, FMC Corporation, Philadelphia, PA); both insecticides are not used in cornfields in PR. However, they are used in several countries against a wide range of pest, including lepidopteran pests, beetles, and various types of flies and bugs, in crops such as citrus, vegetables, and rice (Zhang et al., 2021). Thus, they are good candidates to understand the cross-resistance in FAW among diamides compounds.

Determining inheritance of resistance

F_1 crosses between the two FAW populations (PR and SUS) were tested to determine the inheritance of resistance to chlorantraniliprole and flubendiamide. Forty reciprocal pairs of FAW were separated to create the F1 crosses in heterozygous (H) populations, creating two F1 crosses defined as H1 = ♂ SUS × ♀ PR) and H2 = (♀ SUS × ♂ PR), which were bioassayed in the same way as the parental strains. Four replicates were use in seven to nine concentrations (S1Table) to cover a range of mortality from 5% to 95%.

The degree of dominance at the LC_{50} level was calculated as follows using Stone's equation (1968),

$$D = \frac{2X_2 - X_1 - X_3}{X_1 - X_3} \qquad (1)$$

where X_2, X_1, and X_3 were the log10 LC_{50} of the F_1 (H1 or H2 strain), PR strain, and SUS strains. When *D* values = -1 signify complete recessive, *D* values = -1 < *D* < *0* signify incomplete recessive, *D* values = 0 < *D* < 1 signify incomplete dominant and *D* = 1 signify complete dominant.

Dominance level (D_{ML}) using concentrations that span the log concentration mortalities was calculated using the method described by Bourguet et al. (2000),

$$D_{ML} = \frac{M_{RS} - M_{SS}}{M_{RR} - M_{SS}} \qquad (2)$$

where M_{SS}, M_{RS}, and M_{RR} were the mortalities of the SUS, F_1 (H1 or H2 strain), and PR strains, respectively, at different concentrations of each diamide. D_{ML} values close to 0 were considered completely-recessive inheritance, D_{ML} values approaching 1 —completely-dominant inheritance. This method considered testing a range of concentrations covering the parental and F_1 crosses log concentration responses to understand the trend of dominance/recessiveness.

Role of detoxification enzymes

To determine the role of detoxification enzyme in the resistance of diamides, synergists bioassays were performed as the following: (i) a cytochrome P450s inhibitor, piperonyl butoxide (PBO) (91.3%, SynerPro^{TM} Control Solutions Inc. Pasadena, Texas, USA); (ii) an esterase inhibitor, S,S,S-tributyl phosphorotrithioate (DEF) (98.1%, Sigma-Aldrich, Saint Louis, Missouri, USA); (iii) a glutathione S-transferase inhibitor, diethyl maleate (DEM) (97%, Sigma-Aldrich, Saint Louis, Missouri, USA); (iv) an ABC transporters inhibitor (±)-verapamil hydrochloride (VER) (99%, Sigma-Aldrich, Saint Louis, Missouri, USA). Control treatment consisted of synergist application over the diet without pesticide. Prior to the bioassay with the diamides, the maximum non-lethal concentration of each synergist was assessed on third instars using the diet overlay bioassay method. The criteria to identify the maximum nonlethal concentration of each synergist was that which did not account for significantly higher mortality or loss of fitness (weight) in the larva four days after application compared to water control. The maximum non-lethal concentrations determined for PBO, DEF, DEM and VER per diet surface were 4.5 µg/cm^2, 1.5 µg/cm^2, 0.45 µg/cm^2 and 0.45 µg/cm^{2,} respectively.

Using the maximum non-lethal concentrations, synergists bioassays were performed. Stock solutions of synergist compounds were prepared by diluting in distilled water. A non-ionic surfactant (Triton X-100, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was added in a concentration of 0.05%. A total of 30 µL of synergist was applied over the diet surface of each well and left to air dry for 1 hour. Then, 30 µL of each insecticide concentration was applied over the same surface well and left to air dry. Finally, one third instar was placed in each well treated. Mortality was assessed four days after application. The experimental design consisted of four replicates of five to seven concentrations, and a single replication consisted of twelve wells with one third instar per well.

Scoring of mortality probit analyses and data plotting were estimated following the procedure mentioned before. Synergist ratios (SR_{50} and SR_{90}) were calculated by dividing LC_{50} and LC_{90} values of the diamide alone by the LC_{50} and LC_{90} values of the diamide plus synergist combination.

Results

Inheritance of resistance

The PR strain showed high levels of resistance to flubendiamide and chlorantraniliprole, RR_{50} = 2,762-fold, and RR_{50} = 96-fold over the susceptible, respectively. The F_1 progenies from reciprocal crosses (H1♀ PR × ♂ SUS and H2 ♂ PR × ♀ SUS) presented similar susceptibility to chlorantraniliprole with overlapping confidence levels for the LC_{50} (95% CI) 0.126 (0.07, 0.19) and 0.155 (0.10, 0.22). In contrast, susceptibility of F_1 progenies from reciprocal crosses (H1♀ PR × ♂ SUS and H2 ♂ PR × ♀ SUS) to flubendiamide were different with LC_{50} of 2 (1.2, 3.5) and 7 (4.5, 8.9), respectively (Table 1.1). Equality tests for chlorantraniliprole (X^2 = 14.63, d.f. = 4, $P<0.05$) and flubendiamide (X^2 = 95.93, d.f. = 4, $P<0.05$) demonstrated that the heterozygote strains were different to each other. Compared to the SUS strain, resistance ratios (RR_{50}) for H1 and H2 were 10-fold and 12-fold for chlorantraniliprole and 37-fold and 111-fold for flubendiamide, respectively (Table 2.1). The overlapping of the LC_{50} between confidence intervals (95% CI) of H1 and H2 strains in chlorantraniliprole suggested an autosomal inheritance of resistance. For flubendiamide the results suggested an X-linked inheritance of resistance (Fig. 1.1).

Table 2.1. Dose-response of a resistant (PR) strain and a susceptible (SUS) of FAW and their reciprocal crosses to chlorantraniliprole and flubendiamide.

Strain	**active ingredient**	**n**	**[e]b**	**SE**	**[a]LC_{50} (95% CI)**	**[a]LC_{90} (95% CI)**	**[b]RR_{50}**	**[b]RR_{90}**	**X^2**	**[c]df**	**[d]*D***
PR	chlorantraniliprole	284	1.3	0.2	1.26 (0.92, 1.69)	12.82 (7, 26)	96	287	5.4	4	
SUS		236	2.4	0.3	0.013 (0.11, 0.016)	0.045 (0.03, 0.08)	1	1	3.2	3	
H2 ♀ SUS × ♂ PR		332	1.0	0.1	0.155 (0.10, 0.22)	3.072 (1.7, 7.3)	12	69	2.6	5	0.079
H1 ♂ SUS × ♀ PR		427	1.5	0.3	0.126 (0.07, 0.19)	0.857 (0.4, 3.2)	10	19	21.6	7	-0.011
PR	flubendiamide	333	1.4	0.2	162.42 (128, 210)	1,375 (808, 3,359)	2,762	10,316	4.8	5	
SUS		304	3.6	0.4	0.0588 (0.04, 0.06)	0.13 (0.11, 0.16)	1	1	8.5	5	
H2 ♀ SUS × ♂ PR		383	1.0	0.1	6.51 (4.5, 8.9)	122.44 (71, 271)	111	919	6.2	6	0.018
H1♂ SUS × ♀ PR		384	1.2	0.2	2.149 (1.2, 3.5)	24.04 (12, 74)	37	180	11.7	6	-0.092

[a] LC_{50} or LC_{90} (μg/cm^2)

[b] Resistance ratio (RR), LC_{50} of resistant strain / LC_{50} of susceptible strain or LC_{90} of resistant strain / LC_{90} of susceptible strain

[c] df = degrees of freedom

* chlorantraniliprole (Altacor® 35 WG, 35 g a.i./kg, FMC Corporation, Philadelphia, PA)

* flubendiamide (Belt® 480 SC, 480 g a.i./L, Bayer CropScience LP, Research Triangle Park, NC)

[d] degree of dominance using Stone's equation (1968)

[e] slope

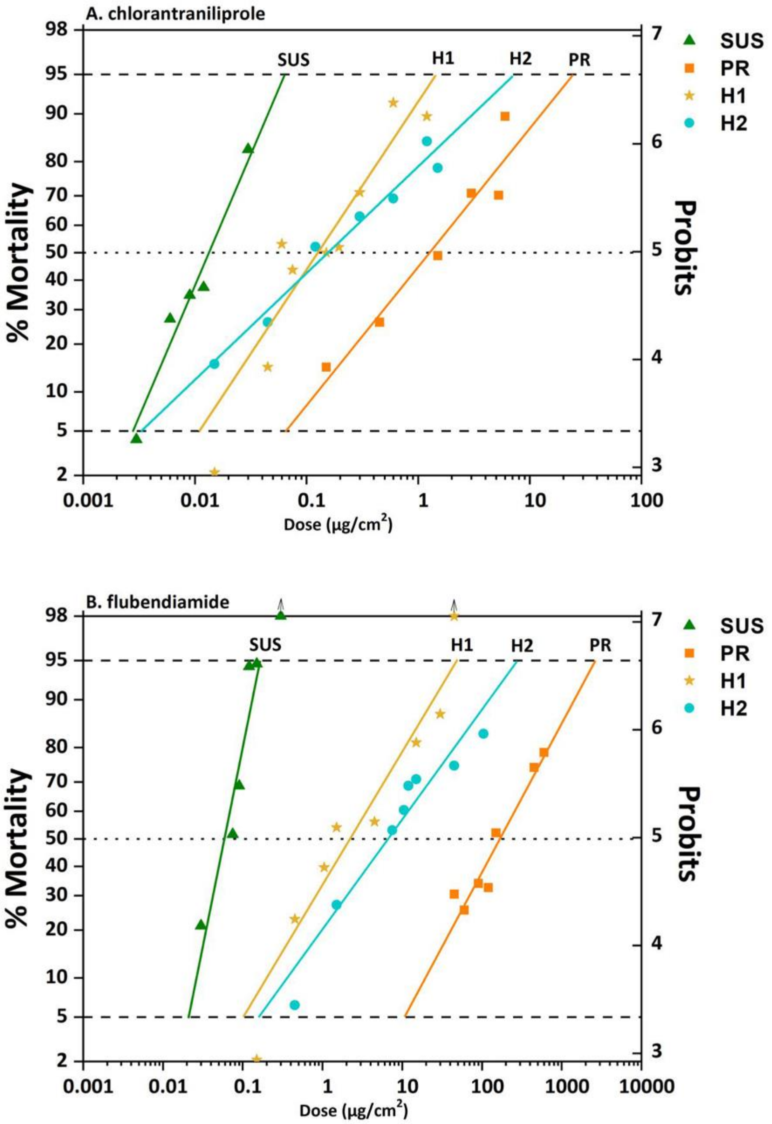

Figure 1.1. Mortality of a susceptible (SUS) and resistant (PR) strain and their reciprocal crosses (H1, ♂ SUS × ♀ PR) + (H2, ♀ SUS × ♂ PR) to A. chlorantraniliprole and B. flubendiamide.

Dominance of resistance

The degree of dominance (*D*) at LC_{50} was -0.011 and 0.079 for chlorantraniliprole and -0.092 and 0.018 for flubendiamide using the method proposed by Stone (1968), suggesting incompletely recessive trait for H1 strain (♂ SUS × ♀ PR) and incompletely dominant trait for H2 strain (♀ SUS × ♂ PR), in both chlorantraniliprole and flubendiamide, respectively. For both active ingredients, dominance decreased indirectly proportional to the concentration. At low doses of active ingredients, dominance is incompletely dominant, while at high doses, is incompletely recessive (Fig. 1.2.). For instance: at the low concentration tested (0.01 µg/cm^2 of chlorantraniliprole) showed a value $D_{ML}<0.83$, whereas (0.3 µg/cm^2 of flubendiamide) showed a value of $D_{ML}<0.92$. At the highest concentration tested (3 µg/cm^2 of chlorantraniliprole) showed a value of $D_{ML}<0.41$, whereas (100 µg/cm^2 of flubendiamide) showed a value of $D_{ML}<0.20$ (Fig. 1.2.).

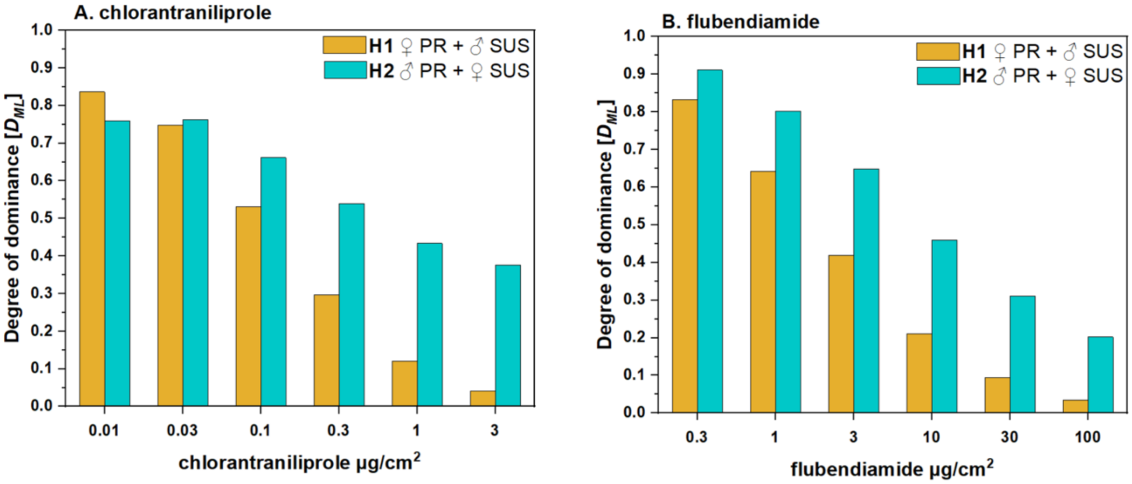

Figure 1.2. Degree of dominance (D_{ML}) of resistance of FAW to chlorantraniliprole and flubendiamide.

Role of detoxification enzymes

In the chlorantraniliprole-synergist bioassays, the LC$_{50}$, LC$_{90}$ values with or without exposure to PBO, DEF, DEM, and VER did not differ significantly based on the overlap between the LC$_{50}$ confidence intervals (95% CI) in each PR and SUS strains (Table 2.2.). However, there was a slight increase in toxicity with the esterase inhibitor (DEF) and the glutathione S-transferase inhibitor (DEM) in the PR strain of chlorantraniliprole (2-fold) compared to the insecticide without synergist.

Table 2.2. Effect of synergists on mortality of Puerto Rico's (PR) and Susceptible (SUS) strains of FAW to chlorantraniliprole.

Strain	**Synergists**	**n**	**[d]b**	**SE**	**[a]LC$_{50}$ (95% CI)**	**[a]LC$_{90}$ (95% CI)**	**[b]SR$_{50}$**	**[b]SR$_{90}$**	**X^2**	**[c]df**
PR	chlorantraniliprole	284	1.3	0.2	1.2659 (0.92, 1.69)	12.82 (7, 26)	-	-	5.4	4
	+ PBO	287	1.7	0.2	1.461 (1.13, 1.83)	8.16 (5.8, 13)	1	1	6	4
	+ DEM	236	0.9	0.2	1.19 (0.69, 1.8)	33.99 (13, 260)	1.2	0.2	3.8	3
	+ DEF	307	1.2	0.2	1.49 (0.62, 2.6)	19.39 (8, 116)	1	0.4	10	5
	+ VER	288	1.0	0.1	1.043 (0.7, 1.47)	17.84 (9, 48)	1.4	0.5	7	4
SUS	chlorantraniliprole	236	2.4	0.3	0.0132 (0.11, 0.016)	0.045 (0.03, 0.08)	-	-	3.2	3
	+ PBO	240	2.1	0.3	0.0150 (0.012, 0.019)	0.06 (0.04, 0.12)	1	1	5.4	3
	+ DEM	336	1.5	0.3	0.0078 (0.004, 0.013)	0.056 (0.026, 0.34)	2	1.1	14	5
	+ DEF	240	2.0	0.2	0.0073 (0.005, 0.009)	0.032 (0.023, 0.051)	2	2	2	3
	+ VER	336	1.9	0.3	0.0135 (0.007, 0.021)	0.064 (0.038, 0.15)	1.1	1	13	5

[a] LC$_{50}$ or LC$_{90}$ (µg/cm^2)

[b] Synergist ratio (SR) = LC$_{50}$ of chlorantraniliprole without synergist / LC$_{50}$ of chlorantraniliprole + synergist.

[c] df = degrees of freedom

PBO = piperonyl butoxide, DEM = diethyl maleate, DEF = S,S,S -tributyl phosphorotrithioate and VER = (±)-verapamil hydrochloride

[d] slope

In the flubendiamide bioassays, the LC_{50}'s values demonstrated a different response in both the SUS and PR strains. A minor antagonism (<1-fold) was found in the SUS strain with all the synergists. While, in the PR strain, there was no synergism (0.9-fold) with the P450s inhibitor (PBO), this was a case not the case with the other synergists. For instance, the esterase inhibitor (DEF) and the glutathione S-transferase inhibitor (DEM) showed moderate synergism with 2.7-fold and 3.2-fold, respectively, and the ABC transporters inhibitor (VER) demonstrated a moderate-high synergism with 7.6-fold at the LC_{50} dosages. Yet, in all synergist combinations with flubendiamide, the synergism was low at the LC_{90} dosages (Table 2.3.).

Table 2.3. Effect of synergists on mortality of Puerto Rico's (PR) and Susceptible (SUS) strains of FAW to flubendiamide.

Strain	Synergists	n	[d]b	SE	[a]LC$_{50}$ (95% CI)	[a]LC$_{90}$ (95% CI)	[b]SR$_{50}$	[b]SR$_{90}$	X^2	[c]df
PR	flubendiamide	333	1.4	0.2	162.42 (128, 210)	1,375 (808, 3,359)	-	-	4.8	5
	+ PBO	239	1.3	0.2	175.07 (126, 244)	1,715 (992, 4,006)	0.9	0.8	5	3
	+ DEM	239	1.1	0.1	60.04 (40, 89)	907 (482, 2,339)	2.7	1.5	5	3
	+ DEF	335	1.0	0.1	50.01 (24, 102)	1,095 (406, 6,981)	3.2	1.3	10	5
	+ VER	240	0.8	0.1	21.31 (11, 34)	795 (356, 3,130)	7.6	1.7	3	3
SUS	flubendiamide	304	3.6	0.4	0.059 (0.04, 0.06)	0.133 (0.11, 0.16)	-	-	8.5	5
	+ PBO	335	2.0	0.3	0.098 (0.06, 0.13)	0.44 (0.26, 1.40)	0.6	0.3	11	5
	+ DEM	288	1.3	0.5	0.144[*]	1.3614[*]	0.4	0.1	31	4
	+ DEF	238	1.7	0.2	0.141 (0.10, 0.17)	0.812 (0.53, 1.6)	0.4	0.2	4	3
	+ VER	285	3.9	0.7	0.161 (0.11, 0.21)	0.3 (0.25, 0.75)	0.4	0.4	11	4

[a] LC$_{50}$ or LC$_{90}$ (µg/cm^2)

[b] Synergist ratio (SR) = LC$_{50}$ of flubendiamide without synergist / LC$_{50}$ of flubendiamide + synergist

[c] df = degrees of freedom

[*] No confidence intervals could be calculated

PBO = piperonyl butoxide, DEM = diethyl maleate, DEF = S,S,S -tributyl phosphorotrithioate and VER = (±)-verapamil hydrochloride

[d] slope

These results suggest that the metabolic resistance differs between chlorantraniliprole and flubendiamide. In the chlorantraniliprole synergist bioassays indicate that there is little evidence of metabolic resistance while the flubendiamide-synergism results indicate the presence of metabolic resistance in FAW strain from Puerto Rico (PR) at concentrations around the LC_{50}.

Cross-resistant among diamides

The Puerto Rico (PR) strain showed cross-resistance to all tested diamide insecticides, including cyantraniliprole (11-fold) and cyclaniliprole (74-fold) (Table 2.4.). However, LD_{50}s, and LC_{90}s were not significant different in the PR strain between chlorantraniliprole and cyclaniliprole (Table 1.4.). The equality ($X^2 = 45.48$, d.f. = 7, $P<0.05$) and parallelism ($X^2 = 58.95$, d.f. = 5, $P<0.05$) tests for all anthranilic diamide (chlorantraniliprole, cyantraniliprole, and cyclaniliprole) suggests a different response for each diamide in the field-evolved strain from Puerto Rico (PR).

Table 2.4. Dose-response to cyantraniliprole and cyclaniliprole of a Susceptible strain (SUS) and a Puerto Rico's (PR) field-collected FAW strain.

Strain	**Active Ingredients**	**n**	**[d]b**	**SE**	**[a]LC_{50} (95% CI)**	**[a]LC_{90} (95% CI)**	**[b]RR_{50}**	**[b]RR_{90}**	**X^2**	**[c]df**
PR	cyantraniliprole	231	1.5	0.2	0.749 (0.56, 1.02)	5.14 (3.23, 10.08)	11	27	2.8	3
SUS		234	2.8	0.5	0.066 (0.04, 0.07)	0.191 (0.15, 0.27)	-	-	4.3	3
PR	cyclaniliprole	286	1.4	0.2	0.3718 (0.17, 0.66)	3.28 (1.6, 13.2)	74	213	7.9	4
SUS		287	2.7	0.3	0.0051 (0.004, 0.005)	0.0154 (0.012, 0.02)	-	-	6.9	4

[a] LC_{50} or LC_{90} (μg/cm^2)

[b] Resistance ratio (RR), LC_{50} of resistant strain / LC_{50} of susceptible strain or LC_{90} of resistant strain / LC_{90} of susceptible strain

[c] df = degrees of freedom

[d] slope

Discussion

In this study, we characterized the inheritance and the possibility for metabolism in field-evolved resistance in a FAW population from Puerto Rico (PR) to both chlorantraniliprole (96-fold) and flubendiamide (2,762-fold) using diet overlay bioassays which probably estimate more accurate the toxicity of diamide compounds to FAW than laboratory topical bioassay (da Silva et al., 2020). Field-evolved resistance to diamides (flubendiamide and chlorantraniliprole) was first reported in 2017 using topical bioassays (Gutierrez-Moreno et al., 2019); however, the FAW population in our study was collected three years later after 12 cropping seasons subjected to diamide insecticide selection. Since 2017 flubendiamide is no longer used to manage FAW in Puerto Rico; however, the resistance levels are still very high likely due to prior intense selection pressure by flubendiamide and cross-resistance by using chlorantraniliprole in each cropping season. Chlorantraniliprole is still used to manage FAWs in corn, despite recent field observations that efficacy has decreased (i.e. practical resistance) (Mota-Sanchez et al., 2019). FAWs from Puerto Rico also exhibited cross-resistance to other diamides as we found in this study.

Cross-resistance in this case is defined as resistance to compounds of the same chemical family never used before (Yu, 2014). In our unique FAW population from Puerto Rico, we observed cross-resistance to cyantraniliprole (11-fold) and high levels of cross-resistance to cyclaniliprole (74-fold), suggesting the possibility of cross-resistance development to diamides. This is particularly concerning in regions where more than two diamide compounds are deployed to manage FAW, as occurred in Asia with the recent FAW infestations (Krupnik et al., 2021). Resistance and cross-resistance to four diamide compounds suggested an overlapping of the binding sites of RyRs (Tao et al., 2013). We determined that there are high levels of field-evolved resistance of FAW from Puerto Rico without further selection in the laboratory. Similarly, resistance to chlorantraniliprole was reported in Brazil by using an F_2 screening method in overlay diet assays from field populations as well as cross-resistance to flubendiamide (42,000-fold) and cyantraniliprole (26-fold) (Bolzan et al., 2019). Resistance to these insecticides has been reported in other pests including tomato leafminer (*Tuta absoluta*) in Greece, Brazil, and Spain (Roditakis et al., 2017; Silva et al., 2019), in South Korea, to beet armyworm (*Spodoptera exigua*) and diamondback moth (*Plutella xylostella*) (Lai et al., 2011; Troczka et al., 2012), in China to the Asiatic rice borer (*Chilo suppressalis*) (Zhao et al., 2017) and lately reported field-evolved resistance in China to cotton bollworm (*Helicoverpa armigera*) (Wang et al., 2021).

Our inheritance of resistance analysis suggests that resistance to chlorantraniliprole in FAW from Puerto Rico is autosomal. Similar results were documented in the continental area of South America (Bolzan et al., 2019). Inheritance of resistance by autosomal traits in FAW from continental areas of the Americas was previously reported to other classes of insecticides: carbamates (carbaryl) (Yu and Nguyen, 1994) and organophosphate (chlorpyrifos) (Garlet et al., 2021), pyrethroids (lambda-cyhalothrin) (Diez-Rodriguez and Omoto, 2001), nicotinic acetylcholine receptor (nAChR) allosteric modulators, spinosyns (spinosad and spinetoram) (Lira et al., 2020; Okuma et al., 2018), glutamate-gated chloride channel (GluCl) allosteric modulators, avermectins and milbemycins (emamectin benzoate) (Muraro et al., 2021) and inhibitors of chitin biosynthesis, benzoylureas (novaluron and teflubenzuron) (do Nascimento et al., 2022; Stacke et al., 2020).

In contrast to chlorantraniliprole, flubendiamide reciprocal crosses indicates a paternal sex-linked inheritance resistance, that the resistance is conferred by the males of the parent trait. Few records of this type of inheritance have been published to date. For example, in 2016, a field-evolved resistant colony of the convergent lady beetle (*Hippodamia convergens*) (Coleoptera: Coccinellidae) from Georgia, USA presented a recessive inheritance with maternal sex-linkage in to the pyrethroid lambda-cyhalothrin (Barbosa et al., 2016). Furthermore, the PR strain presented incompletely recessive resistance from the PR female strain to both chlorantraniliprole and flubendiamide. Similar results were reported in other Lepidoptera species as diamondback moth (Arruda et al., 2020; Jiang et al., 2021), oriental tea tortrix (*Homona magnanima*) (Kamimuro, 2021) and tomato leafminer (Roditakis et al., 2017); however, the reciprocal crosses demonstrated an incompletely dominant trait from the parental PR strain.

In our reciprocal crosses, we observed that individuals, presumed to be heterozygous, exhibited incompletely recessivity (Fig. 1). A factor that might skew this result is the degree of homozygous resistance of the resistance population because the strain might be a mix of homozygous resistant and heterozygous individuals that carry genes for resistance. However, the resistance levels of FAW to both diamide compounds were very high and there was little overlapping with the susceptible strain to most of the concentrations (Fig 1). Another possibility that might affect the level of heterozygosity is the presence of a lethal gene (Dexter, 1914). Dexter (1914) noticed that in a specific stock of *Drosophila*, offspring from half of the females displayed a distinct pattern of twice as many females as males. This observation was attributed to a gene present in one of the sex chromosomes of these females that inhibits the development of any male inheriting it. We did not observe any drastic fitness cost or skew in sex in our resistant strain meaning the low probability of a lethal gene in our resistant strain. We acknowledge the limitations and assumptions of our analysis, particularly the assumption of homozygosity for resistance in the Puerto Rican (PR) population. The PR specimens could indeed be a mix of homozygotes and heterozygotes, or the resistance could be entirely dominant but also recessive lethal (Wilkie). These scenarios are less likely, but potentially influenced the observed results of partial dominance or partial recessivity in the hybrid crosses, thereby adding a layer of complexity to the inheritance of resistance. This underlines the necessity for further research to fully understand the genetic dynamics at play. For instance, recent studies have shown that when comparing strains with genetically distant backgrounds, there could be an overestimation of the fitness cost. This might create a misleading impression that resistance carries a significant cost, and, as a result, its frequency would naturally diminish in the absence of the insecticide (Padovez et al., 2022).

Despite these complexities, it remains vital to note that individuals carrying a single copy of the resistant allele might survive under conditions of decreased pesticide residue, potentially resulting in increased mutation rates of resistance genes (Gressel, 2011) and that might result in the survival of heterozygotes when chlorantraniliprole residues breakdown days after foliar applications or after many days of the emergence of corn plants that come from diamide treated seed. This is a likely scenario, since FAW moths lay egg masses continuously during the season (Sotelo-Cardona et al., 2021). Therefore, to manage resistance effectively, it is essential that diamide treatments, whether seed treatment or foliar sprays, should not be repeatedly applied within the same season.

Resistance of lepidopteran pests to diamides has been considered mainly through target-site mutations that cause high levels of resistance in field-evolved and lab-selected strains (Richardson et al., 2020). Since ryanodine receptors (RyRs) are determined by a single gene (Takeshima et al., 1994) in *Drosophila*, target-site mutations are expected to occur due to the high selectivity on these receptors. For instance, in diamondback moth, rice stem borer (*Chilo suppressalis*), and tomato leafminer, the same polymorphism is reported (G4946E) located close to the C-terminal of the RyRs gene (Richardson et al., 2020; Steinbach et al., 2015; Troczka et al., 2012; Yao et al., 2017), while in the Noctuidae family just one polymorphism has been reported (I4790M) in beet armyworm and FAW (Boaventura et al., 2019; Zuo et al., 2020). In addition, the frequency of these known mutations has been studied in tomato leafminer and confirmed by CRISPR/Cas9 modification in *Drosophila,* suggesting that the target site mutations confer modifications on the action site of different diamides overlapping at the binding site of the RyRs receptors (Douris et al., 2017). However, recent genotyping sequencing for different target-site mutations in Puerto Rico FAW samples showed the absence of the polymorphisms G4946E and I4790M (Boaventura et al., 2020) suggesting either different polymorphisms or a different mechanism of resistance.

In our PR strains, resistance to chlorantraniliprole is not strongly associated with detoxification enzymes including P450s, esterases, ABC transporters, as reported with for FAW in other studies (Boaventura et al., 2019; Zhang et al., 2020). This result is supported from similar evidence in other Lepidoptera pests including diamondback moth (Wang et al., 2010), beet armyworm (Lai et al., 2011), and oblique-banded leafroller (*Choristoneura rosaceana*) (Sial and Brunner, 2012). Our findings using synergists with flubendiamide showed a moderate role of esterases and glutathione S-transferases and some involvement of ABC transporters in the detoxification process. The resistance mechanisms involved in the downregulation of ABC transporters have been observed in a field-evolved strain of diamondback moth, leading to resistance to specific molecules. Therefore, synergism of verapamil might be the first report of the role of ABC transporters on the resistance of flubendiamide in FAW. ABC transporters mutations have been recorded for resistance to the Bt proteins, Cry1F, in lab colony from Puerto Rico (Abdelgaffar et al., 2019; Banerjee et al., 2017). Also, VER (p-glycoprotein inhibitor) has been used to characterize the involvement of ABC transporters in the resistance of *Rhipicephalus* (Boophilus) to pyrethroids (ivermectin) (Khangembam et al., 2018) and *Anopheles gambiae* to pyrethroids (deltamethrin) (Chouaïbou et al., 2014). Further molecular analysis remains to be performed to understand this particular strain's resistance mechanism. This is critical due to the ongoing migration pathway of the FAW, that has shown a great adaptation to those developing countries where factors such as well-established IRM programs, economic resources to acquire novel active ingredients, and efficient application methods and techniques are current limitation (Nagoshi et al., 2020).

Puerto Rico is considered one of the most important areas around the globe for agricultural research focused on plant breeding (Belay et al., 2012; Storer et al., 2010) due to its favorable all year around weather which allows for continuous farming. Furthermore, the identical seed regulatory frameworks in place in the U.S. and the biotechnology science-friendly environment enable a seamless exchange of seeds and uninterrupted experimentation (PRABIA, 2020). However, favorable conditions for the crops are similar for a high pest pressure resulting in intense use of synthetic pest management tools and resistance development. This intense selection has led to resistance evolution in FAW for multiple insecticides (Gutierrez-Moreno et al., 2019). To strengthen IPM and IRM approaches, IRAC-US and PRABIA have established an area-wide resistance management program with five critical workstreams: 1. Field efficacy trials with a different mode of actions on FAW; 2. Development and maintenance of the area-wide rotation program; 3. Scouting practices, treatments thresholds, and training; 4. Implementation and communication, and 5. Resistance monitoring bioassays (Teran-Santofimio, 2019). Our work on the basic aspects of diamide resistance supports these workstreams and will provide the basic knowledge required for better IPM practices for these critical insect control tools. Additionally, in conjunction with the seed industry, workshops have been conducted with the research and development teams of large and medium seed companies with the same resistance problem in common research fields (Teran-Santofimio, 2019). Restoring FAW susceptibility and enhancing the area-wide resistance management program might take time; however, these are the initial steps in the right direction to address the problem.

Conclusion

FAW from Puerto Rico had developed field-evolved resistance to diamides. Weather and pattern of insecticide use might be similar in other areas of the world where FAW had invaded. The incomplete recessiveness of chlorantraniliprole suggests that rapid resistance might occur in other areas of the world if FAW management is followed by treatments of ryanodine receptors compounds. Also, cross-resistance to siblings' molecules is likely to happen if IPM tools are not implement effectively. For instance, diamide seed treatment followed by diamide foliar sprays, or continuous sprays of diamide treatment. Therefore, it is critical to consolidate effective and long-term IPM programs that would prolong the active seed programs in island-type conditions of Puerto Rico that will ensure the current and near-future demand for food around the world. Lessons learned from field-evolved resistance of FAW to diamides in Puerto Rico have applications in other continents, including Africa, Asia, and Oceania.

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CHAPTER 3:

INHERITANCE AND RESISTANCE MECHANISMS OF FIELD-EVOLVED RESISTANCE TO PYRETHROIDS IN A FALL ARMYWORM (*Spodoptera frugiperda* J.E. SMITH) (LEPIDOPTERA: NOCTUIDAE) STRAIN FROM PUERTO RICO.

Introduction

The Fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), is one of the most economically significant pests of the twenty-first century, causing extensive damage to corn and various other crops. FAW possesses several formidable traits that contribute to its prominent pest status, including a high reproductive rate, multiple generations per year, lack of diapause, rapid adaptation to new environments, and a broad host range encompassing hundreds of species (Barros and de Freitas Bueno, 2010; Goergen et al., 2016; Johnson, 1987; Montezano et al., 2018; Nagoshi et al., 2015). Additionally, FAW has evolved resistance to a wide variety of insecticides (Gutierrez-Moreno et al., 2019). As of now, there are 194 reported cases of FAW resistance to 45 different active ingredients, spanning eight modes of action (Mota-Sanchez and Wise, 2024).

FAW is native to Latin America, the Caribbean islands, and the southernmost US, although it migrates annually as far north as the US Corn Belt and Canada (Biondi et al., 2018; Kasoma et al., 2021). However, in 2016, there was a pivotal shift in the FAW's distribution and economic impact (FAO, 2022). For the first time, infestations were reported in Africa (Goergen et al., 2016; Nagoshi et al., 2017), eventually expanding across the continent (Dai et al., 2019; Feldmann et al., 2019; Wennmann et al., 2021), then to Asia (Republic of Korea, India, China, Japan, Pakistan and Vietnam) and Oceania (Australia) (Deshmukh et al., 2018; Kenis et al., 2022; Kim and Babcock, 2019; Qi et al., 2021; Richardson et al., 2020; Tepa-Yotto et al., 2021). Most recently it was found in Saudi Arabia, the Canary Islands and Turkey.

FAW feeding can result in yield reductions over 60% in corn, a critical for crop global food security (Davis et al., 1992; Hruska and Gladstone, 1988). It also poses an over-looked, but significant, challenge for the seed industry. Puerto Rico plays an essential role in agricultural seed production as both a research and bulk-seed production hub. Given its tropical climate, Puerto Rico can support up to four corn crops per season. Remarkably, approximately 85% of all certified field crop seeds used for global food consumption passes some stage of development in Puerto Rico's fields and nurseries (PRABIA, 2020). At the same time, the tropical conditions create an ideal environment for FAW, resulting in up to ten generations of persistent, high pressure per year (Gutierrez-Moreno et al., 2019; Head, 2016; Storer et al., 2010). Since there is low tolerance for kernel damage in seedcorn production, the industry resorts to intensive pesticide usage, with up to thirty applications per season of products from at least nine modes of action (Head, 2016). Unfortunately, sustained pest pressure and extensive insecticide use have led to the evolution of broad-spectrum pesticide resistance in FAW populations in Puerto Rico. Notably, resistance has been observed to a range of synthetic insecticides, including pyrethroids (Gutierrez-Moreno et al., 2019; Head, 2016; Storer et al., 2010).

Pyrethroids (Group 3, IRAC) have been integral components of integrated pest management (IPM) strategies since the 1970s, favored globally because of their lower mammalian toxicity compared to older conventional pesticide groups. Pyrethroids have an established safety profile because insects are intrinsically more susceptible than mammals. Their activity arises from their interference with neurotransmission at insect voltage-gated Na+ channel recognition sites, blocking Na+ transport, extending the Na+ current duration during depolarization, and eliciting a lingering slow current ("tail current"). This chain of events culminates in instant paralysis (Bloomquist, 1993; Du et al., 2016; Ware, 1989; Zhu et al., 2020).

Pyrethroids are classified in two categories, Type I and Type II, based on chemical structure, sensory neuron activity, and the poisoning symptoms (Bloomquist, 1993; Narahashi, 1986; Yan et al., 2020; Yu, 2014). Type I pyrethroids lack an α-cyano group at the phenylbenzyl alcohol position, while Type II pyrethroids possess this group. Functionally, Type I pyrethroids prompt repetitive discharges in sensory neurons, without initiating neurotransmitter release. In contrast, Type II pyrethroids do not produce these repetitive discharges, leading to an extended tail current decay. Furthermore, Type I pyrethroids exhibit a negative temperature-toxicity correlation, with higher toxicity at lower temperature; Type II pyrethroids display the opposite trend (Corbett et al., 1984; Matsumura, 2012).

Resistance to pyrethroids in FAW has evolved in multiple regions (Table 3.1.). In Puerto Rico, over a decade of reliance on pyrethroids, especially esfenvalerate and deltamethrin (both Type II), has led to the evolution of practical resistance and diminishing efficacy in cornfields (Belay et al., 2012; Gutierrez-Moreno et al., 2019; Head, 2016; Tabashnik et al., 2014). This study elucidates inheritance patterns and mechanisms associated with esfenvalerate and deltamethrin resistance in FAW populations in Puerto Rico. By shedding light on the genetic basis of resistance and understanding the role of synergists, this study contributes to a deeper understanding of FAW resistance and offers insights for the development of effective FAW management strategies in Puerto Rico seed production and elsewhere.

Table 3.1. Incidents of resistance to pyrethroids in FAW (Mota-Sanchez and Wise, 2024).

Compound	**Location**	**Year**	**Slope**	^{a}**RR$_{50}$**	**Source**
bifenthrin	USA	1991	2.9	29.4	(Yu, 1991)
	China	2023	2.05	21.8	(Mao et al., 2023)
cyfluthrin	México	2012	1.04	162.7	(León-García et al., 2012)
cyhalothrin	USA	1991	1.8	12.5	(Yu, 1991)
cyhalothrin-lambda	Venezuela	2001	1.31	19.4	(Morillo and Notz, 2001)
			1.26	41.9	
			1.08	65.7	
			1.23	62	
	Brazil	1998	1.62	12.8	(G. Diez-Rodriguez and C. Omoto, 2001)
	Mexico	2008	1.08	204.5	(León-García et al., 2012)
	Brazil	2008	3.11	28.2	(Carvalho et al., 2013)
	Colombia	2010	4.10	34.62	(Ríos-Díez and Saldamando-Benjumea, 2011)
	Colombia	2010	4.84	50.01	
	China	2021	1.8	31.2	(Hafeez et al., 2021)
	China	2021	0.76	29	(Zhang et al., 2021)
			2.56	317	
			0.58	32	
			0.86	72	
			0.70	26	
	Brazil	2023	2.92	21.5	(do Nascimento et al., 2023)
cypermethrin	USA	1992	0.8	9.3	(Yu, 1992)
	USA	2006	2.61	10.18	(Al-Sarar et al., 2006)
cypermethrin-zeta	Puerto Rico	2018	1.9	35	(Gutierrez-Moreno et al., 2019)
deltamethrin	México	2008	1.04	1002.2	(León-García et al., 2012)
	Puerto Rico	2018	1.9	25	(Gutierrez-Moreno et al., 2019)
	Brazil	2020	1.76	14.23	(Boaventura et al., 2021)
	China	2021	3.21	12	(Zhang et al., 2021)
			3.76	10	
			2.94	12	
			2.31	20	
	China	2023	2.24	13.9	(Mao et al., 2023)
fenvalerate	USA	1992	2.2	15	(Yu, 1992)
	China	2021	0.51	15	(Zhang et al., 2021)
			0.98	33	
			1.72	26	
			1.55	11	
fluvalinate	USA	1991	2.9	216	(Yu, 1991)
permethrin	USA	1981	^{b}n/a	17	(Wood et al., 1981)
	USA	1991	3.3	13.9	(Yu, 1991)
	USA	1992	2	40	(Yu, 1992)
	Mexico	2018	2	19	(Gutierrez-Moreno et al., 2019)
	Puerto Rico	2018	1.6	48	
tau-fluvalinate	USA	1992	1.5	263.9	(Yu, 1992)
tralomethrin	USA	1991	5.4	41.2	(Yu, 1991)

[a] Resistance ratio (RR), LC$_{50}$ of resistant strain / LC$_{50}$ of susceptible strain.

[b] n/a = data not available.

Materials and methods

Insect Populations

A FAW field strain from Ponce, Puerto Rico (PPR) originated from a collection of larvae from an infested cornfield. The larvae were shipped in cups with diet to Michigan State University, where they were identified (Hallman, 1978; Luginbill, 1928; Mahmoud and Neven, 2020; Oliver and Chapin, 1981; Smith, 1797) and separated to initiate the rearing process. A susceptible FAW colony (SUS) was provided by Bayer USA from their research facilities in Memphis, Tennessee.

Throughout all larval cycles, colonies were maintained in 60 mL cups with 10 mL of artificial FAW diet (Southland Products Inc., Lake Village, AR). After pupation, thirty reciprocal pairs were placed in 5 L paper brown bags for mating, and the bags were placed inside mesh cages. Ten ml cups with cotton balls impregnated with a liquid solution of Gatorade® lime or orange flavor were placed in bags to feed adults. The bags were checked twice weekly for food maintenance and oviposition of egg masses on the bag surface. Egg masses were placed in cups with artificial diet until they hatched, then first instars were placed into individual cups using a paintbrush to avoid damage. Temperature and photoperiod conditions of 26 ± 2 ºC and 16:10 h (L:D), respectively, were used for both the PPR and SUS colonies. All insects were checked daily to confirm the correct and healthy development of both strains.

Chemicals and insecticides

For all bioassays, commercial formulations of the pyrethroids esfenvalerate (Asana XL EC, 8.4%, 79 g a.i./L, Valent USA Corporation, Walnut Creek, CA, USA) and deltamethrin (Battalion™ 0.2 EC, 2.86%, 23.96 g a.i./L, Arysta LifeScience, Cary, NC, USA) were used. Analytic grade synergist compounds and organic solvents were used Sigma-Aldrich, Saint Louis, Missouri, USA.

Pyrethroid bioassays

Concentration-response bioassays of esfenvalerate and deltamethrin were done via diet overlay bioassays in 24-well trays (ProCell, Alkali Scientific Inc., Fort Lauderdale, FL. USA). Each well was filled with 1 mL of the FAW artificial diet (Southland Products Inc., Lake Village, AR. USA) treated with 30 uL of each insecticide solution to cover a surface area of 2.0 cm^2. The concentrations of the insecticides varied to cover a range of mortality from 5% to 95%, with five to nine concentrations per insecticide and four replicates per concentration. The control treatment consisted of 30 microliters of distilled water with a surfactant at 0.05% (v/v).

Each replicate included 12 wells, with one third-instar per well. After the application of the solution, the trays were left to dry for approximately one hour before introducing FAW larvae to the treated surface. After four days, mortality was recorded. Individuals showing acute intoxication symptoms (necrotic tissue, slow movement, or interrupted molting) or those that did not respond to stimulation with a small paintbrush or forceps were considered dead.

Inheritance of resistance

Pupae were collected from both populations using sexual dimorphism to separate females and males (Guzmán-Prada et al., 2018). Then reciprocal crosses were made using thirty pairs of adult FAW per each F_1 crosses were defined as H1 (♂ SUS × ♀ PPR) and H2 (♀ SUS × ♂ PPR).

To evaluate the dominance of resistance, larvae from the reciprocal crosses were subjected to the same susceptibility bioassays used for the SUS and PPR populations in section 2.3 The degree of dominance was estimated using the equation from Bourguet *et al.* (2000).

$$D_M = \frac{M_{RS} - M_{SS}}{M_{RR} - M_{SS}} (1)$$

where M_{SS}, M_{RS}, and M_{RR} were the mortalities expressed in μg/cm^2 of the SUS, reciprocal crosses (H1 or H2), and PPR population, respectively, at different pyrethroid concentrations. D_M

values close to 1 were considered completely dominant inheritance, whereas values close to 0 were deemed completely recessive inheritance. To understand the trend of resistance dominance versus concentration, a range of concentrations covering both reciprocal crosses was established, where mortalities were found (2 – 98%). Data were further analyzed using Stone's equation (1968) to determine the degree of dominance at the LC_{50}.

$$D = \frac{2Y_2 - Y_1 - Y_3}{Y_1 - Y_3} (2)$$

where Y_1 and Y_2 represent the log10 LC_{50} values for the reciprocal crosse (H1 or H2 heterozygotes) and Y_3 corresponds to the log10 LC_{50} for the parental populations (PPR and SUS) respectively. D values were interpreted as follows: -1 completely recessive, $-1 < D < 0$ incompletely recessive, $0 < D < 1$, incompletely dominant, and $D = 1$ as completely dominant.

Synergist bioassays

Bioassays combining pyrethroids and synergists were conducted to investigate the function of detoxification enzymes. The following compounds were tested: (1) the cytochrome P450 inhibitor piperonyl butoxide (PBO 91.3%, SynerProTM Control Solutions Inc. Pasadena, Texas, USA); (2) the esterase inhibitor S, S, S-tributyl phosphorotrithioate (DEF 98.1%, Sigma-Aldrich, Saint Louis, Missouri, USA); (3) the glutathione S-transferase inhibitor diethyl maleate (DEM 97%, Sigma-Aldrich, Saint Louis, Missouri, USA); and (4) the ABC transporter inhibitor (±)-Verapamil hydrochloride (VER 99%, Sigma-Aldrich, Saint Louis, Missouri, USA). To determine synergist concentration to use in the combined assay, separate diet overlay bioassays were done to find the maximum non-lethal concentration for each synergist alone in third-instar. The highest concentration of each compound that did not cause mortality or loss of fitness in the larvae 96 h after application were 4.5 $\mu g/cm^2$, 1.5 $\mu g/cm^2$, 0.45 $\mu g/cm^2$, and 0.45 $\mu g/cm^2$ for PBO, DEF, DEM, and VER, respectively.

Pyrethroid + synergist bioassays were conducted using the same procedure as the pyrethroid bioassays in Section 2.3, with mortality rates assessed at four days after application. Mortality probit analyses and data plotting were also estimate, with synergist ratios (SR_{50} and SR_{90}) calculated by dividing the LC_{50} and LC_{90} values of the pyrethroid alone by the LC_{50} and LC_{90} values of the pyrethroid with synergist concentration. Each set included four replicates, and each replicate consisted of 12 wells with five to seven concentrations each. Every well contained a single third-instar larva, resulting in a total of 48 wells per bioassay.

Data analysis

Probit analysis (Finney, 1971) was used to analyze bioassay results using the PROC PROBIT procedure from SAS version 9.4 (2023). This analysis estimated the slope values, standard error, lethal concentrations at 50% (LC_{50}) and 90% (LC_{90}), fiducial limits (95%), and χ^2 for each population. The resistance ratios (RR_{50} and RR_{90}) were obtained by dividing the LC_{50} and LC_{90} values of the PPR population by those of the susceptible population (SUS). Mortality data were adjusted using Abbott's equation (1925). The log concentration detoxification responses of both populations were compared using parallelism and equality tests ($P<0.05$) with PoloJR (2021). Graphs and log concentration responses were generated using Prism GraphPad Software (2023).

Results

Bioassays and inheritance of resistance

The PPR population exhibited a 62-fold RR_{50} for esfenvalerate and 15-fold RR_{50} for deltamethrin compared to the SUS strain. For esfenvalerate, there was no overlap in confidence intervals for LC_{50} (95% CI). Between the H1 and H2 populations (Table 3.2.). The absence of overlapping LC_{50} values suggests an X-linked inheritance of resistance. For deltamethrin, the

LC_{50} (95% CI) did overlap, suggesting an autosomal inheritance of resistance (Figure 2.1.). Compared to the SUS strain, the RR_{50} values for esfenvalerate were 13-fold and 34-fold for H1 and H2, and 7-fold and 15-fold for H1 and H2 for deltamethrin. Comparison of detoxification using parallelism ($\chi^2 = 157$, d.f. = 3, $P<0.05$) and equality ($\chi^2 = 152.7$, d.f. = 4, $P<0.05$) revealed a unique response for each pyrethroid in the field-evolved strain from PPR.

Based on Stone's method (1968), the degree of dominance D at the LC_{50} for esfenvalerate were 0.249 and 0.741 and for deltamethrin 0.791, and 0.986. These results suggest that the resistance was incompletely dominant for H1 and H2 strains for both active ingredients.

The degree of dominance calculated from the Bourguet *et al.* equation (2000) for both active ingredients followed a similar trend between crosses. In the H1 progeny (♂ SUS × ♀ PPR), the response to esfenvalerate shows an initial increase in dominance with concentration, reaching its peak at D_M values just below 0.75 (0.10 μg/cm^2). Beyond this concentration, the dominance level decreases, stabilizing around $D_M = 0.5$, indicating a shift from complete to incomplete dominance. For deltamethrin in the H1 progeny, a different pattern emerges, with dominance levels approaching complete dominance (D_M close to 1.0) across all concentrations, except at the highest tested concentration where it shows a slight reduction. In the H2 progeny (♀ SUS × ♂ PPR), the dominance pattern for both esfenvalerate and deltamethrin is more uniform. For deltamethrin, the D_M values are consistently close to 1.0 across all concentrations, indicating a strong, almost completely dominant inheritance. Similarly, for esfenvalerate in H2, the dominance also approaches complete dominance (D_M close to 1.0) across all concentrations, except at the highest tested dose, where it deviates slightly.

Thus, for deltamethrin, both H1 and H2 progeny exhibit a trend towards complete dominance across the range of concentrations, with a minor exception at the highest dose. For esfenvalerate, while H1 progeny show a trend towards incomplete dominance at higher concentrations, H2 progeny maintain a pattern of complete dominance across most concentrations (Figure 2.2.).

Table 3.2. Concentration-response to esfenvalerate and deltamethrin of fall armyworm from a susceptible lab colony (SUS), a field collection from Ponce Puerto Rico (PPR) and their F_1 reciprocal crosses (H1 and H2).

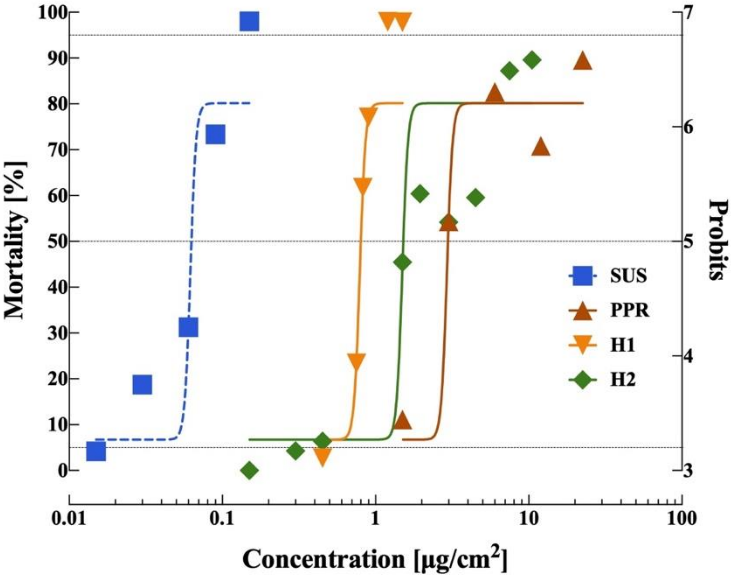
Pyrethroid	**Strain**	**n**	**Slope**	**SE**	**[a]LCs$_{50}$**	**(95% CI)**	**[a]LCs$_{90}$**	**(95% CI)**	**[b]RR$_{50}$**	**[b]RR$_{90}$**
esfenvalerate	PPR	233	1.9	0.4	3.8	(1.3, 6.7)	17	(8.9, 183)	62	123
	SUS	287	3.5	0.6	0.06	(0.04, 0.08)	0.1	(0.09, 0.33)	1	1
	H1 (♂ SUS × ♀ PPR)	369	9.7	1.8	0.8	(0.7, 0.87)	1	(0.97, 1.37)	13	8
	H2 (♀ SUS × ♂ PPR)	424	1.9	0.2	2.2	(1.85, 2.65)	10	(7.9, 14.9)	34	62
deltamethrin	PPR	228	3.9	0.41	0.41	(0.35, 0.46)	0.87	(0.74, 1.07)	15	20
	SUS	240	5.8	1.1	0.03	(0.02, 0.03)	0.04	(0.03, 0.10)	1	1
	H1 (♂ SUS × ♀ PPR)	287	3.7	0.4	0.3	(0.26, 0,34)	0.7	(0.58, 0.84)	12	16
	H2 (♀ SUS × ♂ PPR)	335	2.9	0.40	0.398	(0.3, 0.5)	1.11	(0.79, 2.03)	15	25

[a] LCs$_{50}$ or LCs$_{90}$ (µg/cm^2).

[b] Resistance ratio (RR) = LC$_{50}$ of resistant strain / LC$_{50}$ of susceptible strain or LC$_{90}$ of resistant strain / LC$_{90}$ of susceptible strain.

[c] d.f. = degrees of freedom.

A. esfenvalerate



B. deltamethrin

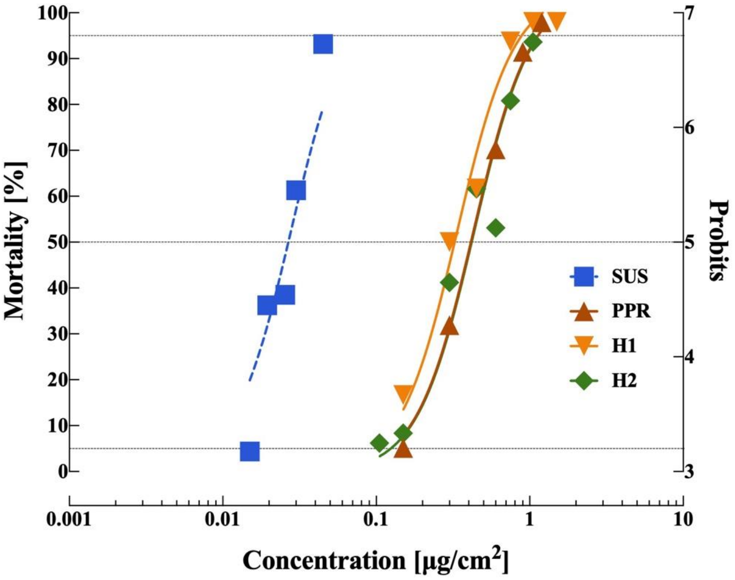


Figure 2.1. Mortality response of fall armyworm from a susceptible lab colony (SUS), a field collection from Ponce Puerto Rico (PPR) and their F_1 reciprocal crosses (H1, ♂ SUS × ♀ PPR) + (H2, ♀ SUS × ♂ PPR) to esfenvalerate and deltamethrin.

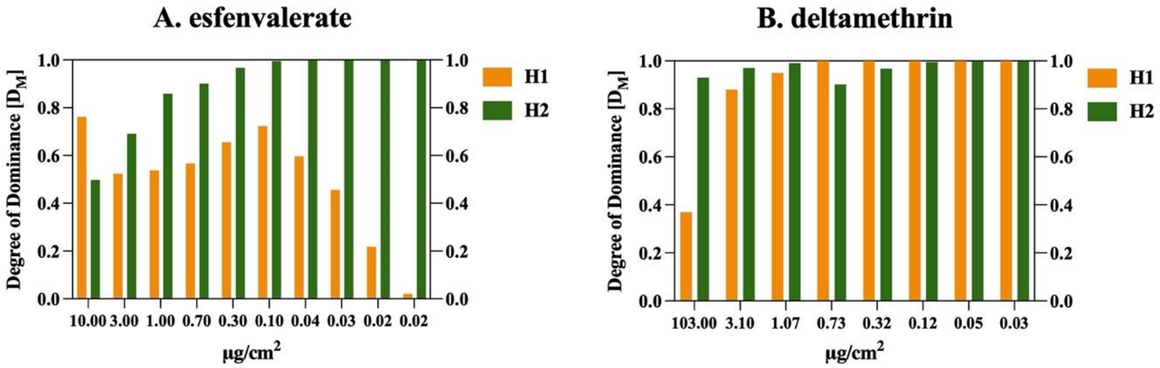


Figure 2.2. Dominance degree [D_M] of resistance to esfenvalerate (A) and deltamethrin (B) in F_1 reciprocal crosses (H1, ♂ SUS × ♀ PPR) + (H2, ♀ SUS × ♂ PPR) of FAW.

Synergism bioassay

Results from the esfenvalerate synergist bioassays demonstrated that both SUS and PPR strains used a detoxification enzyme system to metabolize pyrethroids. In the SUS strain, the P450s inhibitor (PBO) was the most effective, resulting in a 4.6-fold increase in synergism (SR_{50}), followed by the esterase inhibitor (DEF), with a 1.4-fold increase. Glutathione S-transferase inhibitor (DEM) and ABC transporter inhibitor (VER) showed little synergism (<1-fold).

In the PPR strain, all four synergists caused synergism combined with esfenvalerate, indicating the presence of detoxification enzymes. The PBO bioassays showed a 3.5-fold increase in synergism, whereas the VER bioassays showed a 4.7-fold increase. The DEF and DEM bioassays showed a similar trend, with the highest increase in synergism of almost 8-fold. When all synergists were combined in an additional bioassay, there was an accumulative effect, resulting in a 12-fold increase in SR_{50} in the field strain (Table 3.3.).

Table 3.3. Assessing the mortality rate of FAW strains exposed to esfenvalerate with synergists alone and combo - a comparison of susceptible lab colony (SUS) and a field collection from Ponce Puerto Rico (PPR).

Active Ingredient	**Synergists**	**Strain**	**n**	**Slope**	**SE**	**[a]LCs$_{50}$**	**(95% CI)**	**[a]LCs$_{90}$**	**(95% CI)**	**[c]RR$_{50}$**	**[b]SR$_{50}$**	**[b]SR$_{90}$**
esfenvalerate	-	PPR	233	1.9	0.4	3.76	(1.3, 6.7)	17.2	(8.9, 183)	62	-	-
	PBO		327	1.7	0.3	1.07	(0.6, 1.7)	6.0	(3.3, 19)	18	3.5	2.9
	DEM		528	1.8	0.4	0.49	(0.2, 0.76)	2.4	(1.4, 8)	8	7.7	7.1
	DEF		384	1.8	0.1	0.53	(0.4, 0.66)	2.8	(2.1, 4)	9	7.1	6.1
	VER		432	3.0	0.6	0.80	(0.52, 1.2)	2.1	(1.3, 5.3)	13	4.7	8.1
	PBO + DEM + DEF + VER		335	1.9	0.3	0.31	(0.2, 0.45)	1.4	(0.9, 3)	5	12	12
	-	SUS	287	3.5	0.6	0.06	(0.04, 0.08)	0.1	(0.09, 0.33)	1	-	-
	PBO		288	2.6	0.3	0.01	(0.011, 0.015)	0.04	(0.03, 0.06)	0	4.6	3.3
	DEM		432	2.5	1.2	0.33	*-	1.1	*-	5	0.2	0.1
	DEF		240	3.1	0.3	0.04	(0.036, 0.05)	0.1	(0.09, 0.15)	1	1.4	1.3
	VER		479	3.5	0.3	0.12	(0.1, 0.13)	0.3	(0.23, 0.32)	2	0.5	0.5
	PBO + DEM + DEF + VER		430	4.1	1.6	0.07	(0.032, 0.28)	0.1	(0.08, 245)	1	0.9	1.0

[a] LCs$_{50}$ or LCs$_{90}$ (µg/cm^2)

[b] Synergist ratio (SR) = LC$_{50}$ of esfenvalerate without synergist / LC$_{50}$ of esfenvalerate + synergist

d.f. = degrees of freedom

[c] Resistance ratio (RR) = LC$_{50}$ of resistant strain / LC$_{50}$ of susceptible strain

PBO = piperonyl butoxide, DEM = diethyl maleate, DEF = S,S,S -tributyl phosphorotrithioate and VER = (±)-verapamil hydrochloride

[*] No confidence intervals could be calculated

Results of the deltamethrin synergist bioassays indicated similar detoxification enzyme roles. In the SUS strain, the most significant synergism was found in bioassays with the presence of PBO, with a SR_{50} of 3-fold, followed by DEF bioassays with a 1.3-fold. In the DEM and VER bioassays, a minor antagonism was found in the presence of such synergists (<1-fold). However, in the PPR population, higher synergism was found in DEF with a 17-fold, followed by VER with a 4-fold increase, and PBO and DEM bioassays with a 2-fold (Table 3.4.). These findings suggest that both strains (SUS and PPR) have different detoxification mechanisms for esfenvalerate and deltamethrin and that the presence of detoxification enzymes can significantly affect the toxicity of both pyrethroids.

Table 3.4. Assessing the mortality rate of FAW strains exposed to deltamethrin with synergists - a comparison of susceptible lab colony (SUS) and a field collection from Ponce Puerto Rico (PPR).

Active Ingredients	**Synergists**	**Strain**	**n**	**Slope**	**SE**	**[a]LCs$_{50}$**	**(95% CI)**	**[a]LCs$_{90}$**	**(95% CI)**	**[c]RR$_{50}$**	**[b]SR$_{50}$**	**[b]SR$_{90}$**
deltamethrin	-	PPR	228	3.9	0.4	0.406	(0.3, 0.4)	0.9	(0.74, 1.07)	15	-	-
	PBO		239	2.0	0.5	0.184	(0.04, 0.44)	0.8	(0.3, 55)	7	2	1
	DEM		239	2.4	0.3	0.207	(0.16, 0.24)	0.7	(0.54, 0.97)	7.8	2	1
	DEF		335	1.3	0.2	0.024	(0.008, 0.05)	0.3	(0.1, 1.2)	0.92	17	3
	VER		335	2.3	0.4	0.091	(0.05, 0.17)	0.3	(0.17, 1.61)	3.4	4	3
	-	SUS	240	5.8	1.1	0.026	(0.021, 0.034)	0.04	(0.03, 0.106)	1	-	-
	PBO		283	2.6	0.4	0.009	(0.005, 0.012)	0.03	(0.017, 0.07)	0.34	3	1.6
	DEM		336	3.4	0.5	0.026	(0.02, 0.03)	0.06	(0.045. 0.11)	1	1	0.7
	DEF		239	2.3	0.4	0.02	(0.015, 0.023)	0.07	(0.05, 0.14)	0.76	1.3	0.6
	VER		528	2.7	0.3	0.028	(0.02, 0.03)	0.08	(0.063, 0.12)	1.05	1	0.5

[a] LCs$_{50}$ or LCs$_{90}$ (μg/cm^{2}).

[b] Synergist ratio (SR) = LC$_{50}$ of deltamethrin without synergist / LC$_{50}$ of deltamethrin + synergist

[c] Resistance ratio (RR)V = LC$_{50}$ of resistant strain / LC$_{50}$ of susceptible strain

PBO = piperonyl butoxide, DEM = diethyl maleate, DEF = S,S,S -tributyl phosphorotrithioate and VER = (±)-verapamil hydrochloride

Discussion

In this study, we investigated the inheritance of resistance to two pyrethroids in a field evolved resistant FAW population from Puerto Rico and the contribution of detoxifying enzymes to pesticide resistance. The resistance of FAW to esfenvalerate (62-fold) and deltamethrin (15-fold) resulted in “practical resistance.” This is the first report of resistance to esfenvalerate in Puerto Rico. Prior studies on field-evolved resistance to pyrethroids in an FAW strain from the same geographical location in indicated resistance to permethrin, deltamethrin, and zeta-cypermethrin (Gutierrez-Moreno et al., 2019). The continuous use of esfenvalerate to manage FAW over the last decade in all cropping seasons has resulted in high levels of resistance (Teran-Santofimio, 2019). Practical resistance to pyrethroids in the field has been reported, prompting a re-formulation of strategies to rotate action modes and explore innovative IPM programs that utilize all accessible resources.

Reciprocal crosses revealed a noteworthy pattern: heterozygous individuals exhibited an incomplete dominant response to esfenvalerate and deltamethrin, as shown in Figure 1A and 1B. These data suggest that heterozygous larvae can tolerate concentrations akin to their homozygous resistant counterparts, subsequently increasing the gene frequency in field populations (Figure 2.2.). This observed resistance becomes even more pronounced as pesticide residue decays; heterozygous resistant larvae seem to endure and thrive, leading to a swift evolution of resistance in the field. The dynamics of this resistance pattern are especially significant when considering the ubiquity of pyrethroids; they stand out not only for their cost-effectiveness, especially when compared to newer materials such as diamides (Richardson et al., 2020), but also their frequent integration into IPM programs (Hołyńska-Iwan and Szewczyk-Golec, 2020; Yu, 2014). This widespread reliance could intensify selection pressure, resulting in individuals experiencing indirect exposure to sublethal doses at various stages.

With its tropical conditions, Puerto Rico presents a unique environment in which continuous oviposition leads to an ever-present cycle of six larval stages and overlapping FAW generations. Within this context, a compelling hypothesis emerges: the pesticide resistance landscape in Puerto Rico might align with a broader theme of intra-island variation in susceptibility. This pattern implies that the island's gene flow might be insufficient to balance out differences in insecticide susceptibility, a phenomenon echoed in species such as the green aphis (*Aphis gossypii*) (Hollingsworth et al., 1994), whitefly (*Bemisia tabaci*) (Omer et al., 1993), and diamondback moth (*Plutella xylostella*) (Tabashnik et al., 1987), predominantly from Hawaii. However, in order to test this hypothesis, it would be imperative to carry out bioassays involving diverse FAW strains from Puerto Rico.

Resistance to pyrethroids (esfenvalerate and deltamethrin), which is inherited in an incompletely dominant manner, is commonly observed in several species. For instance, a similar inheritance trend has been found in species closely related to the diamondback moth (Sayyed et al., 2005), the predator lady beetle (*Eriopis connexa*) to deltamethrin (Rodrigues et al., 2013), the cotton bollworm (*Helicoverpa armigera*) to cypermethrin (Achaleke and Brevault, 2010) and fenvalerate (Daly and Fisk, 1992; Gunning et al., 1991), the soybean looper (*Chrysodeixis includens*) to lambda-cyhalothrin (Stacke, Godoy, Halberstadt, et al., 2020), the two spotted spider mite (*Tetranychus urticae*) to lambda-cyhalothrin as well (Susurluk and Gurkan, 2020), the tobacco budworm (*Heliothis virescens*) to permethrin (Watson and Kelly, 1991), and the horn fly (*Haematobia irritans*) to cypermethrin.

The inheritance of resistance indicated a sex-linked inheritance pattern for resistance to esfenvalerate in the FAW population from Ponce, Puerto Rico, suggesting that males predominantly transmit this resistance. This type of inheritance pattern is relatively rare in FAW. A previous example included a field-evolved resistant strain of the convergent lady beetle (*Hippodamia convergens*) from Georgia, USA, which demonstrated a sex-linked recessive inheritance pattern for resistance to lambda-cyhalothrin (Barbosa et al., 2016). A similar pattern was observed in the two-spotted spider mite from Antalya, Turkey (Susurluk and Gurkan, 2020). More recently, evidence of a sex-linked flubendiamide resistance pattern has been observed in a population from the same geographic area (Posos-Parra et al., 2024).

In contrast, resistance to deltamethrin in FAW appears to be autosomally inherited, a finding corroborated by studies on a lab-selected FAW population resistant to lambda-cyhalothrin from Guaría, Sao Paulo (G. I. Diez-Rodriguez and C. Omoto, 2001). Previous reports also identified autosomal resistance traits in FAW from other regions of the Americas to a range of other insecticides, including carbamates (carbaryl) (Yu and Nguyen, 1994), organophosphates (chlorpyrifos) (Garlet et al., 2021), pyrethroids (lambda-cyhalothrin) (G. I. Diez-Rodriguez and C. Omoto, 2001), nicotinic acetylcholine receptor (nAChR) allosteric modulators, spinosyns (spinosad and spinetoram) (Lira et al., 2020; Okuma et al., 2018), glutamate-gated chloride channel (GluCl) allosteric modulators, avermectins and milbemycins (emamectin benzoate) (Muraro et al., 2021), and inhibitors of chitin biosynthesis, benzoylureas (novaluron and teflubenzuron) (do Nascimento et al., 2022; Stacke, Godoy, Pretto, et al., 2020). Autosomal inheritance of deltamethrin resistance has also been documented in other species. These include the diamondback moth (Ahrnad et al., 2007), house fly (*Musca domestica*) (Khan et al., 2015), common lacewing (*Chrysoperla carnea*) (Sayyed et al., 2010), tobacco cutworm

(*Spodoptera litura*) (Ahrnad et al., 2007; Huang et al., 2007), and codling moth (*Cydia pomonella*) (Bouvier et al., 2001).

The use of synergists plus pyrethroids indicated the crucial role of detoxification enzymes in resistance mechanisms. This research particularly underscores the importance of a variety of detoxification enzymes when studying esfenvalerate resistance. Enzymes, such as P450s, esterases, glutathione S-transferase enzymes, and ABC transporters, are integral components of the resistance mechanism. Similar detoxification mechanisms have been observed in other instances, such as the soybean aphid's response to lambda-cyhalothrin (Xi et al., 2015), the western flower trips (*Frankliniella occidentalis*) to tau-fluvalinate (Thalavaisundaram et al., 2008), and the cotton bollworm to fenvalerate (Gunning et al., 1991) and cypermethrin (Achaleke et al., 2009; Armes et al., 1996).

Resistance to pyrethroids in FAW is characterized by metabolic enzymes and mutations at the target site (Carvalho et al., 2013; Yu et al., 2003). Owing to their unique chemical structure, pyrethroids undergo phase I detoxification reactions, such as hydrolysis (Yu, 2014). Enzymes such as cytochrome P450s and esterases play critical roles in detoxification in insects. The use of inhibitors of these enzymes, such as PBO for P450s and DEF for esterases, enhances the toxicity of pyrethroids, thereby increasing their susceptibility (Tchakounte et al., 2019). Some of the resistance mechanisms may be triggered by genetic mutations.

The use of synergists with esfenvalerate suggested a high involvement of P450s, esterases, glutathione S-transferases, and ABC transporters in the suppression of resistance, with the combination of all synergists being the most promising factor of resistance (Table 2.3.). For deltamethrin, synergist bioassays indicated reduced involvement of P450s, glutathione S-transferases, and ABC transporters but also a significant presence of esterases in the detoxification process. Given the complexity of these biochemical interactions, we hypothesize that the resistance encountered for both pyrethroids could be polygenic in nature. However, the scope of this investigation did not extend to conducting backcross tests with the field PPR colony. This limitation highlights an area for future research, potentially revealing a more detailed genetic basis for the observed resistance patterns.

More detailed molecular analysis is needed to elucidate other roles in the resistance mechanism of FAW from Puerto Rico (Boaventura et al., 2020). Mutations at the target site have been extensively studied because of the mode of action of pyrethroids in voltage-gated sodium channels (Bradberry et al., 2005; Eldefrawi and Eldefrawi, 2020; Soderlund, 2012). Knockdown (*kdr*) resistance has been investigated since it was first observed in a strain of houseflies that survived DDT exposure (Dong et al., 2014; Quarterman, 1950; Williamson et al., 1993). Cases of pyrethroid resistance featuring *kdr*-type mutations have been elucidated, and scientists have found strong associations with point mutations in the *para*-type sodium channel gene (Davies et al., 2007; Hu et al., 2011). Point mutations have also been reported in other species, such as whiteflies, German cockroaches (*Blattella germanica*), and tobacco budworms (Soderlund and Knipple, 2003). Given the scope of the present study, we cannot conclusively fail to reject or reject the hypothesis that the presence of point mutations results in site-of-action resistance in conjunction with the observed enzyme-mediated metabolic resistance. Nevertheless, unraveling the resistance mechanisms exhibited by this FAW strain is paramount for the reevaluation and reformulation of current integrated resistance management (IRM) programs in Puerto Rico.

Implications

It is increasingly clear that areas experiencing recent FAW invasions face a heightened risk of pyrethroid resistance, as suggested by Nagoshi et al. (2022). This risk is compounded by a lack of established knowledge and resources necessary for implementing effective IRM strategies (Nagoshi et al., 2020; Wang et al., 2023). Consequently, immediate and concerted efforts are needed to equip these regions with the tools and expertise required to mitigate the rapid evolution of resistance and safeguard agricultural outputs. By addressing these challenges proactively, we can better prepare these vulnerable regions to manage the threat of FAW globally more effectively.

Puerto Rico is a globally significant location for plant breeding research because of its year-round favorable climate, thereby enabling continuous farming, regulatory frameworks, and science-friendly environment for biotechnology (Belay et al., 2012; Storer et al., 2010; Storer et al., 2012). However, these assets also create conditions conducive to high pest pressure, leading to the extensive use of synthetic pest management tools and subsequent resistance development, especially in FAW. To enhance IPM and IRM strategies, IRAC-US and PRABIA (2020) have implemented an area-wide resistance management program comprising five work streams: field trials, rotation programs, scouting practices, implementation, and resistance monitoring. Research on pyrethroid resistance in FAW supports the need for these efforts and may contribute to improved IPM practices. Collaborative workshops with the seed industry further aim to address FAW pyrethroid resistance issues (Teran-Santofimio, 2019). While improving area-wide resistance management programs may take time, such actions are necessary given the broad implications for the global food system posed by pesticide resistance in FAW in Puerto Rico.

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CHAPTER 4:

FIELD-EVOLVED RESISTANCE OF FALL ARMYWORM (*Spodoptera frugiperda* J.E. SMITH) (LEPIDOPTERA: NOCTUIDAE) TO SPINOSYNS IN PUERTO RICO: RESISTANCE RATIOS, MECHANISMS, AND CROSS-RESISTANCE.

Introduction

Spodoptera frugiperda, the fall armyworm (FAW), is a significant threat to global agriculture, particularly in corn production (Barros and de Freitas Bueno, 2010; Nagoshi et al., 2015). Originating from the Americas (Kenis et al., 2023; Rwomushana, 2022; Smith, 1797), this adaptable and prolific pest has expanded its range from the Americas into Africa, Asia, Australia, and Europe, impacting agriculture production worldwide (Deshmukh et al., 2018; Kenis et al., 2022; Kim and Babcock, 2019; Qi et al., 2021; Richardson et al., 2020; Tepa-Yotto et al., 2021; Wang et al., 2023). Its wide host range, combined with swift adaptation to various environments (Barros and de Freitas Bueno, 2010; Nagoshi et al., 2015; Gutierrez-Moreno et al., 2019), accentuates the need to develop of innovative pest management strategies against it. Further complicating this challenge is its resistance to an extensive number of insecticides (Boaventura et al., 2019; do Nascimento et al., 2023; Gutierrez-Moreno et al., 2019; Muraro et al., 2021; Okuma et al., 2018; Padovez et al., 2022; Posos-Parra et al., 2024). To date, there are 250 cases of resistance by FAW against 45 different active ingredients (Mota-Sanchez and Wise, 2024). Thus, FAW has emerged as a significant concern in many countries.

The direct losses to food and feed corn due to FAW can be substantial. For example, yield reductions in corn can reach nearly 60% (Hruska and Gladstone, 1988; Deshmukh et al., 2020; Koffi et al., 2020). Less recognized is the impact of FAW on the global seed corn industry. Puerto Rico plays a critical role as a hub for corn seed breeding and production. The island boasts advantages such as skilled labor, a tropical climate, quality soil, proximity to the U.S. mainland, efficient transportation, and strong intellectual property laws, which have attracted major agricultural companies like Corteva Agriscience, Bayer, Syngenta, Mycogen Seeds, and BASF. These companies make a significant contribution to the local economy. According to the Puerto Rico Agricultural Biotechnology Industry Association, more than 85% of the seeds used in global biotechnological agriculture are developed in Puerto Rico, taking advantage of its optimal conditions at some stage of their development (PRABIA, 2020). These factors underscore Puerto Rico's leading position in the agricultural biotechnology sector.

Puerto Rico favorable climate for seed corn increase is also favorable to FAW, allowing for up to ten generations per year and four plantings of corn annually (i.e. continuous presence of a host crop) (Gutierrez-Moreno et al., 2019; Head, 2016; Posos-Parra et al., 2024; Storer et al., 2010). However, if uncontrolled, FAW can cause a total destruction of the crop. This high pest pressure necessitates extensive pest management efforts in seed corn fields. However, extensive pesticide use is required to manage larval injury in seed production, with up to 30 applications per season from at least nine different modes of action (Head, 2016), has led to the evolution of resistance in local FAW populations. This resistance encompasses a wide array of synthetic insecticides plus *Bacillus thuringiensis* (Bt) proteins in genetically modified (GM) corn, further complicating management efforts and threatening the sustainability of seed production (Diego et al., 2019; Gutierrez-Moreno et al., 2019; Head, 2016; Posos-Parra et al., 2024; Storer et al., 2010; Tandy et al., 2023).

Spinosyns, derived from the aerobic fermentation of *Saccharopolyspora spinosa* isolated from soil, are a groundbreaking class of macrolide insecticides. Spinosad and spinetoram, prominent members of this class, had sales approaching $600 million USD in 2018 (Sparks et al., 2020; Sparks et al., 2021). Their increased use is partly attributed to their eco-friendly profile, which shows negligible effects on non-target species and ecosystems (Thompson and Sparks, 2002). For example, the lethal concentration (LC_{50}) for the honeybee, *Apis mellifera*, is higher for spinosad (11.5 ppm) than for the pyrethroid cypermethrin (1.2 ppm) (Durkin, 2016; Thompson et al., 2005). Spinosyns also have a unique mechanism of action (group 5), disrupting nicotinic acetylcholine receptors and GABA-gated ion channels in insects, leading to rapid nervous system excitation, involuntary muscle contractions, tremors, paralysis, and death (Fulton et al., 2013; IRAC, 2020; Sparks et al., 2012). This makes them valuable in cropping systems where pests are resistant to insecticides with older conventional modes of action. In Puerto Rico, spinosad and spinetoram have been used to control FAW for the past 10 years. Resistance cases have been documented in spinetoram but not for spinosad (Head, 2016; Portillo et al., 2018; Gutierrez-Moreno et al., 2019). Consequently, ensuring the prolonged, safe, and efficient use of spinosyns is crucial for their continued success as an IPM solution in seed corn production.

This study aims to determine the genetic and biochemical factors contributing to resistance to spinosyns in a FAW population from the seed production area in Puerto Rico. Synergist bioassays were used to unravel the inheritance patterns and to dissect the mechanisms of resistance. Additionally, this study investigated resistance and cross-resistance between spinosyns and two other insecticides: abamectin, which is derived from a fermentation process using *Streptomyces avermitilis* and is typically used to control soft-bodied insects such as thrips and aphids in soybeans, but not FAW; methoxyfenozide, an insect growth regulator (IGR) that acts as an ecdysone receptor agonist (group 118), used against FAW where field failures have been recorded in recent years (Teran-Santofimio, 2019); and novaluron, an inhibitor of chitin biosynthesis by impacting chitin synthase 1 (group 15), an IGR not registered for use in corn fields in Puerto Rico. Given that FAW might be exposed to other compounds due to neighboring applications in different crops, it is relevant to evaluate the hypothesis that there is not uniform resistance to all IGRs in FAW.

Materials and methods

FAW strains and rearing

Larvae from corn fields in Ponce, Puerto Rico (PPR), where spinosad and spinetoram were used to control FAW, were collected and shipped to Michigan State University in 2019, where a colony was established. A known insecticide susceptible strain of FAW (SUS) was obtained from Bayer USA's research facilities in Memphis, Tennessee. Larvae of both strains

were reared individually in 60 mL plastic containers with 20 mL of a specialized artificial diet for FAW (Southland Products Inc., Lake Village, Arkansas) until pupation. At pupation, pupae were separated into females and males based on sexual dimorphism, following the methodology outlined by (Guzmán-Prada et al., 2018), and pairs were placed in 5 L paper bags for mating. Paper bags were confined inside of mesh cages to avoid escaping. Adults were fed with cotton balls soaked in lime or orange-flavored Gatorade®. Cages were monitored biweekly for food replenishment and to collect egg masses, which were then incubated on the artificial diet until hatch. Newly hatched individuals were carefully transferred to separate containers using a paintbrush. Both strains were maintained under optimal conditions at a temperature of 26 ± 2 °C and a 16:10 h light-dark cycle, with daily checks to monitor healthy development.

In addition to the original PPR and SUS strains, male and female pupae from both populations were separated and used to make reciprocal crosses (30 mated pairs of each), resulting in two F_1 hybrid groups: H1 (♀ SUS × ♂ PPR) and H2 (♂ SUS × ♀ PPR). Larvae from the crosses were handled and maintained in a similar way as the parent colonies.

Spinosyns bioassay

To assess response of the four FAW populations (two parental strains and two hybrid groups) to spinosyns, we used a diet overlay method (Posos-Parra et al., 2024) in 24-well trays (ProCell, Alkali Scientific Inc., Fort Lauderdale, FL). In each tray, 1 mL of FAW artificial diet, (Southland Products Inc., Lake Village, AR), was dispensed into each well, followed by the application of 30 µL of a specific insecticide or control solution, to achieve coverage of the 2.0 cm^2 surface area well. The experimental design aimed to span a larval mortality range from 5% to 95%, with insecticide concentrations adjusted accordingly. The formulated products of two spinosyns insecticides used in Puerto Rico were tested: spinosad (Entrust® SC; 22.5% and 239.65 g a.i./L; Corteva Agriscience LLC, Indianapolis, IN, USA) and spinetoram (Delegate® WG; 25% and 250 g a.i./kg; Corteva Agriscience LLC, Indianapolis, IN, USA). For each FAW population, five to ten concentrations were tested for each insecticide, and each concentration was replicated four to five times. The control consisted of 30 µL of distilled water mixed with a surfactant at a concentration of 0.05% (v/v). Following the application of treatment, trays were left to dry for approximately one hour. The experimental units comprised of 12 wells each of 24-well trays, having two units in one tray. Then a single early-third instar FAW was placed in each well. Mortality was assessed four days after treatment, recording the number of larvae displayed symptoms of acute intoxication (necrosis and interrupted molting) or lack of respond to stimulation with a small paintbrush or forceps.

Synergism bioassays

To investigate the role of detoxification enzymes in spinosyn resistance, PPR and SUS larvae were exposed to spinosad or spinetoram in combination with four high-purity synergists: piperonyl butoxide (PBO), inhibiting cytochrome P450 enzymes; S,S,S-tributyl phosphorotrithioate (DEF), inhibiting esterases; diethyl maleate (DEM), inhibiting glutathione S-transferase; and (±)-verapamil hydrochloride (VER), an ABC transporter inhibitor. All synergists and organic solvents were obtained from Sigma-Aldrich (Saint Louis, MO, USA). The synergist applications were done in two ways, either as part of the diet overlay or topically. Preliminary experiments were conducted to establish the highest non-lethal concentration for each synergist when applied alone to third-instar larvae. The determined non-lethal concentrations for residual application were 4.5 $\mu g/cm^2$ for PBO, 1.5 $\mu g/cm^2$ for DEF, 0.45 $\mu g/cm^2$ for DEM, and 0.45 $\mu g/cm^2$ for VER (Posos-Parra et al., 2024). For topical application, the concentrations were 0.3 μg/μL for PBO, and 1 μg/μL for DEF, DEM, and VER.

The overall method was similar to that used in the spinosyn bioassay, with five to seven concentrations of the insecticides applied to diet, and the plates left to dry before larvae were introduced. When synergists were applied as part of the diet overlay, insecticides and synergist were applied sequentially using 30 μL of each one. After application, trays were left to dry for 1 to 2 hours. Following this, individual early third instars were moved into wells.

When synergists were applied topically, the dorsal thoracic region of each third instar received 1 μL of synergist via a 50 μL micro-syringe, facilitated by a PB600-1 repeating dispenser (Hamilton Company, Reno, NV, USA) as described by Gutierrez-Moreno et al. (2019). After two-hours, larvae were moved into the wells overlaid with the spinosyn insecticides as described by Bolzan et al. (2019). The experimental design included four replicates per set, with each replicate comprising 12 wells. Wells were prepared with five to thirteen different concentrations of the insecticide, and each well housed a single third instar, with a total of 48 wells for each concentration, having at least over 240 insects per bioassays. For this type of synergist application, there was an independent bioassay for the SUS strain applying the synergist topically, but for the PPR, results from the residual bioassays were used. Mortality was assessed after four days, as described in the spinosyn bioassay.

Cross-resistance bioassays

Cross-resistance was assessed using the same methodology described for the spinosyn bioassay, exposing the PPR and SUS strains to formulations of three other biopesticides: abamectin (Agri-Meck® SC; 8% and 83.88 g a.i./L; Syngenta Crop Protection LLC), novaluron (Radiant® SC; 11.7% and 120 g a.i./L; Corteva Agriscience LLC), and methoxyfenozide (Intrepid 2F®; 22.6% and 239.65 g a.i./L; Corteva Agriscience LLC). Although abamectin and novaluron are not used in corn production in Puerto Rico, methoxyfenozide has been applied in conjunction with pyrethroids and botanical, such as neem (Head, 2016).

Data analysis

The analysis of bioassay and synergism results was conducted using probit analysis (Finney, 1971) employing the PROC PROBIT function of SAS software (version 9.4 2023). This statistical approach facilitated the estimation of several key parameters: slope values, standard errors, median and 90% lethal concentrations (LC_{50} and LC_{90}), 95% fiducial limits, and the chi-square (χ^2) value for parent and hybrid populations. Resistance ratios for 50% mortality (RR_{50}) and 90% mortality (RR_{90}) were calculated by dividing the LC_{50} and LC_{90} values of the PPR population by those of SUS population. To account for natural mortality, adjustments to the mortality data were made using Abbott's formula from (1925).

Synergism was quantified through the calculation of synergist ratios (SR_{50} and SR_{90}). These ratios were derived by dividing the LC_{50} and LC_{90} values obtained for the insecticide alone by those achieved when the insecticide was combined with the synergist.

Further, the detoxification response to log concentrations for both populations was assessed for parallelism and equality, setting the significance threshold at $P<0.05$, through analyses conducted with PoloJR (2021). Visualization of the data, including graphs and log concentration response curves, was accomplished using Prism (GraphPad Software, 2023) and (OriginLab, 2023).

Using Stone's equation (1968) was used to assess the degree of dominance at the median lethal concentration (LC_{50}). Here, X1 and X2 represent the log10 LC_{50} values for the reciprocal crosses (H1 or H2 heterozygotes), and X3 is the log10 LC_{50} for the parental populations (PPR and SUS). The interpretation of D values ranged from -1, indicating completely recessive, to +1, indicating completely dominant, with values in between representing varying degrees of incomplete dominance or recessiveness.

Following this, the degree of dominance (D_M) of resistance to spinosyn was quantified using the formula by Bourguet et al. (2000):

$$D_M = \frac{M_{RS} - M_{SS}}{M_{RR} - M_{SS}} (1)$$

In this equation, M_{SS}, M_{RS} and M_{RR} denote mortality rates, in µg/cm², for the susceptible (SUS) population, the reciprocal crosses (H1 or H2), and the field strain (PPR), respectively. A D_M value approaching 1 indicated a pattern of complete dominance in inheritance, whereas a value near 0 suggested completely recessive inheritance. To further elucidate the relationship between resistance dominance and insecticide concentration, a concentration range was defined across which mortalities for both sets of reciprocal crosses were observed (2 – 98%).

Results

Spinosyns bioassay

The PPR population demonstrated a significant resistance to spinosad, with a 213.7-fold increase in resistance at RR_{50} and an even-more-pronounced 2,140-fold increase at RR_{90} (Table 4.1). For spinetoram, the resistance levels were lower, with a 63.3-fold increase at RR_{50} and a 111-fold increase at RR_{90} (Table 4.1). The analysis of the spinosad LC_{50} values for the H1 and H2 populations revealed that the 95% confidence intervals (CIs) overlapped (Table 4.1.) and Figure 3.1.), suggesting that resistance is inherited autosomally. In contrast, for spinetoram, the distinct lack of overlap in LC_{50} values (95% CI) between the H1 and H2 populations suggests that the inheritance of resistance may be X-linked and thus based on a different mechanism.

Table 4.1. Concentration-response of fall armyworm from a field collection from Ponce Puerto Rico (PPR), susceptible lab colony (SUS), and F$_1$ reciprocal crosses (H1 and H2) to spinosad and spinetoram.

Spinosyns	**Strain**	**[d]n**	**Slope**	**[c]SE**	**[a]LCs$_{50}$**	**(95% CI)**	**[a]LCs$_{90}$**	**(95% CI)**	**[b]RR$_{50}$**	**[b]RR$_{90}$**
spinosad	PPR	286	0.7	0.1	6.2	(3.8, 11)	321	(110, 1974)	213.7	2140
	SUS	238	1.8	0.2	0.029	(0.02, 0.03)	0.15	(0.10, 0.26)	1	1
	H1 (♂ SUS × ♀ PPR)	478	1.6	0.1	0.06	(0.05, 0.8)	0.4	(0.29, 0.59)	2.1	2.7
	H2 (♀ SUS × ♂ PPR)	336	1.4	0.3	0.09	(0.04, 0.15)	0.7	(0.32, 4.39)	3.1	4.7
spinetoram	PPR	334	1.7	0.2	0.095	(0.072, 0.116)	0.51	(0.4, 0.8)	63.3	111
	SUS	285	2.5	0.3	0.0015	(0.0012, 0.0017)	0.0046	(0.003, 0.007)	1	1
	H1 (♂ SUS × ♀ PPR)	432	1.5	0.2	0.004	(0.002, 0.006)	0.03	(0.017, 0.077)	2.6	6.5
	H2 (♀ SUS × ♂ PPR)	432	1.7	0.3	0.018	(0.01, 0.03)	0.1	(0.04, 0.62)	12	21.7

[a] LCs$_{50}$ or LCs$_{90}$ (μg/cm^2).

[b] Resistance ratio (RR) = LC$_{50}$ of resistant strain / LC$_{50}$ of susceptible strain or LC$_{90}$ of resistant strain / LC$_{90}$ of susceptible strain.

[c] SE: standard error.

[d] n: total number of insects used for the probit analysis.

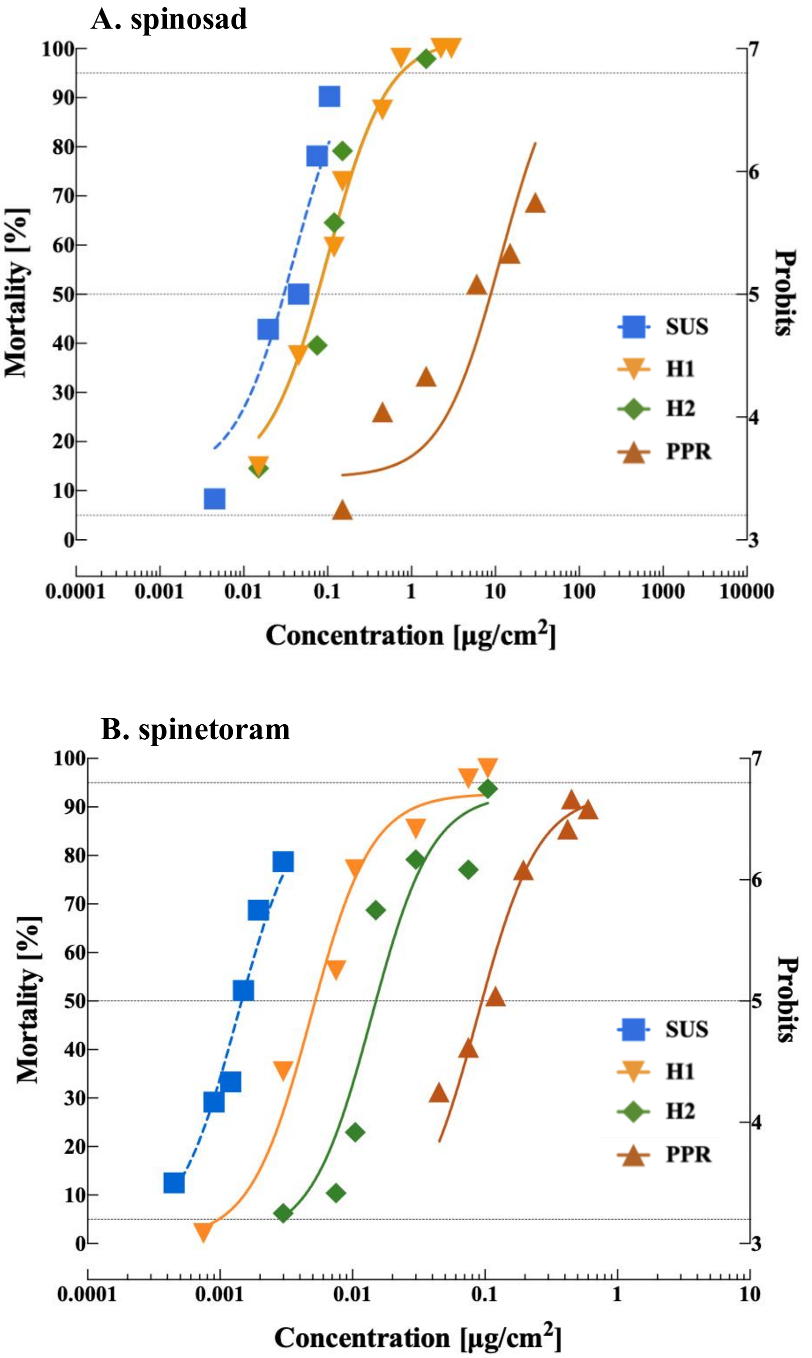

Figure 3.1. Log-concentration mortality response of fall armyworm from a susceptible lab colony (SUS), a field collection from Ponce Puerto Rico (PPR) and their F_1 reciprocal crosses (H1, ♂ SUS × ♀ PPR) + (H2, ♀ SUS × ♂ PPR) to spinosad and spinetoram.

Comparisons of RR_{50} against the SUS strain for spinosad revealed a reduction in resistance by 2.1-fold in the H1 population and by 3.1-fold in the H2 population (Table 4.1). For spinetoram, the reduction in resistance was more varied, with a 2.6-fold decrease in the H1 population and a 12-fold decrease in the H2 population (Table 4.1). The unique response of the PPR strain to detoxification assays was statistically significant, as demonstrated by tests of parallelism ($\chi^2 = 82.70$, degrees of freedom (d.f.) = 3, $P < 0.05$) and equality ($\chi^2 = 177.19$, d.f. = 4, $P < 0.05$), indicating a distinctive detoxification mechanism against each insecticide.

Utilizing Stone's method (1968) to determine the degree of dominance (*D*) at the LC_{50} level, for the value for spinosad was -0.729 for H1 and -0.578 for H2, indicating that resistance was incompletely recessive for both crosses. For spinetoram, the values were -0.527 for H1 and 0.198 for H2; the H1 strain was incompletely recessive but the H2 strain shifted towards incompletely dominant.

Degree of dominance

D_M determined through the Bourguet et equation for both insecticides demonstrated a consistent trend for reciprocal crosses (H1 and H2). For spinosad, the highest D_M value for both H1 (0.85) and H2 (0.7) was at the lowest tested concentration of 0.002 μg/cm² (Fig 3.2A). Then the dominance level declined to nearly 0 at the highest concentration tested (10 μg/cm²). This suggests a transition from complete to incomplete dominance, in alignment with the findings derived from Stone's method. For spinetoram, a similar pattern was noted for H1 progeny, with dominance levels nearing 1.0 at the lowest concentration, then decreasing as the dose increased Fig 3.2B. For H2 progeny, the dominance pattern followed an inverted U-shaped curve, with lower D_M values at the lowest and highest doses tested, meaning that medium and low doses of spinosyns could increase the frequency of resistance alleles in both females and males from the PPR strain.

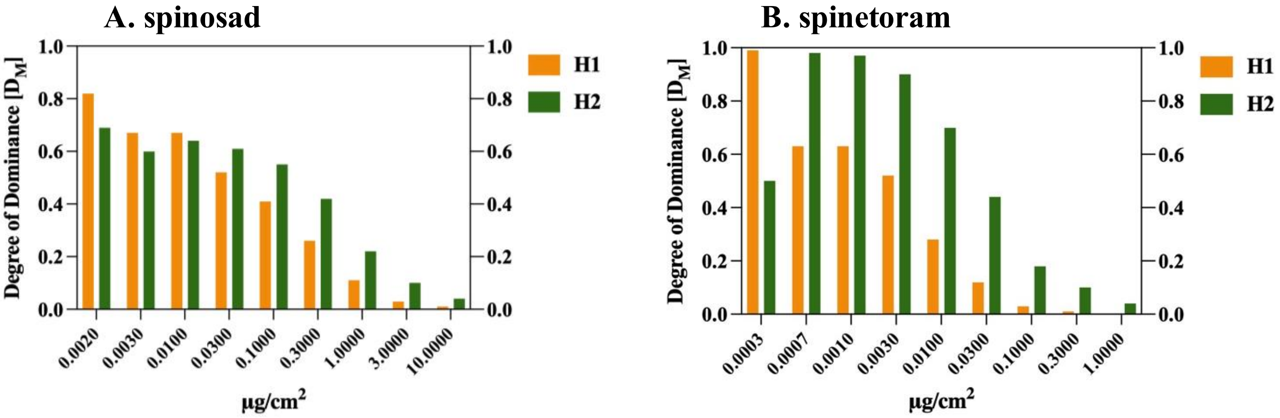

Figure 3.2. Degree of dominance [D_M] of resistance against spinosyns in F1 reciprocal crosses (H1, ♂ SUS × ♀ PPR) + (H2, ♀ SUS × ♂ PPR) of FAW.

Synergist bioassays

The PPR strain exhibited a significant toxicity to spinosad when applied with the synergists. DEM showing the highest synergistic ratio (SR_{50} = 155), followed by DEF (SR_{50} = 122), VER (SR_{50} = 52), and PBO (SR_{50} = 4), meaning a higher role of of glutathione S-transferase, esterases, ABC transporters, and a slightly role of cytochrome P450s in detoxification of spinosyns (Table 4.2.).

Table 4.2. Concentration response of FAW from a susceptible strain (SUS) and field collection in Ponce PR (PPR) exposed to spinosad with and without synergists to block detoxification enzymes: synergists were co-applied with spinosad in a diet overlay.

Active Ingredient	**Synergist**	**Strain**	**^{e}n**	**Slope**	**^{d}SE**	**$^{a}LCs_{50}$**	**(95% CI)**	**$^{a}LCs_{90}$**	**(95% CI)**	**$^{c}RR_{50}$**	**$^{b}SR_{50}$**	**$^{b}SR_{90}$**
spinosad	none	PPR	286	0.7	0.1	6.2	(3.8, 11)	321	(110, 1974)	213.7	-	-
	PBO		287	1.4	0.2	1.5	(0.16, 1.9)	12	(7, 26.2)	52	4	27
	DEM		384	1.1	0.3	0.05	(0.004, 0.12)	0.7	(0.27, 12.21)	1.8	122	459
	DEF		663	1.2	0.2	0.04	(0.024, 0.061)	0.5	(0.25, 1.35)	1.4	155	642
	VER		327	2.2	0.4	0.12	(0.063, 0.18)	0.45	(0.26, 1.60)	4.1	52	713
	All		371	1.1	0.1	0.08	(0.05, 0.11)	1.27	(0.76, 2.44)	2.8	77.5	253
	none	SUS	238	1.8	0.2	0.029	(0.02, 0.03)	0.15	(0.10, 0.26)	1	-	-
	PBO		337	1.1	0.1	0.004	(0.002, 0.005)	0.1	(0.03, 0.11)	0.1	7.3	1.5
	DEM		240	1.0	0.1	0.008	(0.005, 0.0124)	0.2	(0.08, 0.54)	0.3	3.6	0.8
	DEF		288	1.2	0.1	0.02	(0.017, 0.034)	0.3	(0.16, 0.53)	0.7	1.5	0.5
	VER		336	2.1	0.5	0.01	(0.005, 0.021)	0.05	(0.027, 0.3)	0.3	2.9	3
	all		289	2.2	0.2	0.085	(0.69, 0.10)	0.3	(0.23, 0.48)	2.9	0.3	0.5

[a] LCs_{50} or LCs_{90} (μg/cm^2).

[b] Synergist ratio (SR) = LC_{50} of insecticide without synergist / LC_{50} of insecticide + synergist applied residually.

[c] Resistance ratio (RR) = LC_{50} of resistant strain / LC_{50} of susceptible strain

[d] SE: standard error.

[e] n: total number of insects used for the probit analysis.

PBO = piperonyl butoxide, DEM = diethyl maleate, DEF = S,S,S -tributyl phosphorotrithioate and VER = (±)-verapamil hydrochloride

In contrast, the SUS strain exhibited a more moderate response, with the most significant increase in toxicity observed when using PBO (SR_{50} = 7.3). With other synergists, this strain showed only a slight or no increase in toxicity. Notably, when all synergists were combined in the PPR strain, an accumulative effect was observed that markedly enhanced toxicity (SR_{50} = 77.5)—a phenomenon not seen in the SUS strain, where the combination of synergists led to slight antagonism. Topically applied synergists consistently increased the toxicity in the PPR strain across all tested compounds, with SR_{50} values ranging from 41 to 51. In contrast, the SUS strain showed only a minor increase in toxicity with PBO, underscoring the different impacts of detoxification enzymes between strains as shown in Table 4.3.

The use of both dietary and topical applications of synergists was intended to demonstrate the presence and varying effects of detoxification enzymes in each strain.

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Table 4.3. Concentration response of FAW from a susceptible colony (SUS) and field collection in Ponce PR (PPR) exposed to spinosad with and without synergists to block detoxification enzymes: synergists were applied topically to larvae.

Active Ingredient	**Synergists**	**Strain**	[e]**n**	**Slope**	[d]**SE**	[a]**LCs$_{50}$**	**(95% CI)**	[a]**LCs$_{90}$**	**(95% CI)**	[c]**RR$_{50}$**	[b]**SR$_{50}$**	[b]**SR$_{90}$**
spinosad	[f] none	PPR	286	0.74	0.1	6.2	(3.8, 11)	321.1	(110, 1974)	206	-	-
	PBO		332	2.03	0.2	0.13	(0.11, 0.16)	0.58	(0.43, 0.83)	4.3	48	553
	DEM		287	2.4	0.3	0.12	(0.08, 0.18)	0.41	(0.26, 0.97)	4	51	783
	DEF		235	2.72	0.5	0.15	(0.08, 0.25)	0.44	(0.26, 1.8)	5	41	730
	VER		238	1.9	0.3	0.131	(0.05,0.4)	0.61	(0.2, 21)	4.4	47	526
	none	SUS	240	1.9	0.2	0.03	(0.02, 0.04)	0.14	(0.10, 0.23)	1	-	-
	PBO		286	1.66	0.4	0.013	(0.002, 0.035)	0.076	(0.02, 2.43)	0.4	2.2	2
	DEM		288	1.58	0.2	0.03	(0.02, 0.07)	0.23	(0.10, 1.5)	1	1	0.7
	DEF		287	1.56	0.3	0.038	(0.01, 0.12)	0.25	(0.09, 6)	1.3	0.8	0.6
	VER		288	1.87	0.3	0.06	(0.04, 0.15)	0.32	(0.14, 3.3)	2	0.5	0.5

[a] LCs$_{50}$ or LCs$_{90}$ (μg/cm^2).

[b] Synergist ratio (SR) = LC$_{50}$ of insecticide without synergist / LC$_{50}$ of insecticide + synergist applied topically.

[c] Resistance ratio (RR) = LC$_{50}$ of resistant strain / LC$_{50}$ of susceptible strain.

[d] SE: standard error.

[e] n: total number of insects used for the probit analysis.

[f] results from the residual bioassay (Table 4.2.).

PBO = piperonyl butoxide, DEM = diethyl maleate, DEF = S,S,S -tributyl phosphorotrithioate and VER = (±)-verapamil hydrochloride

The spinetoram-synergist bioassays reinforced these findings, with the PPR strain exhibiting high increase in in toxicity, particularly with DEM (SR_{50} = 15.8) and VER (SR_{50} = 11.9), further emphasizing the strain's enhanced susceptibility after applying these synergists (Table 4.4.).

Table 4.4. Concentration response of FAW from a susceptible colony (SUS) and field collection in Ponce PR (PPR) exposed to spinetoram with and without synergists to block detoxification enzymes: synergists were co-applied with s spinetoram in a diet overlay.

Active Ingredients	Synergists	Strain	[e]n	Slope	[d]SE	[a]LCs_{50}	(95% CI)	[a]LCs_{90}	(95% CI)	[c]RR_{50}	[b]SR_{50}	[b]SR_{90}
spinetoram	-	PPR	334	1.7	0.2	0.095	(0.072, 0.116)	0.51	(0.4, 0.8)	63.3	-	-
	PBO		335	1.3	0.2	0.029	(0.017, 0.05)	0.29	(0.12, 1.6)	19.3	3.3	1.8
	DEM		384	1.7	0.3	0.006	(0.003, 0.009)	0.04	(0.02, 0.11)	4	15.8	12.8
	DEF		335	1.7	0.3	0.043	(0.017, 0.09)	0.25	(0.11, 1.60)	28.7	2.2	2.0
	VER		336	1.2	0.3	0.008	(0.0008, 0.02)	0.10	(0.029, 25)	5.33	11.9	5.1
	-	SUS	285	2.5	0.3	0.0015	(0.0012, 0.0017)	0.0046	(0.003, 0.007)	1	-	-
	PBO		286	2.0	0.2	0.0008	(0.0005, 0.0007)	0.0027	(0.002, 0.003)	0.53	1.9	1.7
	DEM		240	2.8	0.3	0.0021	(0.0017, 0.0024)	0.0059	(0.004, 0.008)	1.4	0.7	0.8
	DEF		336	3.0	0.6	0.0035	(0.002, 0.005)	0.0096	(0.006, 0.024)	2.33	0.4	0.5
	VER		336	2.4	0.5	0.0023	(0.0013, 0.0038)	0.0079	(0.004, 0.028)	1.53	0.7	0.6

[a] LCs_{50} or LCs_{90} (µg/cm^{2}).

[b] Synergist ratio (SR) = LC_{50} of insecticide without synergist / LC_{50} of insecticide + synergist.

[c] Resistance ratio (RR) = LC_{50} of resistant strain / LC_{50} of susceptible strain.

[d] SE: standard error.

[e] n: total number of insects used for the probit analysis.

PBO = piperonyl butoxide, DEM = diethyl maleate, DEF = S,S,S -tributyl phosphorotrithioate and VER = (±)-verapamil hydrochloride

Cross-resistant to abamectin, novaluron and resistance to methoxyfenozide

The PPR strain exhibited cross-resistance to abamectin (13.7-fold) and resistance to methoxyfenozide (62.5-fold). However, the RR_{90} value for abamectin was only 1.12. This lower RR_{90} value can be attributed to a smaller slope value (1.2) observed in the susceptible populations compared to a higher slope value (4.8) in the PPR strain, leading to an intersection of the log-dose response curves at higher concentrations. In contrast, no significant cross-resistance to novaluron was detected, with only 1.6-fold increase noted (refer to Table 4.5).

Table 4.5. Dose-response to abamectin, novaluron and methoxyfenozide of a susceptible lab colony (SUS) and Ponce Puerto Rico field collection (PPR).

Active Ingredients	**Strain**	**n**	**slope**	**SE**	**[a]LC_{50} (95% CI)**	**[a]LC_{90} (95% CI)**	**[b]RR_{50}**	**[b]RR_{90}**
abamectin	PPR	323	4.8	0.6	881 (794, 979)	1621 (1391, 2038)	13.7	1.12
	SUS	286	1.2	0.2	64 (28, 140)	727 (280, 6737)	1	1
novaluron	PPR	238	3.3	1.3	[*]0.01	[*]0.02	1.6	2
	SUS	239	5.5	0.6	0.006 (0.0051, 0.0062)	0.01 (0.0085, 0.011)	1	1
methoxyfenozide	PPR	234	0.9	0.2	25 (11, 40)	506 (263, 1882)	62.5	216
	SUS	238	1.6	0.3	0.4 (0.3, 0.64)	2.3 (1.19, 9)	1	1

[a] LC_{50} or LC_{90} (μg/cm^2).

[b] Resistance ratio (RR) = LC_{50} of resistant strain / LC_{50} of susceptible strain or LC_{90} of resistant strain / LC_{90} of susceptible strain.

[c] df = degrees of freedom.

[*] No confidence intervals could be calculated.

Discussion

Our study confirms a significant shift in field-evolved resistance to spinosyns by the FAW in Puerto Rico. We report very high resistance ratios (RR_{50}) of 213-fold and (RR_{90}) of 2,140-fold for spinosad, and 63-fold and 111-fold, respectively, for spinetoram. These results represent a significant increase from the levels reported by Gutierrez-Moreno et al. in 2019, who found RR ratios under 14 for both insecticides. Practical resistance has already been reported in a field strain from Ponce, Puerto Rico. This is likely a consequence of the prolonged, continuous use of spinosad over the past decade in Puerto Rican seed corn production. According to Head (2016) and Teran-Santofimio (2019), this practice played a critical role in the development of these high resistance levels.

In FAW population from Ponce, Puerto Rico, we observed a specific pattern of cross-resistance to abamectin, with a 13.7-fold increase at the RR_{50}, although no significant resistance was evident at the RR_{90}. This distinct partial resistance is critical, especially considering the broad use of diverse pesticides for managing FAW. Such findings highlight the complexity of resistance management and the need for carefully crafted pest control strategies to prevent further resistance development. Previously, abamectin cross-resistance was noted at a lower magnitude, showing only a 7-fold increase. This current study marks the first report of a higher, more pronounced level of cross-resistance to abamectin in FAW. These findings underscore the necessity for well-designed pest control strategies that preempt the development of resistance. Although previous instances showed a 7-fold in abamectin resistance, suggesting no cross-resistance in 2015 (Gutierrez-Moreno et al., 2019). For this reason, this is the first report of such a distinct level of cross-resistance, which contrasts with the resistance patterns to other pesticides like methoxyfenozide, which exhibited a 62.5-fold increase. Additionally, while cross-resistance to the chitin biosynthesis inhibitor triflumuron was observed at a 20-fold increase in a 2015 field strain from Puerto Rico (Gutierrez-Moreno et al., 2019), novaluron, a similar acting agent, did not show resistance, thereby challenging the hypothesis that this particular Puerto Rican field strain of FAW is universally resistant or cross-resistant to all insect growth regulators (IGRs).

Moreover, resistance to spinosad is not confined to FAW but has been observed in other Lepidoptera species, including cotton bollworm (*Helicoverpa armigera*) (Alvi et al., 2012; Qayyum et al., 2015; Wang et al., 2009), beet armyworm (*Spodoptera exigua*) (Ahmad et al., 2018; Gao et al., 2014; Ishtiaq et al., 2014; Su and Sun, 2014; Wang et al., 2021; Zuo et al., 2020), diamond backmoth (*Plutella xyllostela*) (Agboyi et al., 2016; Jiang et al., 2015; Pudasaini et al., 2022; Tamilselvan et al., 2021; Wang et al., 2016; Wang et al., 2022; Zhang et al., 2016), Mediterranean climbing cutworm (*Spodoptera litura*) (Ahmad et al., 2008; Rehan and Freed, 2014; Saleem et al., 2016; Sayyed et al., 2008a; Shad et al., 2012) and tomato leafminer (*Tuta absoluta*) (Campos et al., 2015; Grant et al., 2019; Reyes et al., 2012; Silva et al., 2016), underscoring its widespread nature. Resistance to spinetoram is less frequent, perhaps due it is much expensive than spinosad (Congreve, 2023). However, notable cases have been recorded in diamondback moth and beet armyworm (Tamilselvan et al., 2021; J. Wang et al., 2021; Zuo et al., 2020) indicating a need for vigilance and ongoing research to better understand and manage insecticide resistance.

Autosomal recessive inheritance of resistance to spinosad in various species, such as the western flower thrips (*Frankliniella occidentalis*) (Bielza et al., 2007), the diamond backmoth, the tomato leafminer (Campos et al., 2014), and the beet armyworm (Zuo et al., 2022). This mode of inheritance involves resistance alleles located on autosomes—the non-sex chromosomes—which means that both male and female individuals must inherit these alleles from both parents to express resistance. The consistent observation of autosomal recessive inheritance across diverse species suggests a common genetic mechanism underpinning resistance to this insecticide, indicative of a broader biological pattern in the evolution of insecticide resistance across taxa. Against this backdrop, our study on reciprocal crosses between the PPR and SUS strains of FAW provided new insights into the complex genetics of pesticide resistance. Heterozygous F_1 individuals exhibited incompletely recessive and autosomally-inherited resistance pattern to spinosad, consistent with broader observations. However, for spinetoram, resistance patterns varied, showing incompletely recessive inheritance in the H1 cross but incomplete dominance in the H2 cross, as depicted in Figures 3.1.A. and 3.1.B. This suggests a potential X-linked genetic basis for spinetoram resistance, and a different inheritance mechanism across sex. These findings align with previous observations by Okuma et al. (2018) regarding spinosad resistance. However, they contrast with Lira et al.'s (2020) documentation of an autosomal incompletely recessive pattern of resistance to spinetoram in a lab selected Brazilian FAW strain. Additionally, they differ from the patterns observed by Wang et al. (2020) in a lab-selected tomato leafminer strain.

Our research emphasizes the significant impact of spinosad concentration on the dominance of resistance in FAW. At high concentrations of spinosyns, resistance manifested as co-dominant. However, spinosad concentration towards the recommended field rate led to a noticeable shift from incomplete to complete recessive resistance. This critical finding underscores an essential aspect of resistance management: utilizing spinosyns at below-recommended concentrations may inadvertently support the survival of heterozygous insects. This, in turn, boosts the frequency of resistant alleles, leading to control failures and challenges in restoring susceptibility without selective pressure (Gressel, 2011). Consequently, it is vital to apply the recommended rate of spinosyns to FAW, ensuring effective mortality of heterozygous individuals crucial for preventing resistance development in field conditions.

Spinosyns are distinguished by their beneficial biological properties, notably their minimal impact on populations of beneficial insects (Romi et al., 2006; Sparks et al., 1998), and a unique mode of action that sets them apart from other classes of insecticides (Nauen et al., 2019; Sparks et al., 2020), thus bolstering their significance in Integrated Resistance Management (IRM) strategies (Sparks et al., 2012). The versatility and distinctive chemical composition of spinosyns lead us to hypothesize that their broad applicability across different agronomic practices (conventional and organic farming) might inadvertently facilitate the development of 'practical resistance' (Posos-Parra et al., 2024; Schnaars-Uvino & Baker, 2021; Tabashnik et al., 2014). This emerging resistance pattern suggests that gene flow within island ecosystems may not be robust enough to mitigate variations in insecticide susceptibility. This situation is reminiscent of observations in other species in similar geographical scenarios (Hollingsworth et al., 1994; Omer et al., 1993; Tabashnik et al., 1987). For the robust validation of this hypothesis, conducting regular bioassays with a variety of FAW strains from different geographical areas in Puerto Rico is essential.

Since its introduction to the market in 1997, spinosad's effectiveness against lepidopteran pests has been closely monitored, with resistance becoming a significant concern (Sparks et al., 2021). Investigations have identified that resistance to spinosad primarily arises through target site mutations in approximately two-thirds of cases, while the remaining instances involve metabolic resistance, engaging multiple genes and mechanisms (Sparks et al., 2012). Focusing on the mechanism of resistance, particularly for spinosad, target site mutations have been a common theme. The Dα6 subunit of the nicotinic acetylcholine receptor (nAChR) has been implicated in the resistance observed in the diamondback moth, fruit fly (*Drosophila melanogaster*), and housefly (*Musca domestica*), illustrating a pattern of target site mutation (Perry et al., 2007; Watson et al., 2010). Additionally, specific receptor transmembrane point mutations, notably the G275E mutation, have been identified in several species, including the melon thrips (*Thrips palmi*), Western flower thrips (*Frankliniella occidentalis*), and the tomato leafminer, further evidencing the diverse genetic foundations of resistance to spinosyns (Puinean et al., 2013; Shi et al., 2021; Silva et al., 2016). The Arthropod Pesticide Resistance Database (APRD) has documented 363 instances of resistance to spinosyns across approximately 36 species. Notably, resistance to spinosad and spinetoram in the FAW has been observed since 2017 in several countries, including Brazil (Kanno et al., 2021; Lira et al., 2020; Okuma et al., 2018), China (Chen et al., 2023; Zhang et al., 2021), and Puerto Rico (Gutierrez-Moreno et al., 2019). Despite the relatively rare occurrence of field-evolved resistance—highlighted by significant events in China (2021 and 2022) and Puerto Rico (2019)—the mechanisms underlying this resistance often remain unexplored.

Our research has uncovered that the PPR strain of FAW exhibits a notable resistance to spinosyns, predominantly mediated by detoxification enzymes. Detailed assays have pinpointed the significant contribution of detoxification mechanisms to this resistance, particularly when spinosad is used in conjunction with synergists such as a glutathione S-transferase inhibitor (DEM), an esterase inhibitor (DEF), an ABC transporter inhibitor (VER), and to a somewhat lesser degree, a cytochrome P450 inhibitor (PBO). Topical application trials have also shown a high activity of these detoxification enzymes, with synergists ratios (SR_{50}) observed to be between 41 and 51-fold. While metabolic mechanisms have been less frequently identified as a source of resistance compared to target site mutations, similar phenomena have been documented in other pests including the diamondback moth (Sayyed et al., 2008b), cotton bollworm (Wang et al., 2009), beet armyworm (Wang et al., 2006; Wang et al., 2021b), and Western flower thrips (Herron et al., 2014).

Moreover, the synergistic effect of ABC transporters on spinosyn resistance in FAW was demonstrated through CRISPR/Cas9-mediated knockout of the *SfABCC2* and *SfABCC3* genes in lab-selected FAW eggs (Jin et al., 2021). This adds to the growing evidence of ABC transporter mutations contributing to resistance against Bt proteins, Cry1F, in a laboratory colony from Puerto Rico collected in 2007 (Abdelgaffar et al., 2019; Banerjee et al., 2017), and the identification of transposable elements encoding ATP-binding cassette (ABC) transporters in *Helicoverpa armigera* (Klai et al., 2020). Notably, this study presents the first in vivo test using VER as a synergist in residual and topical application for spinosad characterization through bioassay methodology in this colony, expanding upon previous findings of flubendiamide resistance metabolism due to ABC transporter genes (Posos-Parra et al., 2024). Despite this, the use of VER, a p-glycoprotein inhibitor, to investigate ABC transporter involvement in resistance has been previously applied to species such as *Rhipicephalus* (*Boophilus*) and *Anopheles gambiae* against pyrethroids (Chouaïbou et al., 2014; Khangembam et al., 2018). The necessity for further molecular analysis to unravel the resistance mechanism of this FAW strain is underscored by its significant migratory patterns and adaptability, especially to developing countries facing challenges such as the lack of comprehensive IRM programs, economic constraints, and suboptimal pesticide application techniques.

For this reason, I cannot conclusively fail to reject or reject the hypothesis that the presence of point mutations leads to site-of-action resistance. This complexity is compounded by our findings of enzyme-mediated metabolic resistance, marking an intricate interplay between genetic mutations and biochemical defense mechanisms. These observations collectively constitute the first documented instance of a high prevalence of synergistic effects across four distinct detoxification pathways (Yu, 2014). Such intricacy in the resistance mechanisms demonstrated by the FAW strain underscores the urgent need to revisit and refine the strategies employed in IRM programs within Puerto Rico. This nuanced understanding is vital for developing more effective approaches to counteract resistance and ensure the sustainability of pesticide efficacy.

Impact

The combination of environmental factors such as geographic isolation, the presence of a tropical climate, selection pressure, practical resistance to 'older' insecticides, and the introduction of a unique active ingredient like spinosyns, among others, can create the perfect scenario for the rapid emergence of field-evolved resistance (Posos-Parra et al., 2024). The specific case of spinosad in relation to these factors has been documented previously, for example, the instance of field-evolved resistance of the diamondback moth in Hawaii (Zhao et al., 2006), a scenario sharing similarities with the FAW population in Puerto Rico. Another instance of selection pressure and rapid resistance evolution shortly after spinosad was registered for use on potatoes was observed in Long Island, US (Mota-Sanchez et al., 2006; Schnaars-Uvino and Baker, 2021). Lastly, the indiscriminate use of spinosad in controlled environments such as greenhouses led to the quick appearance of practical resistance cases in Almeria and Murcia, Spain (Bielza et al., 2007).

Recent findings highlight the urgent risk of spinosyns resistance in new FAW territories, with a critical need for enhanced IRM strategies due to gaps in knowledge and resources (Nagoshi et al., 2020; Wang et al., 2023). Immediate action is required to equip affected regions with strategies to mitigate resistance and ensure agricultural productivity. Puerto Rico, a key site for plant breeding research due to its ideal climate and supportive scientific environment, faces increased pest pressures that accelerate resistance development, including to spinosad in FAW. To combat this, IRAC-US and PRABIA (2020) have initiated an area-wide resistance management program, focusing on field trials, pesticide rotation, scouting, implementation, and monitoring efforts to curb spinosad resistance. This comprehensive approach aims to enhance global food security by developing effective countermeasures against the spread of resistance to spinosyns and other chemical groups of insecticides (Posos-Parra et al., 2024) in FAW populations.

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CHAPTER 5:

CONCLUSIONS AND FUTURE DIRECTIONS.

I characterized the resistance of FAW colony from Puerto Rico to three major groups of insecticides: diamides, pyrethroids, and spinosyns. Additionally, I examined the inheritance of resistance to these insecticides, as well as their potential cross-resistance to active ingredients within the same group. FAW exhibited resistance evolution to both synthetic insecticides and Bt proteins (Abdelgaffar et al., 2019; Banerjee et al., 2017; Blanco et al., 2010; Gutierrez-Moreno et al., 2020; Gutiérrez-Moreno et al., 2019; Nagoshi et al., 2017). Notably, my research was pioneering in demonstrating the resistance mechanism of the Puerto Rican population to diamides, pyrethroids, and spinosyns, which appears to be mediated by detoxification enzymes in pyrethroids and spinosyns and a minor detoxification role in diamide compounds. Furthermore, my study was able to detail the inheritance patterns of resistance, providing evidence to support or challenge the hypothesis that the inheritance was autosomal and incompletely recessive for each insecticide group. This finding is crucial, as the dominance or recessiveness of resistance genes plays a significant role in the speed at which resistance develops. Chapter 1 offers an in-depth review of the current literature on the topic, beginning with the significance of agriculture and its contribution to the control challenge posed by continuous use of both synthetic insecticides and those derived from microorganisms *Saccharopolyspora spinosa*, alongside the migration patterns of this pest over the past six years.

In Chapter 2, I characterized the inheritance of resistance and the metabolic roles of detoxification enzymes of diamides (chlorantraniliprole, flubendiamide) and cross-resistance to sister molecules like cyclaniliprole and cyantraniliprole. By breeding a resistant strain with a susceptible colony, I obtained an F_1 hybrids that enabled me to study the role of alleles in the resistance of the PR strain. Notably, the inheritance of the chlorantraniliprole resistance is autosomal, whereas flubendiamide resistance is X-linked in the Puerto Rican colony, suggesting a differential inheritance of the resistance of each diamide compound. Furthermore, the study examined potential cross-resistance mechanisms, revealing varying degrees of resistance to cyantraniliprole and cyclaniliprole. To uncover the metabolic mechanism of resistance I used synergist of cytochrome P450s (PBO), esterases (DFE), glutathione-S-transferases (DEM), and ABC transporters (Verapamil). Notably, the participation of detoxification enzymes such as glutathione S-transferases, ABC transporters and esterases was implicated in suppressing at the LC_{50}s flubendiamide but not chlorantraniliprole ($SR_{50} <2$), indicating disparate metabolic resistance pathways from other studies (Boaventura et al., 2020a; Boaventura et al., 2020b; Zuo et al., 2020a). Consequently, several research questions remain open, additional hypotheses need proved, and some questions require further investigations. For instance, how stable is the resistance of the fall armyworm populations to diamides under tropical conditions? Could suspending the use of diamides as a part of Integrated Pest Management (IPM) strategies eventually counteract resistance in FAW populations? Moreover, what is the FAW allelic frequency of resistance in the FAWs from the Puerto Rican corn fields. It is crucial to emphasize that probit analysis did not refute the hypothesis that different diamides elicit distinct detoxification mechanisms in the Puerto Rican field strain, as evidenced by the results of the equality and parallelism tests.

In Chapter 3, I focus on characterizing the inheritance of resistance to type II pyrethroids (esfenvalerate and deltamethrin) and accept the null hypothesis that the resistance to these pyrethroids is due to a metabolic mechanism, such as: the presence of esterases, cytochrome P450s, glutathione-S-transferases, or ABC-transporters. Our initial premise was the high presence of esterase-type enzymes (carboxylesterases) because, as synthetic esters, pyrethroids are substrates for these enzymes, allowing esterases to recognize and act on ester bonds (R-COO-O-R'), forming less active and less toxic compounds. However, the results indicated a complex of metabolic enzymes responsible for the resistance of FAW to pyrethroids, including esterases and P450s, as well as glutathione S-transferase and ABC transporter, and possibly others metabolic mechanism of resistance, which were not possible to evaluate within the scope of this research. On the other hand, the inheritance of resistance to pyrethroids concluded being highly autosomal for deltamethrin and X-linked for esfenvalerate. The understanding the resistance, inheritance of resistance, and mechanism of defense of the FAW colony to sodium channel disruptors like pyrethroids is critical since farmers in many tropical and sub-tropical regions of most continents use pyrethroids as an inexpensive option to manage FAW.

The origin of FAW that invaded Africa and the Asian continents is tracked down to a Caribbean origin (Nagoshi et al., 2017), and it is likely that genes responsible for pyrethroid resistance are also carry in this migration to other continents. Furthermore, my insights into FAW pyrethroid resistance unveils a potential catastrophic panorama of resistance in other regions of the world that are under tropical conditions, low genetic recombination due to geographical isolation, non-crop rotation, high number of applications, and a high socioeconomic interest by the farmers in using inexpensive and broad-spectrum compounds. In addition, FAW is a pest with a polyphagous profile, potent flyer and short cycles between generations due to tropical conditions. In the geographical range of FAW expansion there are areas with conditions similar to those of Puerto Rico, where FAW has just migrated and IPM strategies are scarce, as is access to information (Laminou et al., 2022). In these areas, pyrethroids are one of the most accessible chemical tools for FAW management because they are available, inexpensive, and have a broad spectrum of action. However, if pyrethroids are not under an IPM scheme the possibility of practical resistance is high (Houndete et al., 2023). Some unresolved questions regarding FAW resistance to pyrethroids in Puerto Rico include: How many genes are involved in resistance? Is the kdr mutation present in the Puerto Rican colony? What is the extent of cross-resistance to other pyrethroids? These research avenues could be explored through molecular biology techniques, such as genotyping field samples and sequencing known resistance regions (Boaventura et al., 2020b; Okuma et al., 2022).

In the concluding chapter of my dissertation, Chapter 4, I conducted a comprehensive analysis of the resistance inheritance patterns to spinosyns (spinosad and spinetoram) and the effectiveness of synergistic bioassays in countering this resistance. The inheritance patterns were found to be autosomal for spinosad and X-linked for spinetoram. Spinosyns, originating from the soil bacterium *Saccharopolyspora spinosa*, are renowned for their broad insecticidal efficacy and lower toxicity to non-target organisms. Through rigorous residual and topical/residual bioassays, I found that the resistance mechanisms were indeed metabolic, involving esterases, P450 enzymes, glutathione-S-transferases, and ABC transporters. This study uncovered such detoxification mechanisms in a field-evolved FAW strain, aligning with our hypothesis and contrasting with other research that found resistance primarily due to target site mutations (Sparks, 2021; Lira et al., 2020; Zuo et al., 2020b).

My research sheds light on the intricate resistance mechanisms that FAW has developed against spinosyns, highlighting the pivotal role of detoxification enzymes and specific inheritance patterns. Although I did not specifically examine mutations in the Dα6 subunit, it is possible that such mutations exist, as suggested by Sparks et al. (2012) and Lira et al. (2020) in other studies. This investigation into a resistant FAW strain from Puerto Rico uncovers a significant threat to global agricultural sustainability, particularly for essential crops like corn. Spinosyns, renowned for their minimal environmental footprint and recognized for Green Chemistry due to their disruption of key insect neural pathways, stand as pillars of sustainable pest management (Sparks, 2012). The study paves the way for future research, such as examining specific genetic markers in the Puerto Rico colony and exploring the fitness costs associated with resistance. Prior studies have documented these costs in relation to Bt proteins and xenobiotics (Kanno et al., 2021), underscoring the unique insights a field-evolved resistant population can provide over lab-selected strains due to its direct agricultural impact.

A promising future direction for scientific inquiry involves the identification and characterization of microRNAs (miRNAs) and their roles in post-transcriptional modifications that contribute to the detoxification of specific active ingredients in insect populations. Recent studies have unveiled the expression of certain miRNAs associated with pesticide resistance. For instance, research has indicated that diamide resistance in FAW is due to a complex development involving multiple detoxification genes and miRNAs in post-transcriptional regulation (Mahalle et al., 2024a). Similar studies have been conducted on other species, such as the diamondback moth (*Plutella xylostella*), in relation to chlorantraniliprole resistance (Li et al., 2015). Notably, current research on this topic highlights the potential role of miRNAs in coordinating detoxification mechanisms in response to pesticide exposure (Mahalle et al., 2024b). Recent advances have identified the insulin signaling pathway as a key player in xenobiotic detoxification in arthropods, with significant implications for pesticide resistance. Studies on *Drosophila melanogaster* revealed that targeting this pathway, particularly through proteins like PEPCK and GSK3β-PO, can significantly reduce DDT resistance. This discovery highlights the insulin signaling pathway as a potential "Achilles' heel" for combating pesticide resistance

(Zhang et al., 2021). These findings open new avenues for exploring the molecular mechanisms underlying insect resistance to pesticides and may inform the development of more effective pest management strategies.

As the sequencing and annotation of the FAW genome progresses, it will become possible to better understand the role of various detoxification processes from a molecular perspective, providing greater clarity and certainty to the results observed in lab bioassays to characterize the phenotypic responses. Despite the spread of FAW to other continents and extensive research performed in this species, there is much work to be done in characterizing the detoxification of various xenobiotics, especially in colonies that exhibit practical resistance in field conditions. The development of rapid tests (Mao et al. 2023) and the complete annotation of the FAW genome will facilitate valuable behavior estimations for designing management strategies against this pest in countries with limited resources (Banerjee et al., 2017; Wennmann Jörg et al., 2021; Abdelgaffar et al., 2021; Zhong et al., 2024).

Entomologists have long maintained that our struggle against insect pests is an enduring one, unlikely to be conclusively won. My conviction is that as we delve deeper into understanding the evolution of insect resistance to xenobiotics, each discovery will inevitably lead to solution and open new questions. The resistance evolution of FAW to insecticides in Puerto Rico demands immediate attention, not only by identifying effective strategies to curb the rate of resistance evolution, but also to heighten awareness of the factors that might foster resistance in the notorious FAW pest, known in Mexico as el *gusano cogollero*.

This research examines into the global challenges of managing insecticide resistance in the FAW, with a focus on resistance to three primary classes of insecticides: diamides, pyrethroids, and spinosyns. The scarcity of established knowledge and resources for executing extensive IRM strategies highlights the pressing need for concerted efforts to equip affected regions with the necessary tools and expertise. Such endeavors are vital for retarding the swift progression of resistance and safeguarding agricultural productivity.

Puerto Rico's unique combination of a year-round favorable climate for agriculture, supportive regulatory frameworks, and biotechnology-friendly environment has made it a prominent player in global plant breeding research. However, high pest pressures and widespread use of synthetic pest management tools in the region have resulted in the development of resistance, particularly in FAW. In response, the integration of comprehensive strategies, such as field trials, rotation programs, scouting practices, and resistance monitoring, is crucial to strengthen Integrated Pest Management IPM and IRM strategies. In addition, research on FAW mechanisms of defense and collaboration in the seed industry by having discussion meetings and implementing workshops are essential steps to refine IRM plans. While the initiation of area-wide resistance management programs may require time, their establishment is critical to address the far-reaching implications of pesticide resistance in the global food system, particularly in Puerto Rico.

Thus, this research holds global significance, providing valuable insights and methodologies that can be universally applied to address pesticide resistance in FAW. I believe that this research contributes to global food security and promotes sustainable agricultural practices.

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