The evaluation of the toxicity of *Bacillus thuringiensis* Cry proteins against reference and field populations of fall armyworm, *Spodoptera frugiperda* (J.E. Smith), in South Africa

by

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DECLARATION

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DEDICATION

This thesis is dedicated to my parents.

I am the person I want to be because of your unconditional support, love, and confidence in me.

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ABSTRACT

Fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith), is a major Western Hemisphere pest that has established and damaged maize crops in South Africa since 2016. The cultivation of *Bacillus thuringiensis* (Bt) maize that expresses insecticidal Bt Cry proteins is a control method of FAW in the Western Hemisphere and is also a control method prevalent in South Africa. However, the toxicity of purified Bt Cry proteins expressed by Bt maize for the control of FAW in South Africa has not been evaluated. Thus, the main aim of the study was to evaluate the toxicity of agronomically important Bt Cry proteins against a reference and field populations of FAW in South Africa. FAW is characterised by two genetically distinct strains which are the rice strain (RS) and the corn strain (CS). The two strains have been reported to exhibit strain-specific responses to Bt Cry proteins. Thus, all populations of FAW in the study were first genetically characterised to assess whether the susceptibilities of FAW populations to Bt Cry proteins are strain-related. The majority of FAW specimens (73%), characterised by strain-diagnostic single nucleotide polymorphisms (SNPs) in the downstream segment of the *mitochondrial cytochrome oxidase subunit I* gene (*COIB)* and the *triosephosphate isomerase* (*Tpi)* exon and intron regions, were found to be hybrids of CS and RS FAW, with variations in the *Tpi* exon and intron regions. To evaluate the toxicity of Bt Cry proteins (Cry1Ac, Cry1Ab, Cry2Ab2.820 and Cry1A.105) to first and second instar FAW larvae, the mortality responses, and growth-inhibitory responses of first and second instar FAW larvae were evaluated by droplet-feeding and diet-overlay bioassays. The toxicity of the Bt Cry proteins against a reference FAW population (V1) was compared with five field populations that were sampled 3 years later. Variation in the susceptibilities of FAW larvae to Bt Cry proteins was observed for all six populations, however, the V1 population was the least susceptible to all the Bt Cry proteins when compared to the field populations. The degree of toxicity of Bt Cry proteins also differed between the different FAW populations. The variability in the susceptibilities of FAW to Bt Cry proteins may be due to the fitness costs associated with resistance alleles, regional selection pressures due to geographical location differences, or strain-related genetic differences. Despite the variation in the susceptibilities between the FAW populations, Cry2Ab2.820 and Cry1A.105 were the most toxic to all the populations in terms of both mortality and growth inhibitory responses. These two most toxic proteins in the present study are expressed together in the MON89034 event in Bt maize. Thus, the toxicity of Cry2Ab2.820 and Cry1A.105 was further evaluated by assessing the interaction between Cry2Ab2.820 and Cry1A.105 at different ratios, using

isobolographic analysis and the calculation of the synergy factor (SF). At five different ratios of Cry2Ab2.820:Cry1A.105, four ratios (1:1, 1:2, 1:3 and 2:1) were synergistic and one ratio (3:1) was antagonistic. Thus, when expressed in Bt maize at ratios that enable synergistic interactions, the Cry2Ab2.820 and Cry1A.105 proteins would be more toxic to FAW larvae than each of the proteins alone. Overall, the toxicity of Bt Cry proteins to reference and field populations from three different provinces in South Africa revealed that FAW populations can be expected to be mildly susceptible to Cry1Ab and highly susceptible to Cry2Ab2.820 and Cry1A.105. The study sets a baseline of the toxicity of Bt Cry proteins to FAW in South Africa and provides an important foundation for future resistance screening programmes in South Africa.

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LIST OF ABBREVIATIONS

ABCA2	ATP-binding Cassette Subfamily A Member 2
ABCC2	ATP Binding Cassette Subfamily C Member 2
ALP	Alkaline phosphatase
APN	Aminopeptidase-N
BBMV	Brush border membrane vesicles
BLAST	Basic Local Alignment Search Tool
Bt	*Bacillus thuringiensis*
CA	Concentration addition
CAPS	N-cyclohexyl-3-aminopropanesulfonic acid
COA	Certificate of Analysis (COA)
COIA	*Cytochrome oxidase subunit I*
COIB	Downstream segment of *COIA*
CS	Corn strain
CABI	Centre for Agriculture and Bioscience International
CIMMYT	International Maize and Wheat Improvement Centre
Cyt	Cytolytic toxin
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EC_{50}	Median effective concentration
EDTA	Ethylenediaminetetraacetic acid

EFSA	European Food Safety Authority
EPPO	European and Mediterranean Plant Protection Organization
FAO	Food and Agriculture Organization of the United Nations
FAW	Fall armyworm
GDARD	Gauteng Department of Agriculture and Rural Development
GPI	Glycosylphosphatidyl-inositol
IC_{50}	Median inhibitory concentration
ID_{50}	Median inhibitory dose
LC_{50}	Median lethal concentration
LD_{50}	Median lethal dose
RS	Rice strain
sdH_2O	Sterile distilled water
SF	Synergy factor
Sip	Secreted insecticidal protein
SNPs	Single nucleotide polymorphisms
Tpi	*Triosephosphate isomerase*
TpiE4	Fourth exon of *Tpi*
TpiI4	Fourth intron of *Tpi*
USD	US dollar
Vip	Vegetative insecticidal proteins

Chapter 1:

General Introduction

1.1. The fall armyworm

1.1.1. Spread and impact of fall armyworm

The fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith), originates from the tropical-subtropical regions in North and South America. Outbreaks of FAW have been documented as early as 1797 (Luginbill, 1928; Sparks, 1979). While the next outbreak was only properly documented in 1856, FAW outbreaks became more regular and more devastating since then. In the years 1975, 1976 and 1977, outbreaks of FAW in the south-eastern U.S. were estimated to be between 61.2 and 31.9 million (USD), and an estimated loss of 137.5 million (USD) in Georgia during 1977 alone (Sparks, 1979). Estimations of annual yield loss caused by FAW subsequently increased to 300 and 500 million (USD)(Mitchell, 1986). Presently, in North and South America, FAW continues to be one of the most economically important pests of major agricultural crops (Overton et al., 2021).

Devastations caused by FAW amplified when the pest spread beyond the Western Hemisphere. The first reported outbreak of FAW outside of the Americas was in the western regions of Africa in the maize fields of south-western Nigeria, northern Nigeria, Benin and Togo (Goergen et al., 2016; Prasanna et al., 2018). The infestations then rapidly spread throughout several western and central African countries including SãoTomé, Príncipe, Edo and several adjacent states, Ghana and Rwanda (Cock et al., 2017; Goergen et al., 2016; Uzayisenga et al., 2018). As of February 2018, the Food and Agriculture Organization of the United Nations (FAO), had reported outbreaks of FAW throughout most of Africa, including South Africa (FAO, 2020a). A study that evaluated the impact of FAW across six maize farms in Gauteng, South Africa, in 2017 found that damage during the maize tasselling stage caused by FAW ranged between 62% and 83% in farms without refuge strategies (Bengyella et al., 2021). In Zimbabwe, smallholder maize fields estimated FAW infestation of 25 to 50% and a reduced yield of 11.57% (Baudron et al., 2019; Chimweta et al., 2020).

Similarly, FAW in Kenya and Ethiopia has been responsible for crop damage estimated at 32 to 47% of crops (Kumela et al., 2018). Damage to maize crops has been projected to equate

to yield losses of 8.3 to 20.6 million tonnes per annum, which is a loss of 2.5–6.3 billion (USD) across 12 African countries (Day et al., 2017). In addition to establishing itself in Africa, FAW has recently spread to Asia India and Oceania. FAW is now deemed a global pest where the FAO maintains a map of the worldwide spread of FAW (see http://www.fao.org/ fall-armyworm/monitoring-tools/faw-map/en/) and the European and Mediterranean Plant Protection Organization (EPPO) maintains a referenced list of the pest status of countries across the world (Figure 1). At the time of writing, FAW has invaded 47 African countries, 18 Asian countries and Oceania (Australia and surrounding islands) (see https:// gd.eppo.int/taxon/LAPHFR/distribution). At present, FAW is classified as an A1 quarantine pest by EPPO, with global trade restrictions and additional phytosanitary measures on maize stipulated by the European Food Safety Authority (EFSA) to minimise the spread of FAW into the European Union (Jeger et al., 2017; Kasoma et al., 2021).

1.1.2. Biology of fall armyworm

Fall armyworm is a highly polyphagous pest that feeds on 350 different plant species, with several being economically important crops such as maize sorghum, rice, wheat and sugar cane (FAO, 2020b, 2018; FAO and CABI, 2019; Montezano et al., 2018). The damage manifests as loss of photosynthetic area, structural damage in the whorl, lodging, impaired reproduction, and direct damage to grain (FAO, 2018). In addition to being a polyphagous pest, FAW exhibits a remarkable survival strategy that allows it to travel distances as far as 2000 km in search of warmer climates instead of undergoing diapause (FAO, 2020b, 2018; Johnson, 1987; Pair et al., 1986; Wan et al., 2021).

Fall armyworm may cause more damage than indigenous noctuids of the same genus in Africa. Unlike native armyworm species that build up a large population on wild grasses before moving onto maize plants, females of FAW directly oviposit on maize (Bhandari, 2021; Sparks, 1979; Wan et al., 2021). The larvae of FAW also possess superior mandibles which enable them to feed on tough plant material (Bhandari, 2021; Brown and Dewhurst, 1975). Moreover, the cannibalistic behaviour of older instars enables them to compete with both interspecific and intraspecific competitors. The ability of FAW to dominate amongst other similar pests has given it the status of the most important pest in several countries in the Western Hemisphere (Goergen et al., 2016).

The lifecycle of FAW typically lasts between 38 to 45 days under controlled lab conditions, where artificial diets provide all the essential nutrients for growth (Figure 2) (Schmidt-Durán

et al., 2015). In the field, the length of the FAW life cycle can vary depending on the weather (Prasanna et al., 2018). The larval and pupal stages can each last 30 days during cooler conditions (Prasanna et al., 2018). Female moths can lay up to a total of 1000 eggs, with approximately 100 to 200 eggs per egg mass, on the underside of leaves (Kumela et al., 2018; Prasanna et al., 2018; Schmidt-Durán et al., 2015). The larval stage of FAW is characterised by 6 distinct stages of development that differ significantly in size, colouration of the larvae and number of hairs per segment (Prasanna et al., 2018; Sparks, 1979). While the first two instars of the FAW feed on one side of the leaves, later instars may feed on most plants' parts causing considerable damage (Kumela et al., 2018). FAW can be distinguished from the closely resembled African armyworm *Mythimna unipuncta* (Haworth) and corn earworm *Helicoverpa zea* (Boddie) by a white coloured inverted "Y" on the front of a dark brown head capsule and four dark spots arranged in a square on the top of the eight abdominal segment (Figure 3) (Matova et al., 2020; Prasanna et al., 2018).

1.1.3. Fall armyworm strains

There are two known and accepted strains of FAW, which for simplicity have been distinguished by the preferred diet of each strain. The rice strain (RS) feeds predominantly on rice and various grasses, while the corn strain (CS) feeds predominantly on maize, sorghum and cotton (FAO and CABI, 2019; Montezano et al., 2018; Pashley, 1986). The two strains are morphologically identical. Additional to the difference in host range, the two strains also differ in mating behaviours and pheromone chemistry which contribute to reproductive isolation mechanisms (Groot et al., 2010; Schöfl et al., 2009; Unbehend et al., 2014, 2013; Velásquez-Vález et al., 2011). Most importantly, there are inherent genetic differences that likely arose from reproductive isolation between the two strains, rather than due to host specificity (Pashley, 1986). Thus, the only method of exact strain identification is through molecular techniques (Cock et al., 2017; Goergen et al., 2016; Hajibabaei et al., 2006; Nagoshi et al., 2011).

DNA barcoding can be used to identify lepidopteran pests on a species level. In DNA barcoding, a conserved region of 600-900 bp in the *cytochrome oxidase subunit I* (*COI*) region is used where barcode regions are substantially less variable within specimens of the same species (Hajibabaei et al., 2006; Hebert et al., 2004; Nagoshi et al., 2011). This technique was used to confirm the first outbreak of FAW in Africa in 2016 (Goergen et al., 2016).

Strain classification of FAW relies on single base nucleotide polymorphisms (SNPs) in the lower region of the *COI* segment as well as the fourth exon (TpiE4) and intron (TpiI4) of the *triosephosphate isomerase* (*Tpi*) gene (Murúa et al., 2015; Nagoshi, 2010). An initial investigation into a population of CS FAW from Togo revealed that the haplotype patterns of the Togo CS population most resembled that of the FL-haplotype (Nagoshi et al., 2017). Further studies into the CS FAW specimens from São Tomé and Príncipe, Burundi, Democratic Republic of Congo, Tanzania, and Kenya revealed that the African CS FAW specimens have very little genetic variability which points to the likelihood of a single origin by a small invasive population from Florida (Nagoshi et al., 2018).

SNP sites in the *COI* gene are conventionally termed with an "m" (mitochondria) while SNP sites in the *Tpi* are termed "g" (genomic). This is followed by the gene names and the number of base pairs a SNP is from the *COI* predicted translational start site, 5' start of TpiE4 exon or the 5' start of TpiI4, and the nucleotides that distinguish RS and CS in the Western Hemisphere using the IUPAC convention (R: A or G; Y: C or T; W: A or T; K: G or T; S: C or G; D: A or G or T) (Nagoshi et al., 2021, 2019b). For example, mCOI1164D indicates a site in the mitochondrial *COIB* region where the nucleotide base that is 1164 bases from the *COIB* start site will be a D (A or G or T). An example of a *Tpi* SNP is gTpi183Y, which indicates a site in the genomic *Tpi* gene where the nucleotide base 183 bases from the 5' start of TpiE4 will be either a C or a T.

In the *COIB* region, the site mCOI1164 defines *COI*-based strain identity (Figure 4). mCOI1164T indicates *COI*-RS while mCOI1664R indicates *COI*-CS in the Western Hemisphere. *COI*-CS FAW is further characterized by four haplotypes in the Western Hemisphere based on SNPs at mCOI1164D and mCOI1287R (Nagoshi et al., 2007). Nucleotide bases AA at sites mCOI1164D and mCOI1287R (denoted as $A_{1164}A_{1287}$), are characteristic of the *COI*-CS haplotype *COI*-CSh1. Accordingly, $A_{1164}G_{1287}$ represents *COI*-CSh2, $G_{1164}A_{1287}$ represents *COI*-CSh3 and $G_{1164}G_{1287}$ represents *COI*-CSh4 (Nagoshi et al., 2019a, 2018, 2017). Moreover, haplotypes can be indicative of specific regions due to FAW populations having been found to contain SNPs at region-specific frequencies. The migration of FAW populations has been tracked using this knowledge (Nagoshi et al., 2009, 2008; Nagoshi and Meagher, 2008). For example, *COI*-CSh2 is also called the TX-type because it predominates in Texas and South America, while *COI*-CSh4 predominates in Florida and is also known as the FL-type (Nagoshi et al., 2008, 2007).

However, unlike in the Western Hemisphere, *COI*-RS in Africa is further characterized into four haplotypes (*COI*-RSa1, *COI*-Rsa2, *COI*-Rsa3 and *COI*-Rsa4) and *COI*-CS is further divided into *COI*-Csa1 and *COI*-Csa2 based on SNPs at mCOI1122, mCOI1125, mCOI1215, and mCOI1287 (Figure 4) (Nagoshi et al., 2019b).

The site gTpi183 in TpiE4 defines *Tpi*-based strain identity (Figure 5A). gTpi183T indicates *Tpi*R while gTpi183C indicates *Tpi*C. The *Tpi* gene is located on the Z sex chromosome where females possess one copy while males possess two copies. The two copies of *Tpi* give rise to the possibility of two different nucleotides overlapping at one site. Visible overlapping of C and T at gTpi183Y on a DNA sequence chromatograph is indicative of a new haplotype observed in Africa, deemed *Tpi*H (Nagoshi et al., 2019b). Thus, in Africa, three haplotypes (*TpiR*, *Tpi*C and *Tpi*H) are present (Figure 5A). Additional to gTpi183, seven other sites gTpi129, gTpi144, gTpi168, gTpi180, gTpi192 and gTpi198 distinguish *Tpi*R into one African haplotype (*Tpi*Ra1), *Tpi*C into three African haplotypes (*Tpi*Ca1, *Tpi*Ca2 and *Tpi*Ca1/Ca2) and two *Tpi*H haplotypes (*Tpi*HCa1/Ra1 and *Tpi*HCa2/Ra1) (Figure 5A) (Nagoshi et al., 2019b). Furthermore, SNPs in TpiI4 (between sites 10 and 172 from the start of TpiI4) distinguish TpiRa1 into one haplotype (TpiI4Ra1a), TpiCa1 into two haplotypes (TpiI4Ca1a or TpiI4Ca1a) and TpiCas2 into three haplotypes (TpiI4Ca2a, TpiI4Ca2b and TpiI4Ca2c) (Figure 5B) (Nagoshi et al., 2019b).

1.1.4. Fall armyworm control strategies in South Africa

At the start of the outbreaks, the Gauteng Department of Agriculture and Rural Development (GDARD) promptly established a set of accessible guides on their website for the control of FAW in South Africa. The guides encourage early detection by early scouting. As of February 2017, 29 chemical pesticides had been registered and approved in terms of the Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act, 1947 (Act No. 36 of 1947) (GDARD). The FAO contributed USD 13 million to try to control the FAW invasion in Africa (FAO, 2018).

The FAO, Agricultural Research Council (ARC), the Centre for Agriculture and Bioscience International (CABI) and the International Maize and Wheat Improvement Center (CIMMYT) are all working together to develop the most efficient Integrated Pest Management Strategy (IPM) for FAW in Africa. The latest three-year Global Action for Fall Armyworm Control plan is aimed at mobilizing USD 500 million over 2020–22 to take radical, direct and coordinated measures to strengthen prevention and sustainable pest control

capacities at a global level. Current strategies are aimed at monitoring, surveillance and scouting FAW in infested regions, reinforcing efforts to discourage the widespread use of highly hazardous chemical pesticides, evaluating host plant resistance, evaluating biological control strategies and pesticides and establishing cost-effective agronomic practices and landscape management approaches (FAO, 2020, 2018; FAO and CABI, 2019; Padhee and Prasanna, 2019).

1.2. *Bacillus thuringiensis*

1.2.1. Mode of action of *Bacillus thuringiensis*

Bacillus thuringiensis is a gram-positive, non-capsulated bacterium with peritrichous flagella allowing it to be mobile. Bt exhibits two different phases during the life cycle (Ibrahim et al., 2010; Palma et al., 2014). The vegetative stage is the stage during which vegetative cells divide into uniform daughter cells through the formation of a septum in the middle of the cell membrane (Ibrahim et al., 2010). In contrast, sporulation is a dormant stage that is further characterised into seven stages where stage three of sporulation is the most significant in terms of toxicity as this is the stage during which parasporal crystals, known as Cry proteins, start to form (Ibrahim et al., 2010).

Different Bt strains are lethal against Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera, Orthoptera, Mallophaga, nematodes, mites and protozoa (Bravo et al., 2011; Pardo-López et al., 2013). Insecticidal Cry proteins have been exploited for use in bioinsecticides, especially against lepidopteran species. Based on primary amino acid sequence, Cry proteins are classified into approximately 74 subgroups and 780 members of Cry proteins within these subgroups (Crickmore et al., 1998). More recently, it has been proposed that Bt pesticidal proteins should be classified based on structure, where only Bt pesticidal proteins that consist of 3 domains can be classified as Cry proteins (Crickmore et al., 2021).

The mode of action between Cry proteins of the three-domain family is similar and has been widely accepted. Cry proteins of the three-domain family exhibit insect specificity which in turn is determined by the specific binding of the Cry proteins to the surface proteins of the microvilli of specific larval midgut epithelial cells, such as cadherin-like receptor (CAD), glycosylphosphatidyl-inositol (GPI)-anchored aminopeptidase-N (APN), GPI-anchored alkaline phosphatase (ALP), a 270 kD glycoconjugate and a 250 kDa P252 protein (Bravo et al., 2011; Heckel, 2020; Melo et al., 2016; Pardo-López et al., 2013). Each of the three domains plays a role in the mode of action of the Cry protein. Domain I consists of a 7-α-helix bundle that is responsible for membrane insertion and pore-formation in the insect midgut through an oligomeric structure while domains II and III are composed mainly of β-sheets which facilitate receptor-related interactions (Bravo et al., 2007; Sanahuja et al., 2011; Soberón et al., 2009; C. Wang et al., 2018).

After sporulation, a mature mother cell would release the spore and parasporal crystals as a spore-crystal complex. The spore-crystal complex is ingested by larvae from contaminated vegetation and due to the small size of the complex, it moves through to the midgut (Bravo et al., 2007, 2004; Melo et al., 2016; Soberon et al., 2007; Soberón et al., 2009; Vachon et al., 2012). The exact mode of action of Cry proteins after ingestion is still under debate between two proposed models: the sequential binding (classical) model, which focuses on the sequential binding of Cry proteins to insect midgut receptors which eventually leads to an increase in midgut membrane permeability through pore formation, and the signalling pathway model, which focuses on signalling pathways that result in necrotic cell death independently of midgut membrane pore-formation (Bravo et al., 2007, 2004; Melo et al., 2016; Soberon et al., 2007; Soberón et al., 2009; Vachon et al., 2012).

In the classical, most accepted, sequential binding model, the ingested Cry proteins are protoxins that require several structural changes to become toxic. The protoxins that are ingested by insect larvae are first solubilised due to the high pH of the larval midgut (Bravo et al., 2007; Schnepf et al., 1998). Protoxins such as Cry2 proteins are short, at approximately 60 kDa and are activated by the cleavage of around 40 amino acids at the N-terminal by proteases. Protoxins such as Cry1 proteins are longer, at approximately 130 kDa. The longer protoxins are activated by the cleavage of approximately 500 amino acids from the C-terminal end in addition to the removal of the 40 amino acids from the N-terminal end (Bravo et al., 2007; de Maagd et al., 2001; Heckel, 2020; Pardo-López et al., 2013; Soberón et al., 2016). The activated toxin is resistant to further action of proteases and is responsible for the toxicity of Cry proteins via membrane insertion and pore formation which results in increased permeability of the midgut membrane (Bravo et al., 2011; Heckel, 2020; Soberón et al., 2009).

The C-terminal that is cleaved off of the protoxin has long been thought to play no significant role in the toxicity of Cry proteins and that its only role is to facilitate a conformational change of the protoxin into an active protein (Heckel, 2020; Sanahuja et al., 2011; Soberón et al., 2009). However, it has recently been found that after cleavage, what is left of the C-terminal assembles into distinct structural domains which not only resemble carbohydrate-binding modules but to domains II and III of the activated protein as well, which are responsible for insect midgut receptor-binding (Evdokimov et al., 2014; Peña-Cardeña et al., 2018). The C-terminal domain of inactive Cry1Ab protoxin has been found to bind to ALP

and APN at a higher affinity than activated toxin (Peña-Cardeña et al., 2018). The higher affinity binding was also directly correlated to the protoxin showing higher toxicity than activated Cry1Ab (Peña-Cardeña et al., 2018).

In some cases, protoxins have even been found to cause toxicity to insects that were resistant to the activated toxin (Peña-Cardeña et al., 2018; Soberón et al., 2016). A proposed mechanism involves the C-terminal region of the protoxin binding to APN and ALP at high abundance, thereby enabling the protoxin to aggregate close to CAD receptors before proteolytic cleavage. Thereafter, the protoxin binds to CAD where the C-terminal is finally cleaved, enabling the formation of a robust oligomeric structure that inserts into the membrane forming a pore that increases midgut membrane permeability (Peña-Cardeña et al., 2018). Thus, it has also been proposed that Cry1 proteins may exhibit a dual mode of action where both protoxins and active toxins may result in toxicity independently of each other (Soberón et al., 2016).

Transgenic crops often express only activated Cry toxins as to not rely on plant proteases to activate protoxins into their active form. Thus, the dual mode of action implies that transgenic crops may be lacking the extra spectrum of activity offered by gene expression of the full-length protoxin Cry gene (Soberón et al., 2016). Nevertheless, the now activated monomeric Cry protein first either binds directly to CAD at high affinity or low affinity to APN or ALP (Gómez et al., 2002; Pacheco et al., 2009b, 2009a; Pardo-López et al., 2013; Pigott and Ellar, 2007). The binding of three exposed loops of domain II of the activated toxin to three CAD epitopes causes a conformation change that results in the exposure and subsequent cleavage of a hydrophobic region of domain I that contains α1 helix (Bravo et al., 2007, 2004; Gómez et al., 2002; Soberon et al., 2007).

While insect resistance to Cry proteins has been associated with mutations in either APN and CAD or individual receptors, the binding of the activated toxin to CAD has also been shown to not be necessary for the toxicity of Cry proteins (Vachon et al., 2012). Nevertheless, the cleavage of the α1 helix favours the formation of a pre-pore oligomeric structure from the aggregation of monomeric activated toxins (Bravo et al., 2007, 2004; Gómez et al., 2002). Thereafter, this oligomeric protein binds to APN or ALP at high affinity and is eventually inserted into the insect midgut membrane thereby increasing midgut permeability leading to osmotic shock and eventual cell lysis which has been termed “colloid-osmotic lysis” (Knowles and Ellar, 1987). It has also been suggested that pore-formation may actually occur

after the insertion of the oligomeric structure into the membrane and that the oligomeric structure is not essential to membrane insertion since monomeric toxins have been demonstrated to be able to insert into the membrane causing toxicity as well (Vachon et al., 2012). Nevertheless, pore-formation in the insect midgut is paramount to the mode of action of Cry proteins in the sequential binding model.

In contrast, the signalling pathways model suggests that the binding of Cry proteins to CAD receptors is crucial as it activates magnesium-dependent and adenylyl cyclase/protein kinases signalling pathways that lead to necrotic cell death (Zhang et al., 2006, 2005). The major difference between the signalling pathway model and the classical sequential binding model is that the signalling pathways will lead to cell death irrespective of the formation of oligomeric toxin structures, pore formation or membrane insertion (Zhang et al., 2006, 2005). While most of the evidence of the signalling pathways model remains inconclusive, it should not be dismissed. It is widely known that the binding of proteins to membrane receptors triggers many intercellular and intracellular signalling pathways (Soberón et al., 2009; Vachon et al., 2012). The mode of action of Cry proteins is therefore more than likely, an intricate combination of both proposed models.

The mode of action of Cry1 and Cry2 proteins may differ in terms of binding receptors in the brush border membrane vesicles (BBMV) of the insect midgut epithelium. While Cry1 toxins such as Cry1A.105, Cry1Ab, Cry1Ac and Cry1Fa compete with each other for the same BBMV binding sites, Cry2Ab and Cry2Ae do not compete with Cry1 binding sites (Hernández-Rodríguez et al., 2013; Soberón et al., 2016). Moreover, while Cry1Ab relies on the integral membrane protein ATP Binding Cassette Subfamily C Member 2 (ABCC2) to facilitate pore-insertion, Cry2Ab relies on ATP Binding Cassette Subfamily A Member 2 (ABCA2) (Tay et al., 2015).

1.2.2. Control of lepidopteran pests with Bt Cry proteins

The control of FAW is traditionally through the spraying of chemical pesticides; however, the larvae often escape the effects of the pesticides due to burrowing within the inner whorls of maize plants (Carvalho et al., 2013). Furthermore, FAW populations have been found to be resistant to many insecticides from classes such as organophosphates, carbamates, pyrethroids and benzoylureas (Carvalho et al., 2013; Diez-Rodríguez and Omoto, 2001; Yu, 1992, 1991). Microbial based pesticides using Cry proteins have been in use since 1961, however, again, the spray application of pesticides exhibited highly limited efficiency as the

Fall armyworm does not feed on the leaves for the entire duration of the life cycle, but rather prefers to move inwards and into the ears of the corn (Betz et al., 2000; Castagnola and Jurat-Fuentes, 2012; Sanahuja et al., 2011).

Transgenic crops expressing Bt Cry proteins, also known as Bt crops, have been cultivated since the 1980s and continue to be the preferred method of controlling lepidopteran pests such as the FAW due to its non-toxic effect on humans and animals (Betz et al., 2000; Blanco et al., 2016; Shelton et al., 2002). USA, Brazil and Argentina, where FAW is native, are consistently leading in transgenic maize cultivation, with 71.5 million hectares, 52.8 million hectares and 24 million hectares of transgenic maize planted in 2019 respectively (ISAAA, 2019). South Africa ranks eighth in the world in terms of the adoption of transgenic crops, where 2.7 million hectares were planted in 2018 and 2019 (ISAAA, 2018, 2019). Out of the 2.7 million hectares of transgenic crops, transgenic maize accounted for approximately 2 million hectares, which were dominated by Bt maize expressing Bt insecticidal proteins (ISAAA, 2019, 2018).

Additionally, since Bt Cry proteins are only activated upon proteolysis of the protoxin in the insect midgut, Bt Cry proteins do not persist in the environment like other spray-type pesticides (Betz et al., 2000; Delaney et al., 2008; C. Wang et al., 2018). Bt Cry proteins are highly specific to certain insects and by engineering specific Cry genes into maize, the specificity of Bt Cry proteins is further increased to only target economically important lepidopteran pests (Delaney et al., 2008; C. Wang et al., 2018).

Due to the potential of Bt toxins for use as a pest control agent, many studies have been conducted to determine the specificity of Bt Cry proteins against the agronomically important *Spodoptera* spp. (Figure 6). Cry proteins Cry1Bb, Cry1Ca, Cry1Da and Cry1Fa have been demonstrated to be highly toxic toward FAW, while Cry1Ab and Cry2Aa have been demonstrated to be moderately toxic against FAW (Figure 6). Moreover, the susceptibility of FAW to Cry2Ab is highly variable (Bernardi et al., 2015; Gilreath et al., 2021; Gutierrez-Moreno et al., 2020; Hernández-Rodríguez et al., 2013; Soares Figueiredo et al., 2019). In the Western Hemisphere, Bt crops expressing Cry1Ab, Cry1F, Cry2Ab2 and Cry1A.105 proteins are the predominant crops planted across the United States to control FAW (Hardke et al., 2011; Hernández-Rodríguez et al., 2013).

The most widely cultivated first-generation Bt maize and Bt cotton encoded single events for the expression of Cry1Ab, Cry1Ac and Cry1F respectively (De Buck et al., 2016; Huang, 2020). However, the resistance of pests to Bt Crops expressing single Bt Cry proteins soon emerged. Resistance is defined as a genetically based decrease in susceptibility to a pesticide and can be expanded to the term "field-evolved resistance" which is defined as the resistance to a pesticide caused by exposure to the pesticide in the field (Tabashnik et al., 2014; Tabashnik and Carrière, 2017). This definition includes the possibility of selection in the field by one toxin that causes cross-resistance to another toxin. Furthermore, Tabashnik and Carrière, 2017, classified cases of field resistance to Bt Cry proteins as "practical resistance" if field-evolved resistance that reduces the efficacy of the Bt crop has practical consequences for pest control.

Practical resistance of FAW in Brazil and Puerto Rico against Bt crops expressing Cry1F and Cry1Ab has been documented between 2 to 4 years after the first cultivation of Bt crops (Farias et al., 2014a; Huang et al., 2014; Monnerat et al., 2015; Omoto et al., 2016; Storer et al., 2010). Cases of practical resistance to Bt crops are continuously reported and have been documented for several other lepidopteran pests across numerous countries as well, with cases averaging about 5 years from the time of first cultivation. Practical resistance of *Busseola fusca*, the African maize stalk borer, to Bt crop expressing Cry1Ab was first reported 8 years after Bt crop adoption in South Africa (Kruger et al., 2009). In Argentina, *Diatraea saccharalis,* the sugarcane stalk borer, exhibited practical resistance to Cry1F and Cry1A.105 in 4 years (Grimi et al., 2018). Although resistance development took longer than other cases where practical resistance developed, the western bean cutworm, *Striacosta albicosta*, developed practical resistance 10 years after the cultivation of Bt crops expressing Cry1F (Eichenseer et al., 2008; Ostrem et al., 2016).

Evidently, the development of practical resistance to Bt crops is a global dilemma, especially in the biocontrol of major lepidopteran pests. However, numerous studies have indicated toxicity of Bt Cry proteins towards FAW and new Bt Cry proteins continue to be screened for their potential (Baranek et al., 2020; Crickmore et al., 2021, 1998; Herrero et al., 2016). After the emergence of resistance to these single events, pyramided traits, which enable the expression of two different Bt Cry proteins, became commercialised (Figure 7) (Hernández-Rodríguez et al., 2013; Huang, 2020; ISAAA, 2019; Storer et al., 2012).

Presently there are several commercially available Bt maize that contains the MON 89034 event, which encodes Cry1A.105 as well as Cry2Ab2 (Figure 7) (Drury et al., 2008). Cry1A.105 is a chimeric protein that contains domains I and II from either Cry1Ab or Cry1Ac (domains I and II of Cry1Ab and Cry1Ac share 100% amino acid sequence identity), domain III from Cry1F and a portion of the C-terminal region of Cry1Ac (C. Wang et al., 2018). There is also Bt cotton with pyramided events such as MON15985 enabling the expression of both Cry1Ac and Cry2Ab which have been found to be more toxic towards *Helicoverpa zea*, FAW and *Spodoptera exigua* compared to single toxin Bt cotton (Figure 7) (Arpaia et al., 2014; Stewart et al., 2001). Events such as MON 89034 and MON15895 are crucial in the delay of the development of insect resistance towards Cry proteins because cross-resistance between Cry1 and Cry2 proteins is highly unlikely due to the two proteins sharing different binding sites in the insect midgut (Bernardi et al., 2015; Flagel et al., 2018a; Huang et al., 2016; Niu et al., 2016).

To further contribute to and implement resistance management of FAW larvae, such as pyramided Bt maize, it is essential to evaluate whether Bt Cry proteins interact synergistically, additively, or antagonistically and to ascertain which proportions provide optimal protection against the development of resistance in lepidopteran pests. This is especially because additive, synergistic and antagonistic interactions between different Cry1 proteins have been reported for *Helicoverpa armigera, Earias insulana* and *Chilo partellus* (Chakrabarti et al., 1998; Ibargutxi et al., 2008; Sharma et al., 2010). Additionally, in different lepidopterans, Bt Cry proteins interact with other Bt insecticidal proteins such as vegetative insecticidal proteins (Vip) and Cytolytic (Cyt) toxins synergistically or antagonistically depending on the proportions in the mixtures (Bergamasco et al., 2013; Lemes et al., 2014; Pérez et al., 2005; Z. Wang et al., 2018). Combinations of different strains of Bt have also been reported to interact additively or synergistically in mosquitoes *Aedes aegypti* and *Culex quinquefasciatus* (Sreshty et al., 2011; Wirth et al., 2004).

1.3. Justification of the study

FAW, the most devastating maize pest of the Americas has recently established itself as an economically important pest in Africa, including South Africa (Cock et al., 2017; FAO, 2018; Goergen et al., 2016). In Africa, where maize is the most widely grown crop which accounts for a large portion of dietary calories and proteins for almost 200 million people, it is estimated that the damage from FAW to African maize production could cause a loss of 8 to 20 million metric tonnes of maize annually (Day et al., 2017). Monetary value aside, the loss of maize due to FAW is equivalent to food for 40 to 100 million people (FAO, 2020).

Left unchecked, FAW is a damaging pest that will cause direct economic damage due to the reduction of food crop yield. Indirect impacts such as reduced food and income (potentially increasing food insecurity, malnutrition, and poverty) have also arisen (FAO, 2020b, 2020c). Furthermore, the rapid spread and ability of FAW to cause crop damage, increasing food insecurity and rural poverty are also likely to increase human migration to cities from rural agrarian communities (Matova et al., 2020). The increased migration will put further pressure on food insecurity, poverty, and the socio-economic systems in South Africa (FAO, 2020a, 2020b).

The initial rapid response to FAW infestations in Africa was primarily the use of chemical insecticides, however, intervention by chemical insecticides is not yielding successful control of FAW in Africa (Chimweta et al., 2020; Kumela et al., 2018; van den Berg et al., 2021). Furthermore, long term control and permanent control of FAW with chemical insecticides are not feasible due to the health and environmental risks it imposes (Blair et al., 2015; FAO, 2018; Hua and Relyea, 2014). There is also the threat that the limited amount of effective chemical insecticides and the use of sub-effective insecticides may put selective pressure on the pests and speed up the development of resistance to insecticides. In fact, FAW has already been reported to be resistant to several insecticides in the Western Hemisphere (Diez-Rodrigues and Omoto, 2001; Yu, 1991, Yu, 1992).

In contrast, biocontrol using Bt insect-specific insecticidal Cry proteins have for years proven to be effective as a sustainable pest biocontrol strategy of FAW in the Western Hemisphere. As seen in Figure 7, globally, Bt maize and Bt cotton express Cry1Ab, Cry1Ac, Cry1A.105 and Cry2Ab2, either as single toxins or as pyramided traits, have remained commercially important Bt crops for the biocontrol of lepidopteran pest. Similarly, in South Africa two

types of Bt crops have been cultivated since 1998 to control local lepidopteran pests: Bt cotton which expresses Cry1Ac and Bt maize, with either a transgenic event expressing Cry1Ab or stacked transgenic events expressing Cry2Ab2.820 and Cry1A.105 (Gouse et al., 2005; ISAAA, 2019, 2018). Recently, the efficacy of Bt maize expressing single-toxin Cry1Ab and pyramided Bt maize expressing Cry1A.105 and Cry2Ab2 has been evaluated in South Africa by leaf bioassays (Botha et al., 2019). Botha et al. (2019) reported moderate FAW survival on Bt maize expressing Cry1Ab and low survival on Bt maize expressing Cry1A.105 + Cry2Ab2. However, to the best of our knowledge, toxicity studies of Bt Cry proteins that report mortality and growth-inhibitory responses of FAW based on purified Bt Cry proteins have not been evaluated in South Africa. Moreover, Bt Cry proteins that are effective in the control of FAW in the Western Hemisphere may not be effective in South Africa as the susceptibility of pests to Cry proteins depend on the type of Cry protein and the pest species. The susceptibility will even vary between different populations of the same species, especially if the FAW strains differ (Da Silva et al., 2016; Gutierrez-Moreno, 2017). Furthermore, evidence of resistance in the African stem borer, *B. fusca* (Fuller), in 2007, serves as a warning that resistance to Bt Cry proteins can develop soon after the implementation of Bt crops thereby highlighting the importance of generating proper toxicity data for field populations of FAW (van Rensburg, 2007).

Information on the toxicity of Bt Cry proteins commercially available as Bt crops in South Africa is crucial as it will indicate the suitability of readily available Bt crops for the long-term control of FAW in South Africa. Thus, the overall aim of the study was to evaluate the toxicity of Bt Cry proteins (Cry1Ab, Cry1Ac, Cry2Ab2.820 and Cry1A.105) against reference and field populations of FAW in South Africa. The toxicity of Bt Cry proteins evaluated can be referred to in future studies as a baseline indication of the toxicity of Bt Cry proteins against FAW. Moreover, the study evaluated synergistic interactions between Cry1A.105 and Cry2Ab2.820 in FAW larvae which offered insight into whether the ratio of Cry2Ab2.820 and Cry1A.105 that are expressed in combination by Bt maize, plays a role in the degree of toxicity in current pyramided Bt maize. The study in its entirety should provide novel information that would contribute to long term, effective FAW management programmes in South Africa.

1.4. Aims and objectives

1.4.1. Research aim

The main research aim of the study was to evaluate the toxicity of agronomically important Bt Cry proteins against reference and field populations of FAW, in South Africa.

1.4.2. Research objectives

The main research aim was achieved through the following objectives:

1. To genetically characterise five populations of FAW from South Africa, into strain-specific haplotypes.

2. To evaluate the compatibility of the droplet-feeding method for the bioassay of four agronomically important Bt Cry proteins in highly alkaline buffers against FAW.

3. To compare the toxicity of four agronomically important Bt Cry proteins against reference and field populations of FAW in South Africa.

4. To evaluate the synergistic interactions between Cry2Ab2.820 and Cry1A.105 in FAW.

1.5. Thesis layout

For ease of location and to keep the paragraphs continuous, all tables and figures relating to a chapter are placed at the end of the chapter. Chapter 1, the Literature Review, offers in-depth and crucial insight into the FAW pest as well as the four Bt Cry proteins of interest. The history, current distribution, damage caused and biology of the FAW are reviewed. The mode of action and relevance of Bt Cry proteins in South Africa to be evaluated in the study are discussed. The main aim and research objectives are listed followed by a justification of the research.

Chapters 2 to 5 report on the four research objectives of the study in the format of a scientific manuscript with the following sections: Abstract, Introduction, Methods and Materials, Results, and Discussion. As the research chapters are written in the format of standard scientific papers, some repetition is unavoidable. To aid the reader, abbreviations will also be reintroduced in each chapter. Chapters 2 to 5 are written in a sequential manner where each chapter logically follows on and expands on the previous chapters. Chapter 2 investigated the genetic characterisation of reference and field populations of FAW in South Africa. The strain-diagnostic characterisation of the FAW populations aided in the interpretation of subsequent studies and offered a basis on which to formulate observed findings into sound explanations and conclusions. Thereafter, in the subsequent chapters, the susceptibilities of a reference and field populations of FAW in South Africa to Bt Cry proteins were explored and compared. The last chapter of the thesis, Chapter 6, is the general discussion and conclusion of the thesis where the findings of all the project objectives are discussed in-depth and the final conclusions are drawn.

Figure 1: Map of the worldwide spread of FAW as of May 2021. This map was compiled using information from a range of sources, including FAO, International Plant Protection Convention, CABI, the EPPO, and national governments (https://www.fao.org/fall-armyworm/monitoring-tools/faw-map/en/)

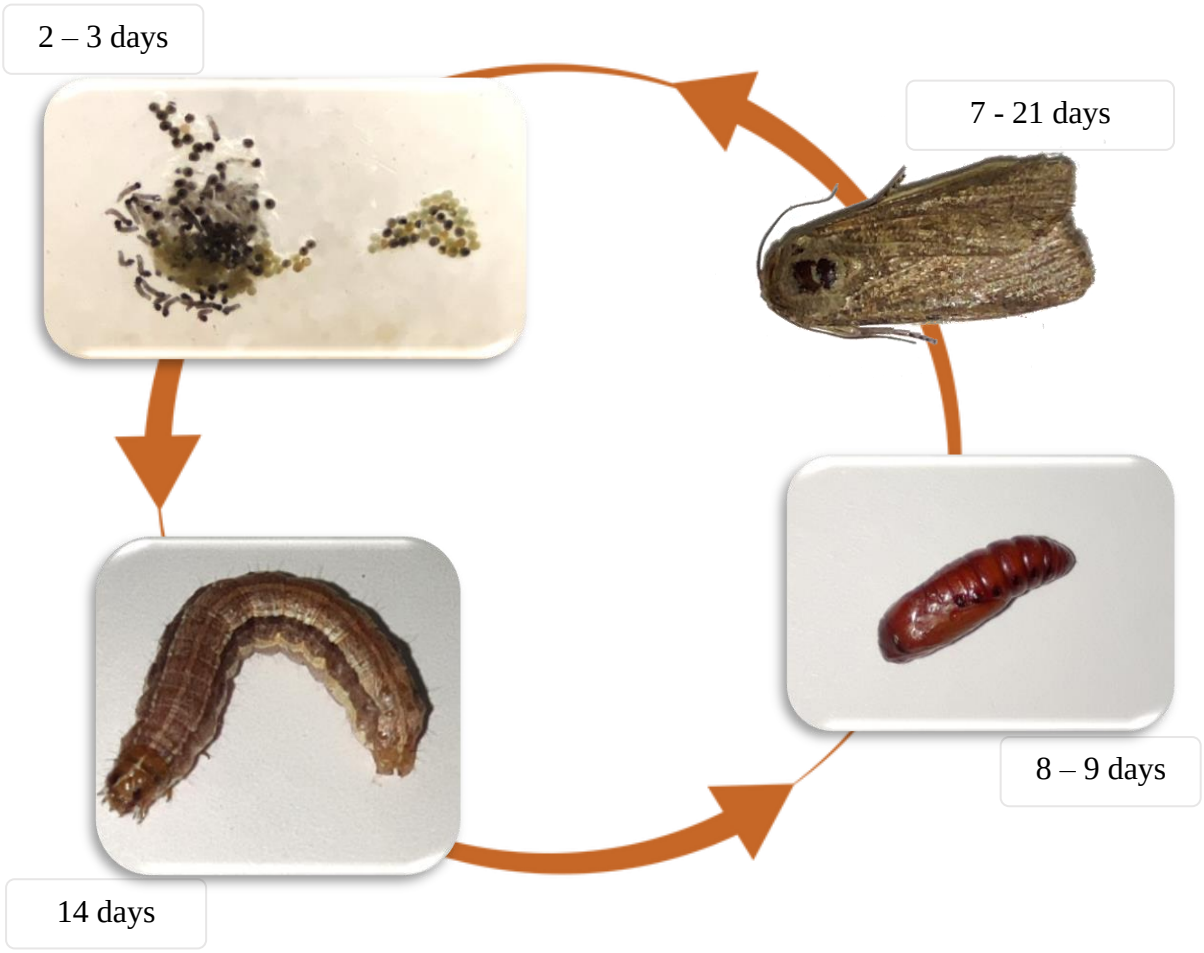


Figure 2: Life cycle of FAW. Duration of the FAW egg stage is 2 to 3 days. Thereafter, larvae hatch and remain in the larval stage for approximately 14 days until pupation. The pupal stage lasts about 8 to 9 days before the adult moths emerge. Adult life of FAW typically lasts between 7 to 21 days.

Figure 3: Third instar FAW larvae photographed and measured using the Zeiss SteREO Discovery.V12 stereomicroscope 10 days after hatching. The images show the characteristic white inverted "Y" on the head capsule and four dark spots on the eighth abdominal segment.

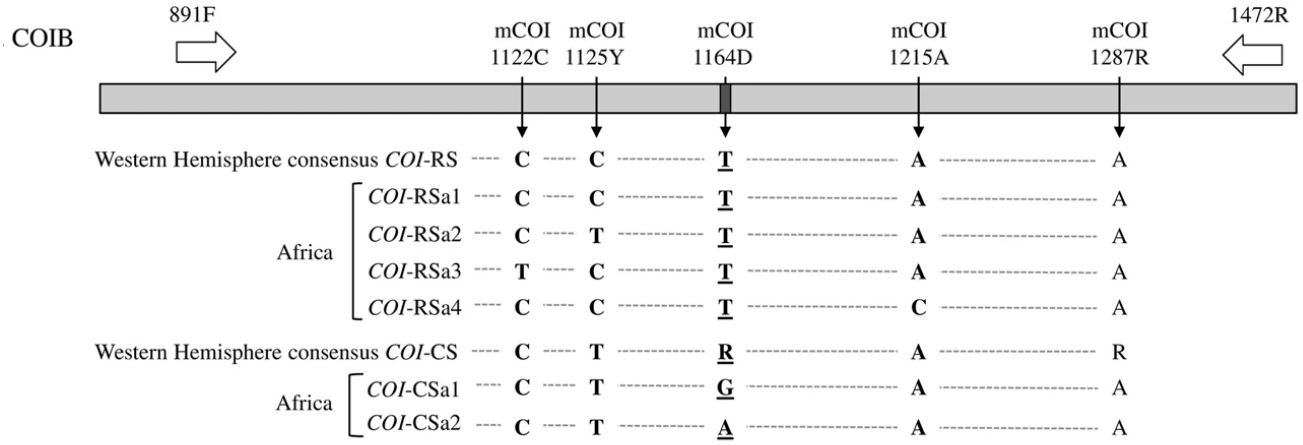


Figure 4: Diagram of the *COIB* gene segment with respect to consensus Western Hemisphere sequences and the six haplotypes (four *COI*-RS haplotypes and two *COI*-CS haplotypes) observed in Africa. The mCOI1164D site, in bold, defines *COIB*-based strain identity. Other polymorphisms define haplotypes.

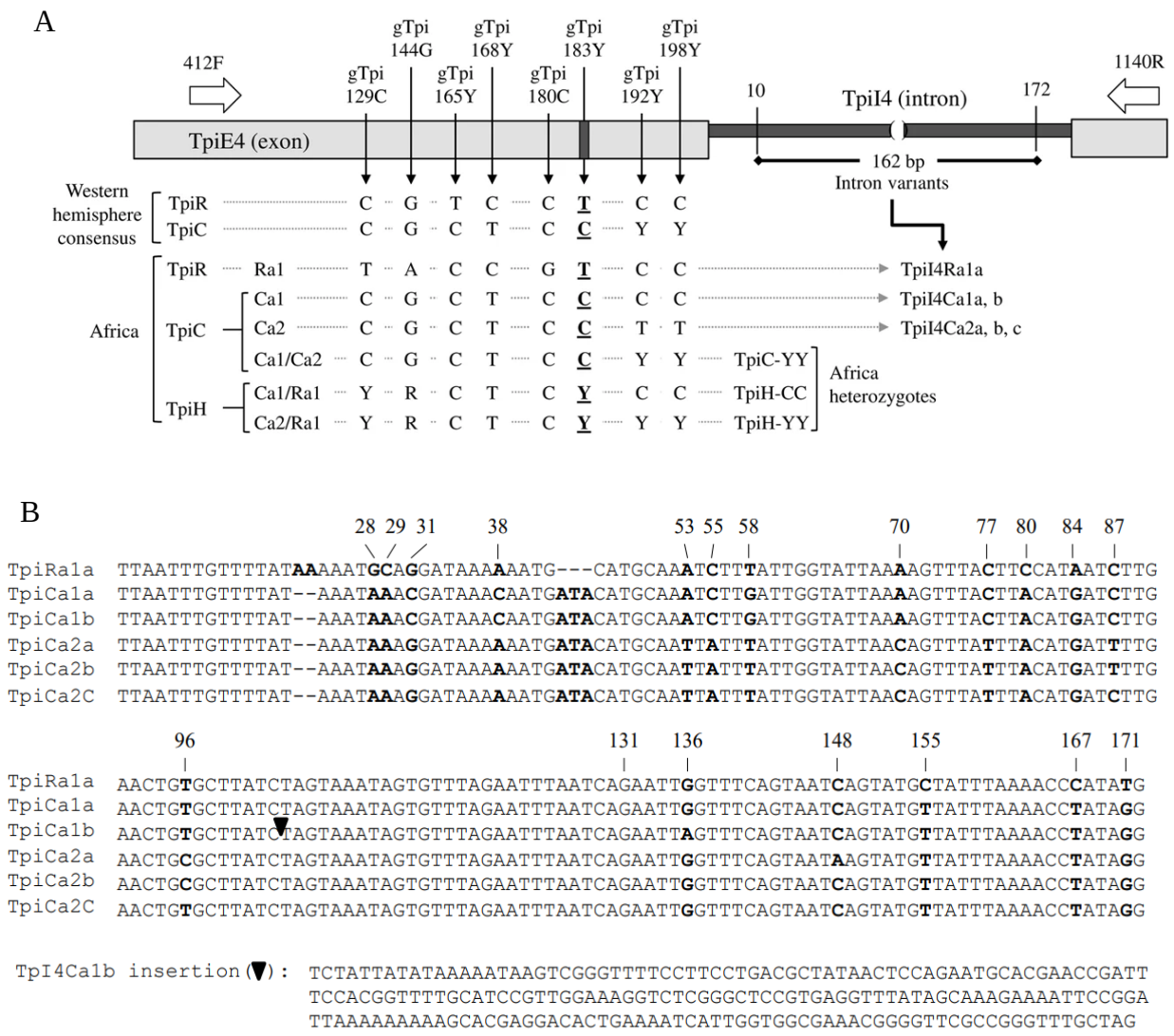


Figure 5: Diagrams of the *Tpi* exon and intron segments with respect to consensus Western Hemisphere sequences and the haplotypes observed in Africa. **(A)** The gTpi183Y site defines *Tpi*-based strain identity. Seven polymorphic exon sites differentiate the consensus strain-specific Western Hemisphere sequences and the three TpiE4 haplotypes from Africa that can be further subdivided by sequence variations in the 162-bp TpiI4 intron segment. **(B)** 162-bp TpiI4 intron segment with sites where additional polymorphic sites are observed.

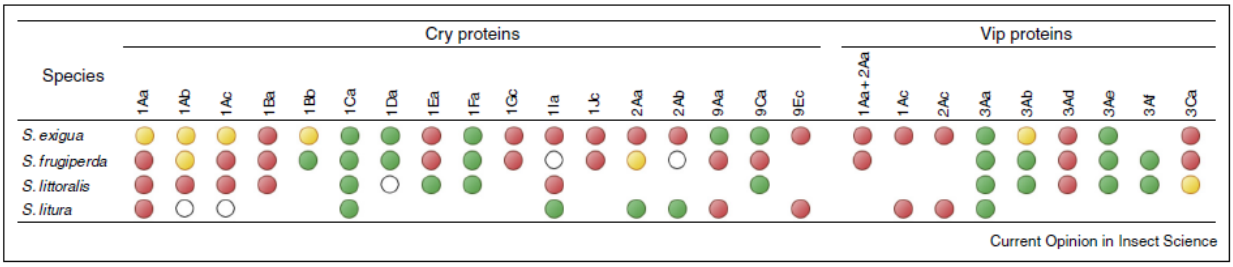


Figure 6: Toxicity of some Bt Cry proteins against *Spodoptera* spp. The activity of the toxins is indicated as strong (green), moderate (yellow), and non-active (red).
This figure was taken from Herrero et al., 2016. Permission to reuse in a thesis has been granted through Copyright Clearance Center's RightsLink®.

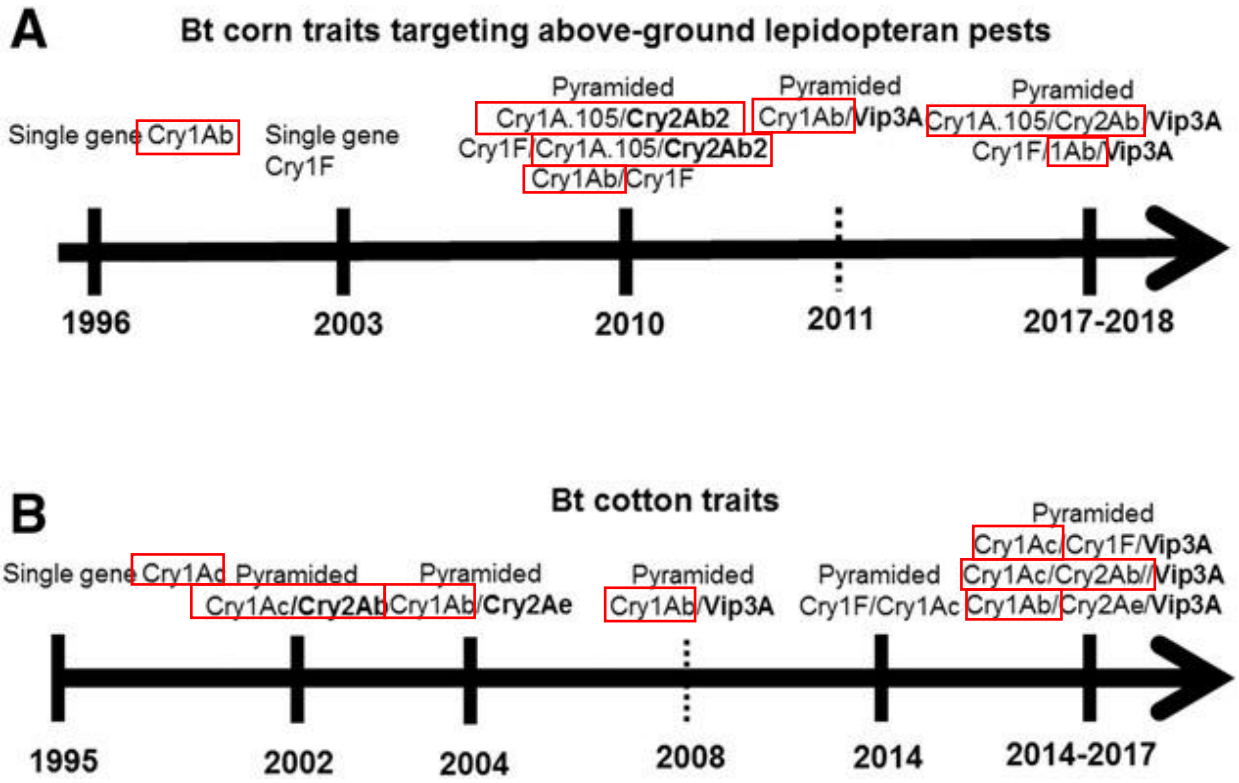


Figure 7: Schematic diagrams to show that, each time, only a single new Bt gene (bold) with a different mode of action was introduced to produce "pyramided" Bt crops. **(A)** Bt corn, **(B)** Bt cotton. Red blocks outline the Bt Cry proteins evaluated in this study.
The diagram is taken from Huang (2020), an open access article distributed under the terms of the Creative Commons CC BY license, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Modification to the diagram was made by the addition of red outlines.

Chapter 2:

Genetic characterisation of five populations of fall armyworm, *Spodoptera frugiperda* (J.E. Smith), from South Africa, into strain-specific haplotypes

2.1. Abstract

The recent invasion of fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith), from the Western Hemisphere into the Eastern Hemisphere has been catastrophic to the agricultural sectors affected by FAW infestations. FAW is characterised by two morphologically identical but genetically distinguishable strains, the corn strain (CS) and rice strain (RS), which have been shown to differ in their responses towards *Bacilllus thuringiensis* (Bt) Cry proteins. Since the toxicity of Bt Cry proteins towards FAW is strain-dependent, the objective of the study was to genetically characterise five populations of FAW in South Africa, which were used in subsequent Bt Cry protein toxicity assessments, into strain-specific haplotypes. The FAW specimens were confirmed at the species level through DNA barcoding using the *mitochondrial cytochrome oxidase subunit I gene* (*COIA*). Thereafter, strain-diagnostic single nucleotide polymorphisms (SNPs) in the *COIB* (downstream segment of *COIA)* and the *triosephosphate isomerase* (*Tpi)* exon and intron regions were used to distinguish between the haplotypes within each strain. Using the *COIB* marker, the incidence of the *COI*-CS haplotype was 27% and the *COI*-RS haplotype was 73%. However, using the *Tpi* marker, only *Tpi*C strains were identified. Thus, 73% of specimens exhibited the overall genetic haplotype of the discordant *COI*-RS *Tpi*C or hybrid haplotype. The findings would assist in assessing the role of FAW strains or haplotypes in any observed differences in the susceptibility of FAW populations to Bt Cry proteins in subsequent studies.

2.2. Introduction

Fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith), is a pest that avoids diapausing by migrating away from winter months and into warmer areas to feed on a wide variety of plants, including agriculturally important crops such as maize and rice (Pashley, 1986; Sparks, 1979; Wan et al., 2021). The great migratory potential coupled with the polyphagous nature of FAW makes it one of the most agriculturally damaging pests in the Western Hemisphere. Since the first reported outbreak of FAW in West Africa in 2016, outbreaks of FAW continues to be identified throughout the rest of Africa (Goergen et al., 2016; Nagoshi et al., 2020, 2019a; Piggott et al., 2021).

While FAW is polyphagous, it has historically been classified into two strains, corn strain (CS) and rice strain (RS) due to the preference of CS for maize, cotton and sorghum, and RS for rice, pasture grasses and millet (Pashley, 1986; Sparks, 1979). However, the preference of the host is not an absolute criterion for the classification of FAW strains since plasticity among strains has frequently been observed (Juárez et al., 2012; Nagoshi et al., 2021; Saldamando and Vélez-Arango, 2010). Thus, the characterisation of FAW into strains does not necessarily infer which crops are in danger of FAW infestations. However, FAW strains do differ in their responses to chemical insecticides such as methomyl and lambda-cyhalothrin and Bt Cry proteins such as Cry1Ab, Cry1Ac, Cry1B, Cry1C and Cry1D and Cry1F, making the genetic characterisation of FAW populations important to Bt Cry protein toxicity studies (Ingber et al., 2018; Monnerat et al., 2006; Ríos-Díez et al., 2012; Ríos-Díez and Saldamando-Benjumea, 2011; Virla et al., 2008).

The two strains are morphologically identical, thus the most accurate method for distinguishing the two strains is the use of molecular techniques. Single nucleotide polymorphisms (SNPs) at specific nucleotide sites in the *mitochondrial cytochrome oxidase subunit I gene* (*COIA)* and the *triosephosphate isomerase* (*Tpi*) genes can be used to distinguish between FAW strains. More specifically, a few key SNPs in the downstream segment of the *COIA* gene, *COIB*, and the fourth exon (TpiE4) and intron (TpiI4) of the *Tpi* gene are diagnostic of the strain while other non-diagnostic SNPs distinguish between different haplotypes within a strain (Nagoshi et al., 2009, 2008; Nagoshi and Meagher, 2008). CS FAW is designated by the term "*COI*-CS" if identified by CS-specific SNPs in the *COIB* gene segment or "*Tpi*C" if identified by CS-specific SNPs in the *Tpi* gene segment. "*COI*-

RS" and "*Tpi*R" are identifiers of FAW with RS-specific SNPs in the *COIB* and *Tpi* segments respectively (Nagoshi, 2010; Nagoshi et al., 2007).

Conventionally, SNP sites in the *COI* gene are termed with an "m" (mitochondria) while SNP sites in the *Tpi* are termed "g" (genomic). This is followed by the gene names and the number of base pairs a SNP is from the *COI* predicted translational start site, 5' start of TpiE4 exon or the 5' start of TpiI4, and the nucleotides that distinguish RS and CS in the Western Hemisphere (Nagoshi et al., 2021, 2019b). Using these terms, *COI*-CS FAW is further characterized into four haplotypes in the Western Hemisphere based on SNPs at mCOI1164D and mCOI1287R, which are *COI*-CSh1, *COI*-CSh2, *COI*-CSh3 and *COI*-CSh4 (Nagoshi et al., 2019a, 2018, 2017). The *COI*-CSh2 is also called the TX-type because it predominates in Texas and South America, while *COI*-CSh4 predominates in Florida and is also known as the FL-type (Nagoshi et al., 2007).

In the Western Hemisphere, the *Tpi* strain markers are usually in concordance with *COI* markers. Discordant *COI* and *Tpi* haplotypes are less common, occurring in <20% of specimens (Nagoshi, 2010; Nagoshi et al., 2019c, 2012). Furthermore, among discordant haplotypes, the *COI*-RS *Tpi*-C discordant haplotype is found in 78% of FAW specimens and predominates over the *COI*-CS *Tpi*-R discordant haplotypes, which is only found in 6% of FAW specimens (Nagoshi, 2010; Nagoshi et al., 2019c, 2012). The discordant haplotypes demonstrate proof that interstrain hybridization is possible between CS and RS, predominantly between RS females and CS males (Nagoshi, 2010). However, the rare FAW discordant *COI*-RS *Tpi*-C hybrid haplotype of the Western Hemisphere has recently been reported to be the dominating haplotype in Africa, India, Australia and southeastern Asia, where FAW has recently established (Acharya et al., 2021; Nagoshi et al., 2020, 2019a, 2019b; Piggott et al., 2021).

Although several studies have genetically characterised FAW strain haplotypes of FAW in South Africa (Jacobs et al., 2018; Nagoshi et al., 2019a, 2019b), the exact haplotypes of the populations used in the present study cannot be inferred based on those studies. The strain identity of the populations in any toxicity studies using Bt Cry proteins should be characterised especially when strain identity may explain differences in the toxicity of Bt Cry proteins against different FAW populations. Furthermore, strain-characterisation of FAW in regions outside of the Western Hemisphere has reported a predominance of the hybrid *COI*-RS *Tpi*C haplotypes. Since hybrid *COI*-RS *Tpi*C haplotypes can only be characterized using

both *COI* and *TPI* markers, the objective of this study was to genetically characterise FAW populations and confirm the presence of FAW strains reported in South Africa, using both *COIB* and *Tpi* strain and haplotype-distinguishing SNPs.

2.3. Methods and materials

2.3.1. Insects

All FAW populations were sampled from maize fields in South Africa. The V1 population was established from larvae collected from White River, Mpumalanga, in April of 2018. Two field FAW populations were sampled from Marble Hall (MH1) and Lehau (LE1) from the Limpopo province. Another three field populations were sampled from Brits (BR1) and Koster (K1F1 and K1F2 were sampled from neighboring fields, in close proximity to each other) from the North West Province. However, since K1F1 and K1F2 were near each other, only specimens from the K1F1 population were sequenced. Field populations were collected during January and February of 2021. FAW larvae were reared on a meridic bean-based diet with the exclusion of formaldehyde (Shorey and Hale, 1965). Pupae were sterilized in 0.25% (w/v) sodium hypochlorite. Adults were fed 5% (w/v) sucrose through soaked cotton rolls.

2.3.2. DNA extraction and PCR amplification

DNA from three second instar FAW larvae per population, frozen at -20 ^{o}C, was extracted as per the standard protocol of the NucleoSpin Tissue XS DNA Extraction kit (Macherey-Nagel, Germany) with the following adjustments: Prelysis was for 1 h; 5 min lysis was followed by manual homogenization by sterile micropestle; 25 µl of DNA was eluted.

Extracted DNA was PCR amplified with the FastStart Taq DNA Polymerase, dNTP pack (Roche, Switzerland) as per the user manual. Each gene segment was amplified separately for 30 cycles: 4 min initial denaturation at 95 ^{o}C, 30 s denaturation at 95 ^{o}C, 30 s annealing at temperatures corresponding to each primer pair (Table 1), 45 s elongation at 72 ^{o}C and 5 min final elongation at 72 ^{o}C.

Amplified DNA samples were visualized through gel electrophoresis with a 1% (w/v) agarose gel at 90 V for 45 min. The DNA samples were sequenced by Sanger sequencing (Inqaba Biotechnical Industries, South Africa). Amplified products were purified and sequenced in both orientations. Consensus DNA sequences were built from pairwise alignment (optimal GLOBAL alignment) of forward and reverse sequences amplified by each primer set, using BioEdit Sequence Alignment Editor (version 7.0.5.3). Consensus sequences for each amplified region were subjected to multiple alignment using MUSCLE (multiple sequence comparison by log-expectation) in MEGA 11 using default parameters.

2.3.3. Strain diagnosis

The *COIA* gene segment was used for DNA barcoding analysis. The *COIA* sequences were compared with FAW barcode sequences using nucleotide BLAST (Basic Local Alignment Search Tool) as well as on the Barcode of Life Database (BOLD, www.barcodinglife.org) (Cock et al., 2017; Goergen et al., 2016; Hajibabaei et al., 2006).

SNPs in the *COIB*, TpiE4 and TpiI4 regions where identified at sites detailed in Figures 4 and 5 (Nagoshi, 2010; Nagoshi et al., 2019b). The sites mCOI1164 and gTpi183 define *COI*-based and *Tpi*-based strain identity respectively, while other sites define haplotypes within each strain (Nagoshi et al., 2019b). Furthermore, SNPs in TpiI4 (between sites 10 and 172 from the start of TpiI4) distinguish TpiRa1 into one haplotype (TpiI4Ra1a), TpiCa1 into two haplotypes (TpiI4Ca1a or TpiI4Ca1a) and TpiCas2 into three haplotypes (TpiI4Ca2a, TpiI4Ca2b and TpiI4Ca2c) (Nagoshi et al., 2019b).

2.4. Results

The *COIA* barcode region is generated by BOLD systems on the Barcode of Life Database (BOLD, www.barcodinglife.org). All barcodes matched the unique Barcode Index Number (BIN) for FAW (BOLD: AAA4532) at 100% similarity and the consensus sequences matched FAW by nucleotide BLAST at 99% similarity. Sequences for all the specimens were therefore used for subsequent analysis.

Based on the strain-diagnostic mCOI1164D SNP, both *COI*-RS and *COI*-CS strains were found (Table 2). Furthermore, based on other SNPs in the *COIB* region, only one haplotype of each strain was observed, which were *COI*-RSa1 and *COI*-CSa1 respectively (Table 2). All specimens from the V1, MH1, BR1 populations, one specimen from the LE1 population and one specimen from the K1 population were identified as *COI*-RSa1 strains (Table 2). Two specimens from the LE1 population and two specimens from the K1 population were *COI*-CSa1 strains (Table 2). Thus, 11/15 specimens were identified as *COI*-RSa1 strains while 4/15 specimens were identified as *COI*-CSa1 strains.

Based on the strain-diagnostic SNP at the gTpi183Y in the TpiE4 region, all the specimens were identified as *Tpi*C strains (Table 3). SNPs in the TpiE4 exon region further identified all three *Tpi*C African haplotypes in the 15 specimens: 10/15 being *Tpi*Ca1, 3/15 being *Tpi*Ca1/Ca2, and 2/15 being TpiCa2 (Table 3). Furthermore, SNPs in the TpiI4 region identified 3 TpiI4 haplotypes where 10 haplotypes were TpiICa1a, one was TpiICa2a and one was TpiICa2b (Table 4).

All TpiCa1 haplotypes based on SNPs in TpiE4 were accompanied by TpiICa1a intron haplotypes (Table 5). The TpiE4 TpiCa2 haplotype of the LE1b specimen was accompanied by the TpiI4 TpiICa2a haplotype while the TpiE4 TpiCa2 haplotype of the BR1c specimen was accompanied by the TpiI4 TpiICa2b haplotype (Table 5). TpiI4 regions of TpiCa1/Ca2 haplotypes were consistently indecipherable based on the DNA sequence chromatographs (Tables 4 and 5).

Overall, discordant *COI*-RSa1 *Tpi*C haplotypes were found in 11/15 of the specimens while concordant *COI*-CSa1 *Tpi*C strains were found in 4/15 specimens (Table 5). Only the V1 population had all specimens with identical COIB, TpiE4 and TpiI4 haplotypes, which were COI-RSa1, TpiCa1 and TpiICa1a (Table 5).

2.5. Discussion

FAW strains are identified by diagnostic SNPs in two independent gene segments, the mitochondrial *COIB* and the nuclear Z-linked *Tpi* gene segments (Acharya et al., 2017, 2021; Nagoshi et al., 2019a; Pigott and Ellar, 2007). In the Western Hemisphere, 70% of *COI*-CS or *TpiC* markers correspond with CS-preferred crops while 95% of *COI*-RS or *Tpi*R markers correspond with RS-preferred crops (Murúa et al., 2015; Nagoshi, 2010). However, the findings of the present study showed that despite all specimens of FAW being collected from maize fields in South Africa, 73% of the specimens in the study were RS FAW as determined by *COIB* markers. Similar findings have been reported where 63% to >80% of FAW specimens from India, Myanmar, Australia, Benin, Cameroon, and 11 countries of sub-Saharan Africa, including South Africa, were *COI*-RS (Haenniger et al., 2020; Kuate et al., 2019; Nagoshi et al., 2020, 2019b, 2019a; Piggott et al., 2021).

Although two *COI*-CS haplotypes and four *COI*-RS haplotypes characterise African FAW populations (Nagoshi et al., 2019b), only *COI*-CSa1 and *COI*-RSa1 were found in this study This is also consistent with other studies where >90% of all *COI*-CS strains and *COI*-RS strains in African countries and India have been *COI*-CSa1 and *COI*-RSa1 (Nagoshi et al., 2019b, 2019a). Furthermore, the *COI*-CSa1 haplotype, characterised by $G_{1164}G_{1287}$, is the same as the CS-h4 or FL-type haplotype of the Western Hemisphere. Thus, in terms of *COI*-CS haplotypes, the *COI*-CS strains in the study likely originated from Florida or the eastern United States and the Caribbean islands where FAW migrates to and from Florida (Nagoshi et al., 2008, 2007). This observation has been reported in other studies as well (Nagoshi et al., 2019b, 2019a). FL-type FAW has been reported to be more susceptible to Bt maize containing a single Cry1F protein event or pyramided Bt maize containing the Cry1A.105 and Cry2Ab2 proteins compared to populations from Brazil, Puerto Rico and Argentina, which are characterised by the TX-type (Chandrasena et al., 2017; Farias et al., 2014a; Storer et al., 2010). Thus, FL-type FAW from the five field populations in the study may be expected to be susceptible to Cry1F, Cry1A.105 and Cry2Ab2.

All the specimens from the five field populations were *Tpi*C. As characterized by Nagoshi et al. (2019b), *Tpi*R haplotypes are rare or non-existent in several African countries, Asia and Australia (Acharya et al., 2021; Haenniger et al., 2020; Nagoshi et al., 2019b; Piggott et al., 2021). Thus, the dominance of *Tpi*C in this study is consistent with other studies. *Tpi*C strains in Africa are further classified into two variants (TpiCa1 and TpiCa2) based on TpiE4 SNPs

(Nagoshi et al., 2019b, 2019a). Moreover, TpiCa1 is linked to TpiI4 SNPs that further distinguish TpiCa1 into TpiICa1a and TpiICa1b and TpiCa2 into TpiICa2a, TpiICa2b and TpiICa2c (Nagoshi et al., 2019b, 2019a). The proportion of the TpiCa1 (82%) in the present study was also comparable with studies from South Africa, India and Myanmar that have reported >80% of TpiI4 TpiCa1 haplotypes being made up of TpiICa1a with the rest being TpiE4 TpiCa2 linked with either TpiI4 TpiCa2a or TpiCa2b haplotypes in FAW populations (Nagoshi et al., 2020, 2019a).

Since 73% of the specimens in the study were RS as identified by *COIB* markers, but CS as identified by *Tpi* markers, 73% of specimens have the overall haplotype of *COI*-RS *Tpi*C with varying TpiE4 and TpiI4 haplotypes. The discordant *COI*-RS *Tpi*C haplotype is rare in the Western Hemisphere, where FAW originates, but is observed in numerous studies of new invasive populations in the Eastern Hemisphere, making it a phenomenon of interest and concern (Haenniger et al., 2020; Nagoshi et al., 2020, 2019b, 2019a; Piggott et al., 2021; Sarr et al., 2021). The predominance of the discordant populations, which has been seen in >80% of specimens from South Africa, India and Myanmar, is theorized to have arisen due to a bottleneck at the point of entry (Nagoshi, 2019; Nagoshi et al., 2020). It is possible these discordant populations represent an interstrain hybrid that arose due to a severe bottleneck and lack of mates between the initial or several small populations of FAW that consisted of both CS and RS. Bottlenecks introduce a higher proportion of deleterious alleles that impact the progeny fitness, as a result, heterozygote interstrain crosses would naturally be favoured (Groot et al., 2016; Nagoshi and Meagher, 2003; Unbehend et al., 2013). A bottleneck situation of field populations of FAW may also explain why the V1 population was more homogeneous compared to the other populations. The V1 population was sampled 2 to 3 years before the sampling of the other populations and has been maintained under laboratory conditions. The V1 population, therefore, was exposed to fewer interbreeding chances compared to the other populations, thereby causing it to accumulate less genetic variation.

While the two different strains in the Western Hemisphere are largely sympatric and are mainly localized within their preferred host types and have been shown to be reproductively isolated, interbreeding studies between the African populations have been observed and documented (Nagoshi and Meagher, 2003, 2008). A study on mating behaviours between the two strains in African populations found no strain mating biases and showed through F crosses that African FAW populations are dominated by pure C-strain or interstrain *COI*-RS

*Tpi*C hybrids, while R strain and *COI*-CS *Tpi*R are rare (<1% of the population) (Nagoshi, 2019, 2010).

The only population where all three specimens carried identical *COIB*, TpiE4 and TpiI4 haplotypes was the V1 population. This observation points toward the likelihood that haplotypes are not confined to specific regions and that within the same population, there are haplotype differences. Although three specimens may seem like a few specimens to base haplotype frequencies on, several papers using 1 or 2 specimens per region (Acharya et al., 2021; Jacobs et al., 2018; Piggott et al., 2021) have reported similar haplotype proportions to studies using >100 specimens in south-eastern Asia and >800 specimens in Africa (Nagoshi et al., 2020, 2019b). Thus, the number of specimens observed in the study may accurately reflect haplotype proportions in South Africa. However, the genetic make-up of more FAW regional specimens would need to be characterised to confirm this observation.

The fact that no concordant RS haplotypes were observed in the study means that the evaluation of the toxicity of Bt Cry proteins in the subsequent studies may be expected to reflect the toxicity of Bt Cry proteins towards CS and hybrid FAW strains rather than to RS FAW. A study conducted to evaluate the toxicity of Cry1Ab, Cry1Ac and Cry1F against CS, RS and hybrid strains found that CS and hybrid FAW strains were more tolerant toward purified Bt Cry proteins as well as to Bt leaf tissue, with Cry1F being the most toxic when compared with RS FAW (Ingber et al., 2021b, 2018). Similarly, in Columbia, a study reported higher toxicity of Cry1Ab against RS compared to CS FAW larvae (Ríos-Díez et al., 2012). Thus, all five populations of FAW in South Africa may be expected to be susceptible to Bt maize expressing Cry1 proteins.

In conclusion, the use of *COIB* markers in conjunction with *Tpi* markers revealed that the majority of FAW strains in the five field populations were hybrid RS and CS at haplotype proportions comparable to other studies in South Africa. The evaluation of strains and haplotypes was necessary since there is strain- and haplotype-specific differences in the susceptibility of FAW to Bt Cry proteins expressed by Bt maize (Chandrasena et al., 2017; Farias et al., 2014b; Ingber et al., 2021b; Ríos-Díez et al., 2012; Storer et al., 2010). *Tpi* exon and intron markers further revealed that slight variation exists even within *Tpi*C strains in this study. Variation of FAW within the same strain is an important characterisation of FAW populations since differences in susceptibility of field populations against Bt maize have also been linked to overall genetic variation within the same strain (Monnerat et al., 2006). This

strain-based and additional genetic variation from the TpiI4 gene may result in variation in the susceptibility of these FAW populations to toxicity studies using Bt Cry proteins.

Table 1: Primer names and sequences with corresponding annealing temperatures used for the strain characterisation of FAW in South Africa

Amplified region	Primer name	Size of amplicon (bp)	Primer sequences (5´ to 3´)	Annealing temperature (°C)
COIA	LepF1[a]	650	ATTCAACCAATCATAAAGATATTGG	53.5
	LepR1[a]		TAAACTTCTGGATGTCCAAAAAATCA	
COIB	891F[b]	603	TACACGAGCATATTTTACATC	51.1
	1472R[b]		GCTGGTGGTA AATTTTGATATC	
Tpi	412F[b]	500	CCGGACTGAAGGTTATCGCTTG	59.6
	1140R[b]		GCGGAAGCATTCGCTGACAACC	

[a] Primers from Goergen et al. (2016); Hajibabaei et al. (2006); Jacobs et al. (2018)
[b] Primers from Nagoshi et al. (2019a, 2019b).

Table 2: Strain-diagnostic SNPs in the *COIB* region of FAW specimens from five different populations in South Africa

FAW specimens[a]	*COIB* SNP sites[b]					Haplotype
	mCOI1122C	mCOI1125Y	**mCOI1164D**	mCOI1215A	mCOI1287R	
V1a	C	C	**T**	A	A	COI-RSa1
V1b	C	C	**T**	A	A	COI-RSa1
V1c	C	C	**T**	A	A	COI-RSa1
MH1a	C	C	**T**	A	A	COI-RSa1
MH1b	C	C	**T**	A	A	COI-RSa1
MH1c	C	C	**T**	A	A	COI-RSa1
LE1a	C	T	**G**	T	G	COI-CSa1
LE1b	C	C	**T**	A	A	COI-RSa1
LE1c	C	T	**G**	T	G	COI-CSa1
K1a	C	T	**G**	T	G	COI-CSa1
K1b	C	C	**T**	A	A	COI-RSa1
K1c	C	T	**G**	T	G	COI-CSa1
BR1a	C	C	**T**	A	A	COI-RSa1
BR1b	C	C	**T**	A	A	COI-RSa1
BR1c	C	C	**T**	A	A	COI-RSa1

[a] Each FAW population was represented by three specimens denoted as a, b, and c.
[b] The mCOI1164D site in bold defines *COIB* SNPs that distinguish between CS and RS FAW. SNPs at other sites define haplotypes.
The same colour shading represents FAW specimens with the same haplotype.

Table 3: Strain-diagnostic SNPs in the TpiE4 region of FAW specimens from five different populations in South Africa

FAW specimens[a]	TpiE4 SNP sites[b]								Haplotype
	gTpi129C	gTpi144G	gTpi165Y	gTpi168Y	gTpi180C	**gTpi183Y**	gTpi192Y	gTpi198Y	
V1a	C	G	C	T	C	**C**	C	C	TpiCa1
V1b	C	G	C	T	C	**C**	C	C	TpiCa1
V1c	C	G	C	T	C	**C**	C	C	TpiCa1
MH1a	C	G	C	T	C	**C**	C	C	TpiCa1
MH1b	C	G	C	T	C	**C**	C	C	TpiCa1
MH1c	C	G	C	T	C	**C**	Y	Y	TpiCa1/Ca2
LE1a	C	G	C	T	C	**C**	Y	Y	TpiCa1/Ca2
LE1b	C	G	C	T	C	**C**	T	T	TpiCa2
LE1c	C	G	C	T	C	**C**	C	C	TpiCa1
K1a	C	G	C	T	C	**C**	C	C	TpiCa1
K1b	C	G	C	T	C	**C**	C	C	TpiCa1
K1c	C	G	C	T	C	**C**	C	C	TpiCa1
BR1a	C	G	C	T	C	**C**	Y	Y	TpiCa1/Ca2
BR1b	C	G	C	T	C	**C**	C	C	TpiCa1
BR1c	C	G	C	T	C	**C**	T	T	TpiCa2

[a] Each FAW population was represented by three specimens denoted as a, b, and c.
[b] The gTpi183Y site in bold defines TpiE4 SNPs that distinguish between CS and RS FAW. SNPs at other sites define haplotypes. IUPAC convention (Y: C or T).
The same colour shading represents FAW specimens with the same haplotype.

Table 4: Strain-diagnostic SNPs in the TpiI4 region of FAW specimens from five different populations in South Africa

FAW specimens[a]	TpiI4 SNP sites[b]										Haplotype
	gTpiI4 31	gTpiI4 38	gTpiI4 53	gTpiI4 55	gTpiI4 58	gTpiI4 70	gTpiI4 77	gTpiI4 87	gTpiI4 96	gTpiI4 148	
V1a	C	C	A	C	G	A	C	C	T	C	TpiICa1a
V1b	C	C	A	C	G	A	C	C	T	C	TpiICa1a
V1c	C	C	A	C	G	A	C	C	T	C	TpiICa1a
MH1a	C	C	A	C	G	A	C	C	T	C	TpiICa1a
MH1b	C	C	A	C	G	A	C	C	T	C	TpiICa1a
MH1c	S	M	W	M	K	M	Y	Y	Y	M	N/A
LE1a	S	M	W	M	K	M	Y	Y	Y	M	N/A
LE1b	G	A	T	A	T	C	T	T	C	A	TpiICa2a
LE1c	C	C	A	C	G	A	C	C	T	C	TpiICa1a
K1a	C	C	A	C	G	A	C	C	T	C	TpiICa1a
K1b	C	C	A	C	G	A	C	C	T	C	TpiICa1a
K1c	C	C	A	C	G	A	C	C	T	C	TpiICa1a
BR1a	S	M	W	M	K	M	Y	Y	Y	C	N/A
BR1b	C	C	A	C	G	A	C	C	T	C	TpiICa1a
BR1c	G	T	A	T	C	T	T	C	C	C	TpiICa2b

[a] Each FAW population was represented by three specimens denoted as a, b, and c.
[b]TpiI4 SNPs that define haplotypes. IUPAC convention (Y: C or T; W: A or T; K: G or T; S: C or G; M: C or A)
Same colour shading represents FAW specimens with the same haplotype
N/A and no shading indicate haplotypes that could not be determined due to consistently indecipherable nucleotide bases at multiple sites on the DNA sequence chromatographs.

Table 5: Overall *COIB*, TpiE4 and TpiI4 haplotypes of each FAW specimen from five different field populations in South Africa

Population	*COIB*	TpiE4	TpiI4
V1a	*COI*-RSa1	TpiCa1	TpiICa1a
V1b	*COI*-RSa1	TpiCa1	TpiICa1a
V1c	*COI*-RSa1	TpiCa1	TpiICa1a
MH1a	*COI*-RSa1	TpiCa1	TpiICa1a
MH1b	*COI*-RSa1	TpiCa1	TpiICa1a
MH1c	COI-RSa1	TpiCa1/Ca2	N/A
LE1a	*COI*-CSa1	TpiCa1/Ca2	N/A
LE1b	*COI*-RSa1	TpiCa2	TpiICa2a
LE1c	*COI*-CSa1	TpiCa1	TpiICa1a
K1a	*COI*-CSa1	TpiCa1	TpiICa1a
K1b	*COI*-RSa1	TpiCa1	TpiICa1a
K1c	*COI*-CSa1	TpiCa1	TpiICa1a
BR1a	*COI*-RSa1	TpiCa1/Ca2	N/A
BR1b	*COI*-RSa1	TpiCa1	TpiICa1a
BR1c	*COI*-RSa1	TpiCa2	TpiICa2b

N/A indicates haplotypes that could not be determined due to consistently indecipherable nucleotide bases at multiple sites on the DNA sequence chromatographs.
Lack of shading indicates FAW specimens where some haplotypes could not be confirmed.

Chapter 3:

The compatibility of the droplet-feeding method for the bioassay of four Bt Cry proteins in highly alkaline buffers against fall armyworm, *Spodoptera frugiperda* (J.E. Smith)

3.1. Abstract

Bioassays are a fundamental part of crop protection science, as they enable the evaluation of insecticidal *Bacillus thuringiensis* (Bt) Cry proteins in terms of lethal and sublethal responses. Compared to other bioassays, the droplet-feeding method is the simplest bioassay method that enables the accurate estimation of lethal doses (LDs) and growth-inhibitory doses (IDs) based on the volumes of the sample imbibed. However, the compatibility of the droplet-feeding method with highly alkaline Bt Cry protein solubilising buffers has not been evaluated before. Thus, the objective of this study was to determine the compatibility of the droplet-feeding method for the bioassay of four agronomically important four Bt Cry proteins in highly alkaline buffers against fall armyworm, *Spodoptera frugiperda* (J.E. Smith), the newly invasive pest in South Africa. The droplet-feeding feeding method was combined with a fluorescent dye to enable the determination of the volumes of each highly alkaline buffer imbibed by first and second instar FAW larvae. Thereafter, the volumes imbibed were used to calculate Bt Cry protein doses in the subsequent droplet-feeding bioassays with Bt Cry proteins. It was found that first and second instar FAW larvae imbibe significantly different volumes of each highly alkaline buffer with both instars showing an aversion towards the Cry1Ac buffer. The droplet-feeding bioassays for the evaluation of Bt Cry protein toxicity revealed that, for both first and second instar FAW larvae, Cry1Ac and Cry1Ab were the least toxic, while Cry1A.105 and Cry2Ab2.820 were the most toxic in terms of both lethal and growth-inhibitory responses.

3.2. Introduction

Bt is a Gram-positive, non-capsulated bacterium that produces toxic proteins during the two phases of its life cycle (Bel et al., 2020; Kumar et al., 2021; Palma et al., 2014). During the vegetative growth stage, vegetative insecticidal proteins (Vip) and secreted insecticidal proteins (Sip) are produced while during the sporulation phase, Cry proteins, along with Cytolytic (Cyt) proteins accumulate in parasporal crystals (Bel et al., 2020; Kumar et al., 2021; Palma et al., 2014). However, only Vip and Cry proteins are used in Bt Crops for the control of insect pests (Bel et al., 2020).

One of the major agricultural pests of economic importance in the North and South Americas is the fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith) (Bhandari, 2021; Overton et al., 2021). Since the first reported outbreak of FAW in Africa, FAW has spread to much of sub-Saharan Africa, including South Africa (Cock et al., 2017; FAO, 2018; Goergen et al., 2016). Long term biological control strategies of FAW in the Western Hemisphere include the use of *Bacillus thuringiensis* (Bt) Cry proteins expressed by Bt maize (Adamczyk et al., 2008; Blanco et al., 2016; Buntin, 2008; Frankenhuyzen, 2009; Siebert et al., 2012; Tindall et al., 2009).

The mode of action of Bt Cry proteins in Bt maize begins with exposure of the host to the bacterial protoxin, followed by the solubilisation and the activation of the protoxin into its active form under the highly alkaline and reducing condition of the insect midgut (Heckel et al., 2007; Kumar et al., 2021; Palma et al., 2014; Soberón et al., 2009). The activated form undergoes processing and transformational changes that are crucial to the recognition and binding of several insect-midgut receptors (Bravo et al., 2011; Heckel, 2020; Ibrahim et al., 2010; Pardo-López et al., 2013; Soberón et al., 2016). Alterations to the functioning of these major steps contribute to the development of resistance to Bt Cry proteins (Bravo et al., 2011; Heckel, 2020; Jurat-Fuentes and Crickmore, 2017; Melo et al., 2016).

First-generation Bt crops encoded single events for the expression of Cry1Ab and Cry1Ac, respectively (De Buck et al., 2016; Huang, 2020). After the appearance of resistance to these single events, pyramided events such as the MON89034 event became commercialised (ISAAA, 2018). The MON89034 event encodes Cry2Ab2 as well as Cry1A.105 (Andersson et al., 2008). Cry1A.105 is a chimeric protein that contains domains I and II of Cry1Ab or Cry1Ac (Cry1Ab and Cry1Ac share 100% amino acid sequence identity in domains I and II),

domain III from Cry1F and a portion of the C-terminal region of Cry1Ac (Andersson et al., 2008; Hernández-Rodríguez et al., 2013; Storer et al., 2012; C. Wang et al., 2018). Cross-resistance between Cry1 and Cry2 proteins has not been documented and is not likely due to a lack of shared binding receptors to insect midgut (Hernández-Rodríguez et al., 2013; Soares Figueiredo et al., 2019). Thus, pyramided traits such as the MON89034 have high efficacy for the control of FAW (Botha et al., 2019; Rule et al., 2014).

In South Africa, Bt maize MON810 (expressing Cry1Ab) and MON8904 (expressing Cry1A.105 and Cry2Ab2.820) and Bt cotton (expressing Cry1Ac) are commercially cultivated to control local pests (ISAAA, 2022, 2018). However, since the establishment of FAW in South Africa, to the best of our knowledge, no studies have indicated the toxicity of Cry1Ab, Cry1Ac, Cry2Ab2 and Cry1A.105 to FAW in South Africa, in terms of lethal doses (LDs) and growth inhibitory doses (IDs).

Assessment of the toxicity of Bt Cry proteins in Bt crops relies on insect bioassays. The preferred bioassay method using Bt Cry proteins are diet incorporation and diet-overlay bioassays for the determination of lethal concentration (LC) and inhibitory concentration (IC) (Luttrell et al., 1999; MarÇon et al., 2000). For diet-based bioassays, it is only possible to determine the LD and ID using the diet plug method (Evans, 1981; Mandava, 2018). The use of small diet plugs, in theory, allows the dose ingested to be known if the entire plug has been consumed (Evans, 1981; Mandava, 2018). However, there are several problems with this method when using early instar larvae. First, for early instar larvae to consume an entire plug, plugs would have to be small. However, small plugs usually dry out quickly, which impacts larval feeding and results in the failure of early instar larvae to consume an entire plug (Chen and Bouwer, 2019). Secondly, toxin suspensions pipetted on the surface of the diet plug during diet-overlay bioassays often run down the sides of the plugs into the diet wells thereby reducing the dose ingested (Chen and Bouwer, 2019). Thirdly, first and second instar larvae seldom finish an entire plug of the treated diet (Chen and Bouwer, 2019).

In contrast, the droplet-feeding method, first proposed by Hughes and Wood (1981), and later modified in 1986 (Hughes et al., 1986), is an easier method for the determination of LDs and IDs compared to diet-based methods. Unlike the diet incorporation and diet-overlay methods, the droplet-feeding method enables the determination of the dose imbibed by the larvae by determining the volume of a solution of interest imbibed by the larvae (Hughes et al., 1986). The volume imbibed can be determined gravimetrically, radioisotopically or by a fluorescent

dye method (Hughes et al., 1986). The fluorescent dye method is, however, superior to gravimetric and radioisotopic methods due to its ease of application and proven accuracy of this method (Hughes et al., 1984; van Beek and Hughes, 1986).

Bt Cry proteins used in bioassays can be purified in laboratories through various protein purification protocols (Höss et al., 2011; Sayyed et al., 2005). Alternatively, Cry proteins can be purified in large agrochemical companies such as the Monsanto Company (Crespo et al., 2008; Gutierrez-Moreno et al., 2020; Jalali et al., 2015, 2010; Marçon et al., 1999; Wu et al., 2009). Regardless, Cry proteins are often solubilised and stored in highly alkaline buffers (de Maagd et al., 2001; Huang et al., 2012). Similarly, the Bt Cry proteins used in the present study to evaluate the toxicity of Bt Cry proteins against FAW in South Africa were supplied by the Monsanto Company and are solubilised in highly alkaline (pH > 10) buffers.

To the best of our knowledge, the compatibility of highly alkaline buffers with the droplet-feeding method has never been evaluated in FAW. Due to the advantages of the droplet-feeding method, this study aimed to evaluate the compatibility of the droplet-feeding method for the bioassay of four agronomically important Bt Cry proteins (Cry1Ab, Cry1Ac, Cry2Ab2.820 and Cry1A.105) in highly alkaline buffers, against FAW in South Africa. To achieve this aim, the volumes of each Bt Cry protein-solubilising buffer imbibed by first and second instar FAW larvae were determined using the droplet-feeding method in combination with a fluorescent dye. Thereafter, the toxicity of the same four Bt Cry proteins was evaluated by the droplet feeding method against a FAW population.

3.3. Methods and materials

3.3.1. Insects

The V1 population was used in this study. The V1 population was reared as per section 2.3.1. The first instar (neonates) used in droplet-feedings were within 12 hours of hatching. Second instars were selected 12-24 hours after head capsule slippage and starved for three hours before droplet-feeding.

3.3.2. Bt Cry proteins

The following agronomically important Bt Cry proteins and their respective buffers were provided by the Monsanto Company: Cry1Ab tryptic core (1.8 mg/ml), Cry1Ab buffer (50 mM sodium bicarbonate, pH 10.25), Cry1A.105 (1.2 mg/ml), Cry1A.105 buffer (25 mM CAPS, pH ~10.3, 1 mM benzamidine-HCl, 0.1 mM EDTA, and 0.2 mM DTT), Cry2Ab2.820 (0.36 mg/ml), Cry2Ab2.820 buffer (50 mM CAPS, pH 11, and 2 mM DTT), Cry1Ac (1.4 mg/ml) and Cry1Ac buffer (50 mM CAPS, pH 10.25, 1 mM EDTA, 2.5 mM DTT, and 1 mM benzamidine-HCl).

Each Bt Cry protein was subjected to SDS-PAGE to visualise each Bt Cry protein at the expected size and to confirm that the proteins have not degraded. As per the Certificate of Analysis (COA) that accompanied each Bt Cry protein from the Monsanto Company, the expected sizes of Cry1Ab, Cry1A.105, Cry2Ab2.820 and Cry1Ac were listed as 58.4 kDa, 131.5 KDa, 61.1 kDa and 131.7 KDa respectively.

Each Bt Cry protein was prepared by boiling for 15 min in 2X sample reducing buffer (0.5M Tris-HCl, pH 8; 4% SDS w/v; 0.2% w/v Bromophenol blue and 20% (v/v) glycerol and 200 µM DTT). The 0.5 mm polyacrylamide gel was set as per the user manual for the TGX Stain-Free FastCast Acrylamide Starter Kit, 7.5% (Bio-Rad, USA). The molecular weight marker, PageRuler Unstained Protein Ladder 26614 (Thermo Fisher Scientific, USA) and the prepared protein samples were subjected to SDS-PAGE at 100 V for 10 min, followed by 80 V for 30 min. After electrophoresis, gels were washed for 30 s in distilled water to eliminate electrode buffer. The gel was stained with 0.1% (w/v) Coomassie Brilliant Blue G-250 dissolved in acetic acid:methanol: distilled water (1:5:4) for 1 h. Thereafter, the gel was destained with distilled water:acetic acid:methanol (17:1.5:20) for 1 h followed by overnight destaining in distilled water.

3.3.3. Determination of the volume imbibed

To calculate an exact Bt Cry protein dose that causes a toxic response, the volumes of Bt Cry protein buffers imbibed by FAW larvae were first determined. The volumes imbibed were determined through a modified fluorescent dye method (van Beek and Hughes, 1986) further modified by Bouwer and Avyidi (2006). Batches of approximately 24 larvae were allowed to imbibe 5 µl droplets of a feeding solution consisting of 0.5 mg/ml erioglaucine (blue dye) and 2.4 mg/ml sodium fluorescein which was diluted with either sterile distilled water (sdH_2O) or one of four alkaline solubilising buffers (section 3.3.2). Larvae that had imbibed the feeding solution (evident by the blue colouring in the foregut and midgut) were homogenised in 1.5 ml of sodium phosphate buffer (50 mM, pH 7.7). The absorbance of the sodium fluorescein in each larval sample was measured using a JASCO Spectrofluorometer FP-8200 set at an excitation wavelength of 481.5 nm and an emission wavelength of 511.5 nm. A sodium fluorescein standard curve and calibration of feeding solutions were constructed and performed according to van Beek and Hughes (1986).

The volume imbibed by each larva was calculated by the formula nl ingested = (ng fluorescein in 1.5 ml of larval homogenate)/ (ng/nl in the feeding solution) (Kunimi and Fuxa, 1996; van Beek and Hughes, 1986). The droplet-feeding of larvae with each buffer and sdH_2O was performed in at least triplicates, as were all the sodium fluorescein standards and calibration solutions.

After an outlier test (Grubbs test at $\alpha = 0.05$) was performed on the $\log_{10}$-transformed volume ingested data, a one-way ANOVA test was used to analyse the data (GraphPad Prism 9.3.1). Statistically significant ANOVA results were followed by *post-hoc* comparisons of the means using the Tukey test. The coefficient of variation (CV) for each buffer was also calculated.

3.3.4. Bioassays

The toxicity of the four Bt Cry proteins against first and second instar FAW larvae was determined by the droplet-feeding method as described above. At least three replicates of 32 larvae per dose were used. Doses were determined based on the volumes of each buffer that was imbibed, as determined in section 3.3.3. For the droplet-feeding bioassay, neonates (first instar) were allowed to imbibe ten doses of Cry1A.105, Cry1Ab and Cry2Ab2.820 and seven doses of Cry1Ac, while second instars were allowed to imbibe four doses of Cry2Ab2.820, ten doses of Cry1A.105 and Cry1Ab and seven doses of Cry1Ab. Cry protein feeding

solutions contained 0.5 mg/ml erioglaucine, with the corresponding solubilising buffers, from the Monsanto Company, as the solvent. Larvae that had imbibed buffers only were used as controls. Larvae that had imbibed the solution were placed individually into individual wells containing a meridic diet.

To evaluate the LD_{50} of the Bt Cry proteins, mortality was scored after 7 days. Survivors were weighed to determine the sublethal effect through the determination of ID_{50}. The scoring of mortality included functional mortality, where larvae that failed to moult to the next instar were considered dead (MarÇon et al., 2000). From our observations of FAW larvae, first instar larvae that failed to moult to the next instar weighed less than 0.5 mg, while second instar larvae that failed to moult to the next instar weighed less than 1 mg. Thus, larvae that weighed less than or equal to 0.5 mg for first instar larvae and less than or equal to 1 mg for second instar larvae were considered dead.

The LD_{50} was determined by probit regression analysis (Finney, 1952). Outliers in weight-response data were removed based on Grubbs tests at $\alpha = 0.05$. Thereafter, weight-response data was normalised using the following settings on GraphPad Prism: the smallest mean in each data set was normalised to 0% and the largest mean in each dataset was normalised to 100%. The ID_{50} values were determined by non-linear regression analysis using the function: [inhibitor] vs normalised response - variable slope, least squares fit (GraphPad Prism 9.3.1). The best fit ID_{50} values for each Bt Cry protein were also compared by the Extra sum of squares F test to determine whether the ID_{50} values were statistically significant ($P < 0.05$) from each other.

3.4. Results

3.4.1. Bt Cry proteins

All Bt Cry proteins corresponded with their expected size. Cry1Ab was visualised at 58.4 kDa, Cry1Ac at 131.7 kDa, Cry1A.105 at 131.5 kDa and Cry2Ab2.820 at 61.1 kDa (Figure 8). Faint bands below Cry1Ac (lane 3), Cry1A.105 (lane 4) and above Cry2Ab2.820 (lane 5) were also observed (Figure 8).

3.4.2. The volume of Bt Cry protein solubilising buffer imbibed by FAW larvae

The volumes of the different feeding solutions imbibed by the first ($F_{(4,369)} = 7.952$, $P < 0.001$) and second instars ($F_{(4,378)} = 9.523$, $P < 0.001$) were statistically different (Table 6). Both instars imbibed the lowest volume of the Cry1Ac buffer (Table 6). First instar FAW larvae imbibed the highest volume of the Cry1Ab buffer while second instar FAW larvae imbibed the highest volume of sdH_2O (Table 6). The CVs for the volumes of buffers imbibed by the first instar larvae were smaller than that of the second instar larvae, ranging between 21.55 – 36.55% and 33.78 – 53.09%, respectively (Table 6).

3.4.3. Toxicity bioassays

As seen in the dose-weight response curves (Figure 9), each Bt Cry protein elicited a statistically different response curve with statistically different best-fit ID_{50} values ($P < 0.001$ for both instars) based on the Extra sum-of-squares F test. Furthermore, for all Bt Cry proteins, the normalised dose-weight response of first and second instar larvae decreased as the dose of Bt Cry protein increased (Figure 9). For second instar FAW larvae, the best-fit ID_{50} values of Cry1Ac could not be accurately determined, since a 50% normalised dose-weight response could not be determined within a 95% confidence interval (Figure 9). Furthermore, the standard error of mean for the dose-weight response of second instar FAW larvae to Cry1Ac, appeared to be significantly larger than that of the other Bt Cry proteins, indicating that the dose-weight response of second instar FAW to Cry1Ac was significantly more variable compared to the other Bt Cry proteins (Figure 9b).

The LD_{50} of first instar FAW larvae could not be determined for Cry1Ac and Cry1b (Table 7). Even at the highest dose of Cry1Ac (6.45 ng) and Cry1Ab (14.04 ng), Cry1Ac and Cry1Ab were not lethal towards first instar FAW larvae (Table 7). None of the four Bt Cry proteins was lethal against second instar FAW larvae at the highest possible dose (undiluted Bt Cry

protein) (Table 7). The highest ID_{50} values for first instar FAW larvae were observed for Cry1Ac (0.95 ng) and Cry1Ab (1.60 ng) (Table 8). An ID_{50} value for Cry1Ac could not be determined in second instars even at the highest dose administered (Table 8). Thus, both Cry1Ac and Cry1Ab were found to be the least toxic towards both first and second instar FAW larvae in terms of both mortality and growth-inhibition.

The LD_{50} value of Cry1A.105 against first instar FAW larvae was statistically different to that of Cry2Ab2.820 (Table 7). Furthermore, Cry1A.105 was the most lethal toward first instar FAW larvae with an LD_{50} of 0.65 ng, which is 3.9-fold lower than that of Cry2Ab2.820 (2.51 ng) and it had a slope that was steeper than that of Cry2Ab2.820 (Table 7). The lowest ID_{50} value for first instar FAW larvae was obtained for Cry2Ab2.820 with an ID_{50} of 0.06 ng, which is 2.5-fold lower than that for Cry1A.105 (0.15 ng) (Table 8). In contrast, for second instar FAW larvae, Cry1A.105 appeared to inhibit the growth of larvae the most, with an ID_{50} of 0.13 ng, which is 13.2-fold lower than that of Cry2Ab.820 (1.71 ng) (Table 8). Furthermore, what was interesting was that in the second instars, the ID_{50} of Cry1Ab (0.54 ng) was 3.2-fold lower than the ID_{50} of Cry2Ab (1.75 ng) (Table 8).

3.5. Discussion

Thick bands corresponding to the size of each Bt Cry protein from the Monsanto Company were observed on the SDS-PAGE gel. However, faint bands below Cry1Ac and Cry1A.105 and above Cry2Ab2.820 were observed. The faint bands below Cry1Ac and Cry1A.105 could be the result of slight protein degradation or it could be variants of the same protein since in the COA from the Monsanto Company, the purity of each Cry protein was a summation of stained bands migrating between and including the full-length Cry protein and the trypsin resistant core. The faint band above Cry2Ab2.820 was likely not a variant since full-length Cry2Ab2.820 protein is 61 kDa and the faint band was heavier. Thus, the faint band above the Cry2Ab2.820 could be the remnant of other proteins leftover from the purification protocol. Regardless, the four Bt Cry proteins had not degraded in a manner that would hinder the toxicity of the Bt Cry proteins and were used in subsequent bioassays.

The volumes of each buffer and sdH_2O imbibed by first and second instar FAW larvae were statistically different as determined through the ordinary one-way ANOVA ($P < 0.001$). The significant difference in volumes imbibed could not be due to the effect of the buffers on the fluorescence of sodium fluorescein as this was accounted for through the calibration of the feeding solution (section 3.3.3.). This observed significant difference is likely due to the acute chemoreception of FAW. Insects rely on chemoreception for crucial behaviours such as foraging and feeding (Mandava, 2018; Xu, 2020). Chemoreception affects the rate of feeding, with less acceptable foods being eaten more slowly (Bernays and Simpson, 1982; Chapman, 1995). Gustatory receptors are involved in sugar and bitterness detection, where sugars can act as phagostimulants and bitterness (bitter compounds) can result in insect aversion (Xu, 2020). Being a highly polyphagous pest, FAW possesses a total of 231 candidate gustatory receptor genes, more than in other studied insects (Xu, 2020).

The sensitivity of FAW to gustatory stimuli may explain the preference or aversion of FAW larvae to certain solutions. In the present study, the feeding solutions were highly alkaline (pH >10) buffers containing reagents such as EDTA, DTT and benzamidine-HCl. While EDTA, DTT and benzamidine-HCl are common components in protein buffers due to their metal-chelating and reducing abilities which prevent the interference of metal-generated free radicals, nucleases, and proteases (Huang et al., 2012; Liu et al., 1997; Sharma and Luthra-Guptasarma, 2009), these reagents are far from what FAW will encounter on the crops they feed on. Thus, the higher concentration of EDTA and DTT in the Cry1Ac buffer compared to

the other buffers in the study could explain the observed aversion of FAW larvae to the Cry1Ac buffer. While both first and second instar showed an aversion towards the Cry1Ac buffer, they differed in their preference of buffer. First instar FAW larvae imbibed the largest volume of the Cry1Ab buffer while second instar FAW larvae imbibed the largest volume of water. This may be due to a change in the preference for solutions during the development of FAW larvae. This is not uncommon as diet preferences have been shown to change throughout developmental stages (Kuřavová and Kočárek, 2015).

The fact that first and second instar imbibe different feeding solutions variably, highlights that the droplet-feeding method can be an extremely accurate method for determining the dose administered to insect larvae. It also shows that FAW larvae used in droplet-feeding bioassays should be assessed for the volumes imbibed for each new feeding solution in order to accurately determine doses.

The droplet-feeding and fluorescent dye method have been used to evaluate the volumes of water imbibed by noctuids. However, to the best of our knowledge, the volumes of highly alkaline buffers imbibed by noctuids have never been evaluated. The volumes of water imbibed by first instar FAW larvae in the present study were comparable with the volumes imbibed by neonates of *Anticarsia gemmatalis*, *Heliothis virescens*, *Pseudoplusia includens*, *Trichoplusia ni*, which were recorded to have imbibed between 5.83 to 10.90 nl (Kunimi and Fuxa, 1996). However, neonates of *Eldana saccharina* and *Helicoverpa armigera* were recorded to have imbibed higher volumes (15.3 to 19.7 nl) of feeding solution (Bouwer and Avyidi, 2006). Similarly, second instar larvae of *A. gemmatalis*, *H. virescens*, *P. includens* and *T. ni* have been reported to imbibe between 50 nl to 78.37 nl, which are much larger volumes than what was observed by second instar FAW in the present study (Kunimi and Fuxa, 1996). This discrepancy is likely due to a reduced starvation time of second instar larvae in the present study. While Kunimi and Fuxa (1996), detailed starvation of 20-24 h before droplet-feeding, the present study starved second instars for 3 h which through many trials, was observed to be a sufficient amount of starving time to enable second instars to readily and quickly imbibe the droplets without duress.

The CVs of the volumes of distilled water imbibed by second instar V1 (33.8%) are comparable to the CVs for droplet feeding assays of distilled water of second instars in other noctuid species (33.80% for *T. ni*, 42.28% for *H. virescens*, 29.99% for *P. includens* and 27.68% for *A. gemmatalis*) (Kunimi and Fuxa, 1996). Similarly, the CVs obtained for

Plutella xylostella, *Mamestra brassicae* and *Crocidolimia binotalis* were between 32.6% to 49.7% as determined through the fluorescent droplet feeding method (Ridout and Fenlon, 1991). The CVs of the volume imbibed by second instar V1 larvae (Cry1A.105 buffer, 46.9%; Cry1Ab buffer, 47.0%; Cry1Ac buffer, 53.1%; Cry2Ab2.820 buffer, 38.78%) were, therefore, all in range with CVs in other studies.

The findings indicated that the droplet-feeding method can be expanded to the inclusion of bioassays with highly alkaline solubilising buffers in which many readily obtainable purified Bt Cry proteins are solubilised and stored. Thus, the volumes of feeding solutions imbibed determined in the present study were used to determine the doses used in subsequent toxicity bioassays.

As determined through the droplet-feeding bioassay, Cry1A.105 was the most lethal towards FAW larvae compared to Cry1AC, Cry1Ab and Cry2Ab2.820, an observation that has previously been observed in FAW from the Western Hemisphere (Hernández-Rodríguez et al., 2013; Herrero et al., 2016). The growth inhibition caused by Cry1A.105 was also the most pronounced compared to Cry1Ab, Cry1Ac, and Cry2Ab2.820, with the lowest ID_{50} values reported for Cry1A.105. The LD_{50}/ID_{50} ratio is an indication of the ability of a Cry protein to cause greater growth inhibition than mortality (Hernández-Martínez et al., 2008). The LD_{50}/ID_{50} ratio of Cry1A.105 was 4.3 for first instar FAW larvae, indicating that Cry1A.105 has a significant effect on the larval growth at doses much lower than those that cause mortality.

Cry2Ab2.820 had the second lowest LD_{50} value for first instar larvae, the lowest ID_{50} value for first instar, but the second highest ID_{50} value for second instar FAW larvae. However, the LD_{50}/ID_{50} ratio of Cry2Ab2.820 (41.83) was considerably larger than that of Cry1A.105 (4.33) indicating that while the LD_{50} of Cry2Ab.820 is lower than that of Cry1A.015, it can inhibit larval growth and development at a dose 41.83-fold less than what is required to cause mortality for first and second instar larvae. The toxicity of Cry2Ab in FAW has long been debated (Frankenhuyzen, 2009; Herrero et al., 2016). Several studies have reported Cry2Ab being less toxic than Cry1Ab and Cry1Ac (Hernández-Martínez et al., 2008; Lu et al., 2013). Another study reported that Cry2Ab had little to no effect on the lab susceptible or the field-resistant populations of FAW (Monnerat et al., 2015). Furthermore, a 50% mortality was unable to be achieved even at a Cry2Ab concentration of >1350 ng/cm^2 (Hernández-Rodríguez et al., 2013). In contrast, the present study was more comparable with Soares

Figueiredo et al. (2019), which reported Cry1Ab to be less toxic than Cry2Ab by 6-fold against FAW larvae. Similarly, Cry1F susceptible larvae have been observed to be the most susceptible to Cry2Ab2, followed by Cry1Ab, and the least susceptible to Cry1Ac in terms of mortality as well as growth inhibition (Gutierrez-Moreno, 2017; Li et al., 2016; Yang et al., 2017).

Cry1Ab and Cry1Ac were only mildly toxic toward FAW larvae compared to Cry1A.105 and Cry2Ab2.820. This was evident by the finding that even at the highest possible dose, LD_{50} values for Cry1Ab and Cry1AC could not be calculated for first and second instar FAW larvae. Furthermore, an ID_{50} value for Cry1Ac could not be calculated for second instar FAW larvae. Similar findings in the Western Hemisphere have been reported where LC_{50} values of Cry1Ab for first instar FAW were the highest compared to Cry1F and Cry1A.105 (Hernández-Rodríguez et al., 2013), and compared to Cry2Ab (Soares Figueiredo et al., 2019). Similar to the present study, Hernández-Rodríguez et al. (2013) reported an LC_{50} value of >4050 ng/cm^2 for Cry1Ac which represented the highest concentration used in the bioassay which produced less than 50% mortality.

In the unpublished United States and European Union documentations, Bt Cry protein in Bt MON810 maize was reported at: 7.59- 15.06 µg/g fresh weight in leaf, 0.09 µg/g fresh weight in pollen and 0.19 to 0.91 µg/g fresh weight in grain (Székács et al., 2010). However, Cry1Ab protein content in MON810 maize has been found to be season-specific, and soil nitrogen dependent in addition to varying across growth stages and plant tissue (Bruns and Abel, 2003; Nguyen and Jehle, 2007). Furthermore, Bt Cry1Ab protein content in MON810 maize also varies under cold/wet and hot/dry environmental stresses (Trtikova et al., 2015). Nguyen and Jehle (2007), reported that Cry1Ab protein content from MON810 maize collected from two sites in Germany was 0.33 to 1.13 in µg/g fresh weight in the stalk, 2.54 to 4.37 µg/g fresh weight in lower leaf, 2.451 to 5.52 µg/g fresh weight in lower leaf and 0.0.57 to 0.509 µg/g fresh weight in the grain during the 2001 to 2003 growing season. Thus, contrary to what documents from the United States and the European Union reported, Cry1Ab protein content in MON810 maize is much lower than expected. Furthermore, Cry1Ab protein content in maize leaves has been found to decrease by approximately 1.6 fold from 2001 to 2008 (Székács et al., 2010). Thus Cry1Ab expressed by MON810 maize is likely, not high enough to be considered a “high-dose” to kill >99% of FAW in South Africa (Botha et al., 2019; Luttrell et al., 1999; Omoto et al., 2016; Sousa et al., 2016).

However, the ID_{50} of Cry1Ab was lower in second instar compared to that of Cry2Ab2.820. Cry1Ab could be more toxic than Cry2Ab2.820 in second instars due to it being an already activated tryptic core. Proteolytic activation of Bt Cry protoxins can act as a limiting step that dictates the susceptibility of an insect to Bt Cry proteins. Activation of a protoxin with *Aedes aegypti* gut extract created an activated toxin with different specificity compared to the same protoxin that was activated with *Phyllosticta brassicae* gut enzymes or trypsin. Furthermore, feeding non-susceptible species with activated toxins can cause toxicity, and feeding susceptible individuals with protease inhibitors could reduce toxicity (Deist et al., 2014). Likewise, improved toxicity is often observed when administering activated toxins instead of protoxin mixtures (Deist et al., 2014).

Ultimately, the present study showed that the droplet-feeding method, which has more advantages than diet-based bioassays in terms of dose determination, was compatible with the evaluation of the toxicity of Bt Cry proteins in highly alkaline Cry protein solubilising buffers. However, while the droplet-feeding method can be used to determine ID_{50} values, it may not be suitable for the determination of LD_{50} values for mildly toxic proteins, such as Cry1Ac and Cry1Ab, in FAW. The overall order of toxicity of Bt Cry proteins against the V1 populations from the least toxic to the most toxic was Cry1Ac < Cry1Ab < Cry2Ab2.820 < Cry1A.105. This order of toxicity indicates what can be expected for the susceptibility of other FAW populations to Bt Cry proteins in South Africa.

Table 6: Volumes of highly alkaline buffers imbibed by first and second instar larvae of FAW population (V1) as determined by the droplet-feeding and fluorescent dye method

Solubilising buffer named after each Cry protein	First instar[1]				Second instar[2]			
	Volume imbibed (nl)[3]	95% Confidence intervals (nl)		CV (%)	Volume imbibed (nl)[3]	95% Confidence intervals (nl)		CV (%)
		Lower	Upper			Lower	Upper	
Cry1A.105	7.06 (69)^{ac}	5.87	8.51	34.64	15.89 (70)^{a}	12.45	20.28	46.88
Cry1Ab	8.57 (71)^{a}	7.63	9.63	21.55	18.03 (70)^{ac}	14.09	23.01	47.03
Cry1Ac	5.06 (68)^{b}	4.35	5.88	27.59	9.72 (73)^{b}	7.44	12.71	53.09
Cry2Ab2.820	5.60 (95)^{bc}	5.01	6.25	23.96	15.17 (96)^{a}	12.74	18.07	38.78
sdH_2O	5.45 (71)^{bc}	4.49	6.61	36.55	25.53 (74)^{c}	21.43	30.41	33.78

[1] $F_{(4,369)} = 7.952$, $P < 0.001$; Values followed by the same letters are not significantly different at $\alpha = 0.05$ (*post-hoc* Tukey test).
[2] $F_{(4,378)} = 9.523$, $P < 0.001$; Values followed by the same letters are not significantly different at $\alpha = 0.05$ (*post-hoc* Tukey test).
[3] Volumes imbibed are presented as the mean (sample size).

Table 7: Median lethal dose (LD_{50}) of first and second instar larvae of the V1 population of FAW to agronomically important Bt Cry proteins as determined by droplet-feeding bioassays

Bt Cry protein	First instar					Second instar				
	LD_{50} (ng)	95% Fiducial limits (ng)		Slope ± SE	X^2	LD_{50} (ng)	95% Fiducial limits (ng)		Slope ± SE	X^2
		Lower	Upper				Lower	Upper		
Cry1Ac	>6.45	-	-	-	-	>6.45	-	-	-	-
Cry1Ab	> 14.04	-	-	-	-	> 14.04	-	-	-	-
Cry1A.105	0.65	0.54	0.77	1.19 ± 0.0	15.17	>6.78	-	-	-	-
Cry2Ab2.820	2.51	1.34	7.32	0.53 ± 0.09	5.51	>1.75	-	-	-	-

> indicates that the maximum dose (undiluted, neat Bt Cry protein) was used
"-" indicates values that could not be determined due to mortality not being observed even at the maximum dose

Table 8: Median inhibitory dose (ID_{50}) of first and second instar larvae of the V1 population of FAW to agronomically important Bt Cry proteins as determined by droplet-feeding bioassays

Bt Cry protein	First instar				Second instar			
	ID_{50} (ng)	95% Confidence intervals (ng)		Slope (95% CI)	ID_{50} (ng)	95% Confidence intervals (ng)		Slope (95% CI)
		Lower	Upper			Lower	Upper	
Cry1Ac	0.95	0.36	1.50	-1.18 (-2.09 to -0.51)	>6.45	-	-	-
Cry1Ab	1.60	1.17	2.15	-1.13 (-1.56 to -0.84)	0.54	0.26	0.89	-0.78 (-1.22 to -0.50)
Cry1A.105	0.15	0.11	0.19	-0.80 (-1.07 to -0.64)	0.13	0.03	0.25	-0.60 (-1.01 to -0.30)
Cry2Ab2.820	0.06	0.04	0.09	-0.7 (-1.02 to -0.60)	1.71	1.06	2.30	-1.69 (-2.82 to -0.82)

> indicates that the maximum dose (undiluted, neat Bt Cry protein) was used

"-" indicates values that could not be determined due to mortality not being observed even at the maximum dose

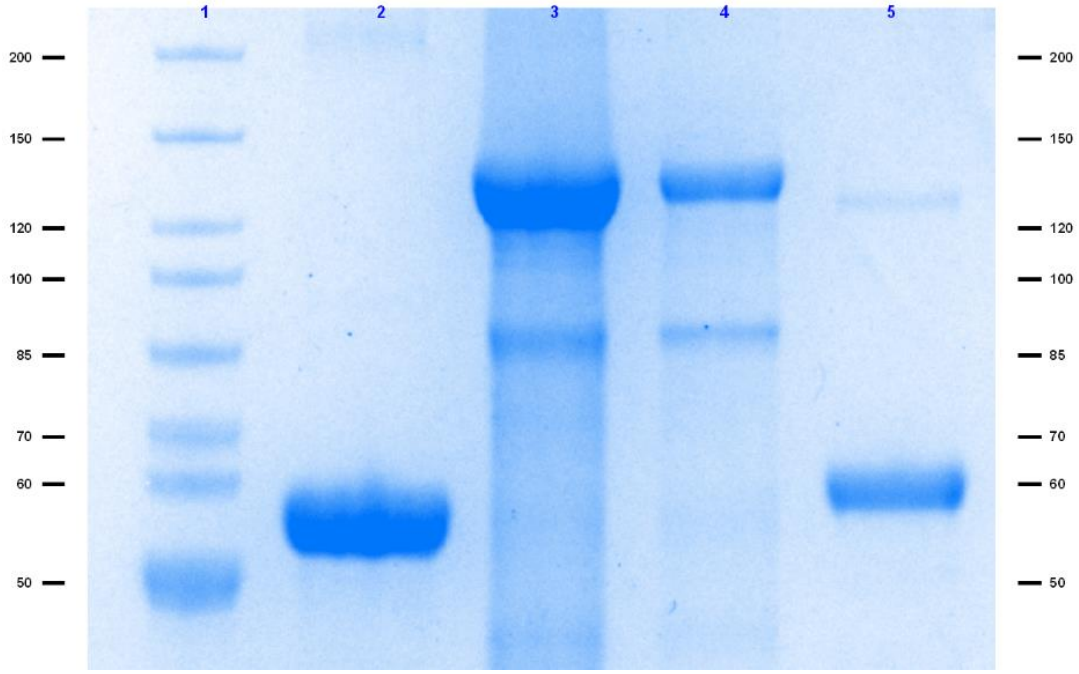
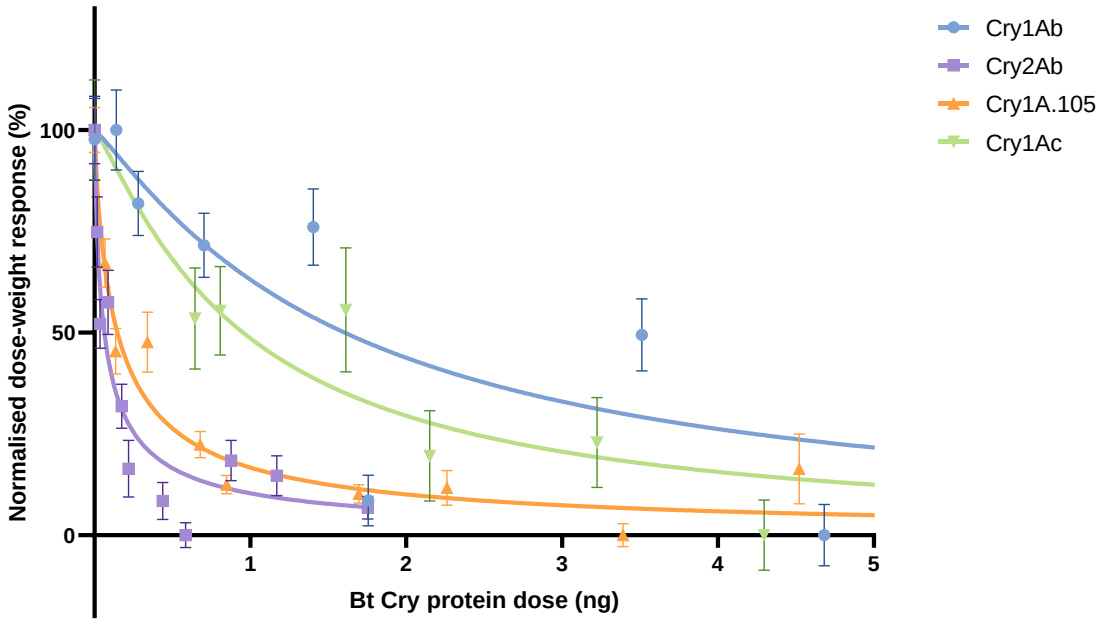


Figure 8: SDS-PAGE of Bt Cry proteins received from the Monsanto Company. Lane 1, PageRuler Unstained Protein Ladder 26614 (Thermo Fisher Scientific, USA); lane 2, Cry1Ab tryptic core at 58.4 kDa; lane 3, Cry1Ac at 131.7 kDa; lane 4, Cry1A.105 at 131.5 kDa; and lane 5, Cry2Ab2.820 at 61.1 kDa.

A



B

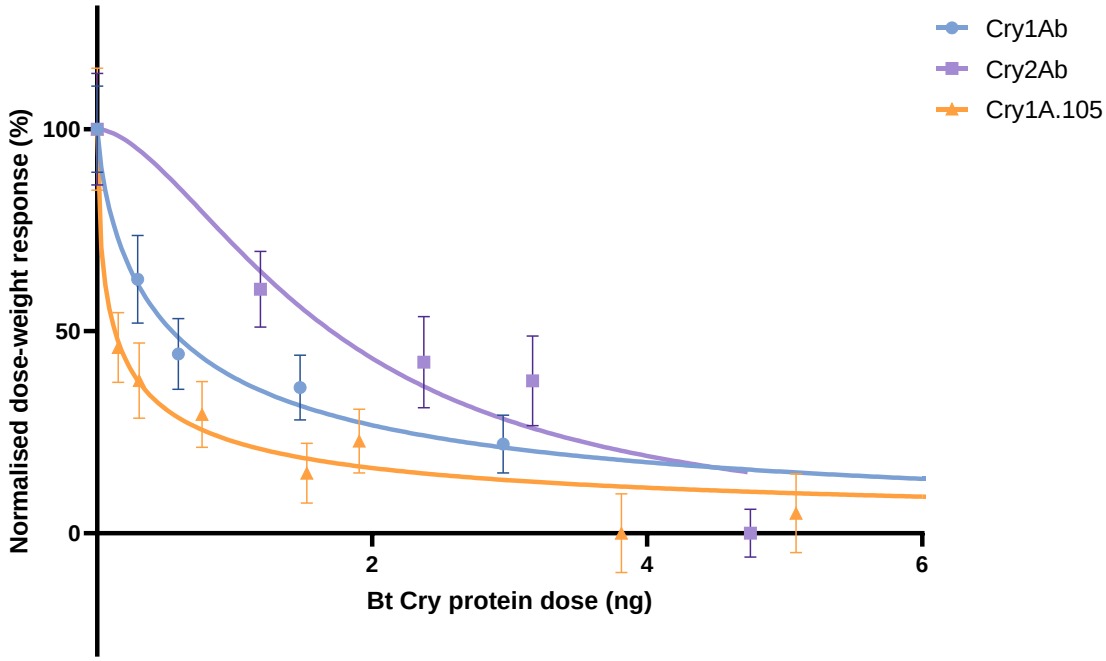


Figure 9: Growth-inhibitory response of FAW larvae based on mean dose-weight response as determined by droplet-feeding bioassays for FAW larvae. **(A)** First instar larvae, $P < 0.001$; 28.3 (6, 2406), and **(B)** Second instar larvae, $P < 0.001$; 4.92 (6, 2097). Since a statistically significant curve could not be fitted for the Cry1Ac protein, the data points and curve are not shown for second instar larvae. Best-fit non-linear regression curves were fitted based on Extra sum-of-squares F-test. P value; F (DFn, DFd) for each instar shows whether best-fit ID_{50} values for each Bt Cry protein were significantly different. Data points represent mean normalised response of each Bt Cry protein with error bars representing the standard error of the mean. To clearly show the dose-response curve of Cry1A.105, which required smaller doses, the full range of doses for Cry1Ac, Cry1Ab and Cry2Ab2.820 is not shown.

Chapter 4:

Toxicity of *Bacillus thuringiensis* Cry proteins against a reference and field populations of fall armyworm, *Spodoptera frugiperda* (J.E. Smith), in South Africa

4.1. Abstract

In countries such as the United States and Brazil, *Bacillus thuringiensis* (Bt) maize expressing Bt Cry1 and Cry2 proteins are important for the control of fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith), the agronomically important maize pest. In South Africa, Bt maize with the MON810 (Cry1Ab), Bt11 (Cry1Ab) and MON89034 (Cry1A.105 and Cry2Ab2.820) events are approved for cultivation. However, to the best of our knowledge, the susceptibility of FAW populations to agronomically important Bt Cry proteins, in terms of the median lethal concentration (LC_{50}) and median inhibitory concentration (IC_{50}) has not been evaluated in South Africa. Thus, the study aimed to evaluate the toxicity of Bt Cry proteins (Cry1Ac, Cry1Ab, Cry1A.105 and Cry2Ab2.820) against first and second instar FAW larvae of a reference (V1) and five field populations of FAW. The LC_{50} and IC_{50} of each Bt Cry protein against first and second instar FAW larvae in each population were evaluated by diet-overlay bioassays. Overall, the majority of LC_{50} and IC_{50} values for first and second instar FAW larvae were the highest for the V1 reference population but the overall susceptibility of all six FAW populations to Bt Cry proteins was highly variable. Despite the variability, the LC_{50} values of Cry1A.105 against first and second instar FAW larvae were the smallest across all six FAW populations, ranging between 0.9 - 6.4 ng/cm^2 and 30.4 - 858.5 ng/cm^2, respectively. The toxicity of Bt Cry proteins evaluated in the present study can serve as a baseline of the susceptibility of the FAW field populations to Bt Cry proteins.

4.2. Introduction

The highly polyphagous agricultural pest, fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith), which is native to the Western Hemisphere, has recently established itself in Africa, including South Africa (Cock et al., 2017; FAO, 2018; Goergen et al., 2016). In the Western Hemisphere, FAW can be controlled by *Bacillus thuringiensis* (Bt) maize expressing Bt insecticidal Cry proteins such as Cry1Ab, Cry1F, Cry1A.105 and Cry2Ab2 (Blanco et al., 2016; Burtet et al., 2017; Drury et al., 2008; Omoto et al., 2016; Rule et al., 2014; Sousa et al., 2016).

In South Africa, Bt maize expressing Cry1Ab through the Bt11 and MON810 events and Cry1A.105 and Cry2Ab2 through the MON89034 event, are approved for cultivation (ISAAA, 2022). The Bt11 and MON810 events are available as single toxin Bt maize or stacked event Bt maize (ISAAA, 2022). Single Bt11 event Bt maize has been approved for cultivation in South Africa since 2003 and stacked Bt maize with Bt11 x GA21 (herbicide tolerance) has been approved for cultivation since 2010. Single MON810 event Bt maize was approved for cultivation in 1997, stacked NK603 x MON810 Bt maize was approved in 2007 and stacked TC1507 x MON810 and TC1507 x MON810 x NK603 Bt maize was approved in 2014, where NK603 confers glyphosphate herbicide tolerance and TC1507 confers Cry1Fa2 (ISAAA, 2022). MON89034 as single and stacked MON89034 x NK603 Bt maize were both approved for cultivation in 2010 while stacked MON89034 x TC1507 x NK603 Bt maize was approved in 2018 for cultivation in South Africa (ISAAA, 2022).

FAW infestations in South Africa have only been observed in maize fields, thus the commercially available Bt maize in South Africa, makes the control of FAW easily achievable. However, the resistance of FAW towards Bt maize has been observed in the Western Hemisphere. As seen in Puerto Rico, field evolved Cry1F resistance in FAW can manifest as quickly as 3 years after the cultivation of Bt maize, and was subsequently identified in, Brazil, the United States of America and Argentina (Chandrasena et al., 2017; Farias et al., 2014a; Huang et al., 2014; Li et al., 2016; Omoto et al., 2016; Storer et al., 2012). Furthermore, cross-resistance of Cry1F resistance has been reported for Cry1Ab, Cry1Ac, and Cry1A.105 in FAW (Bernardi et al., 2015; Huang et al., 2016; Niu et al., 2016).

Since FAW populations likely originated from regions in the Western Hemisphere where the resistance of FAW to Bt Cry proteins is widespread, it is possible that they would, to some

extent, harbour resistant alleles towards Bt Cry proteins expressed by Bt maize as well. Thus, the efficacy of Bt maize for the control of FAW in South Africa will depend on the susceptibility of field populations, with or without Bt Cry protein resistance alleles, to the expression levels of Bt Cry proteins in Bt maize. Furthermore, the difference in susceptibility between different field populations, such as the lower susceptibility of certain FAW populations to Bt maize, may be indicative of resistance alleles (Bernardi et al., 2015; Luttrell et al., 1999).

However, natural variation in the susceptibility of FAW to Bt Cry proteins exists that is not always indicative of resistance development. Natural variation in four field populations of FAW sampled in the same season from four different regions in Brazil was approximately 4-fold between LC_{50} (median lethal concentration) values and 2-fold between EC_{50} (median effective concentration) values (Farias et al., 2014b). In a different study that evaluated the toxicity of Cry1F against 11 populations of FAW, almost an 8-fold difference in LC_{50} values, a 20-fold difference in LC_{90} (maximum lethal concentration) values and a 3- to 5-fold difference in EC_{50} values were observed (Da Silva et al., 2016). Furthermore, in the closely related species, *Spodoptera exigua,* a 6.5-fold difference for Cry1Ab, 1.2-fold for Cry1Ac and 25-fold difference for Cry1Da have been reported for LC_{50} values (Hernández-Martínez et al., 2008).

Variation in the susceptibility of FAW populations to Bt Cry proteins can be indicative of field-evolved resistance development if some populations exhibit much lower susceptibility to a Bt maize event, however, it can also be indicative of innate susceptibility differences between populations. Nevertheless, to detect variation in susceptibility of pests to Bt Cry proteins, toxicity studies using Bt Cry proteins must first be conducted to determine lethal doses (LDs) or LCs, or growth-inhibitory doses (IDs) or ICs. To the best of our knowledge, the toxicity of agronomically important Bt Cry proteins, expressed by commercially available Bt maize has not been evaluated and the baseline susceptibility of different FAW populations to Bt Cry proteins in terms of LDs and IDs or LCs and ICs have not been evaluated. Thus, the present study is the first study aimed at evaluating the toxicity of agronomically important Bt Cry proteins (Cry1Ab, Cry1Ac, Cry2Ab2.820 and Cry1A.105) to six different populations of FAW in South Africa. Furthermore, the susceptibility of a population, designated as a reference population, due to it being sampled three years before the others, served as a benchmark against which the susceptibility of later-sampled populations can be compared.

5.3. Methods and materials

4.3.1. Insects

All FAW populations were reared as per section 2.3.1. The V1 population is a field population that was sampled in April 2018, from White River, Mpumalanga. The V1 population was chosen as the reference population in this study because it was the earliest-sampled population and the toxicity of Bt Cry proteins against the V1 population had already been determined by droplet-feeding bioassays in Chapter 3. Thus, the V1 population served as a benchmark for the comparison of Bt Cry protein susceptibility with other field populations. Furthermore, preliminary trials of the droplet-feeding bioassay method were performed on a population of FAW sampled around the same time as the V1 population. This population (P3) was sampled from Schoemanskloof, Mpumalanga, in 2017, a year before V1 was sampled. However, the population was lost due to unforeseen circumstances. Nevertheless, a few replicates of droplet-feeding bioassays to evaluate the sublethal exposure of Cry1Ab, Cry1Ac and Cry1A.105 were obtained. The ID_{50} values for the P3 population were as follow: 1.18 (0.57 – 2.05) ng for Cry1Ab, 0.21 (0.16 – 0.26) ng for Cry1A.105, and 0.53 (0.18 – 1.13) ng for Cry1Ac. The 95% confidence interval of the ID_{50} values of the P3 population was in range with the values obtained for V1 in Chapter 3. Toxicity evaluation of the Bt Cry proteins against V1 is therefore not an outlier. Thus, the V1 population is a good reference population that reflects other populations sampled around the same time from the Mpumalanga province.

The field populations were sampled from the Limpopo (MH1, LE1) and the North West (BR1, K1F1 and K1F2) provinces during January and February of 2021. The exact locations can be found in section 2.3.1.

4.3.2. Bt Cry proteins

The four agronomically important Bt Cry proteins (Cry1Ac, Cry1Ab, Cry1A.105 and Cry2Ab2.820) and their respective buffers were provided by the Monsanto Company. The composition of the Cry protein-solubilising buffers is as per section 3.3.2.

4.3.3. Bioassays

While Chapter 4 determined that the droplet-feeding method was compatible with the bioassay of Bt Cry proteins in highly alkaline buffers against FAW and that sublethal effects

can be evaluated, lethal effects could not be determined for the Bt Cry proteins in the study. Thus, to try to elicit a mortality response, FAW were subjected to a longer exposure (7 days) to Bt Cry proteins using the diet-overlay bioassay method. Furthermore, the diet-overlay method is a common method in the literature for the bioassay of Bt Cry proteins against FAW larvae (Beegle, 1990; Da Silva et al., 2016; Ferre et al., 1991; Gutierrez-Moreno et al., 2020; Marçon et al., 1999; Wu et al., 2009). The diet-overlay method cannot be used to determine LDs and IDs, however, it would enable the direct comparison of LC_{50} and IC_{50} values with current literature.

At least three replicates of 16 larvae per concentration were used in the diet-overlay bioassays. Aliquots of 75 µl of a range of concentrations for each Bt Cry protein were uniformly dispensed over the meridic diet (7 cm^2 surface area per well) and allowed to air-dry (Marçon et al., 1999). Each of the Cry protein-solubilising buffers, without Cry protein, was dispensed over the meridic diet as controls. To evaluate LC_{50} of the Bt Cry proteins, mortality was scored after 7 days. Larvae that had survived after 7 days were weighed to determine the sublethal effect through the determination of the IC_{50} (Marçon et al., 1999). The scoring of mortality included functional mortality, where larvae that failed to moult to the next instar were considered dead (MarÇon et al., 2000). From our observations of FAW larvae, first instar larvae that failed to moult to the next instar weighed less than 0.5 mg, while second instar larvae that failed to moult to the next instar weighed less than 1 mg. Thus, larvae that weighed less than or equal to 0.5 mg for first instar larvae and less than or equal to 1 mg for second instar larvae were considered dead.

4.3.4. Data analysis

The LC_{50} was determined by probit regression analysis (Finney, 1952). Outliers in weight-response data were removed based on Grubbs tests at $\alpha = 0.05$. Thereafter, weight-response data was normalised using the following settings on GraphPad Prism: the smallest mean in each data set was normalised to 0% and the largest mean in each dataset was normalised to 100%. The IC_{50} values were determined by non-linear regression analysis using the function: [inhibitor] vs normalised response, least squares fit (GraphPad Prism 9.3.1). The best fit IC_{50} values for each Bt Cry protein were also compared by the Extra sum of squares F test to determine whether the ID_{50} values were statistically significant ($P < 0.05$) from each other

4.4. Results

For first instar FAW larvae, the LC_{50} values for Cry1Ac and Cry1Ab against the V1 population could not be determined since mortality was not observed even at the highest concentration (Table 9). The LC_{50} values for first and second instar FAW larvae were significantly the highest for the V1 reference population than the field populations, except for the Cry1A.105 protein (Tables 9 and 10). The LC_{50} of Cry2Ab2.820 against first instar larvae of the V1 population was significantly higher than that of any other field population, by 5- to 22-fold (Table 9). The LC_{50} values for first instar FAW larvae of the field populations ranged from 235.5 - 7 314.3 ng/cm^2 for Cry1Ac, 25.5 - 196.9 ng/cm^2 for Cry1Ab, 20.1 - 80.9 ng/cm^2 for Cry2Ab2.820, and 0.9 - 6.4 ng/cm^2 for Cry1A.105 (Table 9). The LC_{50} values among first instar field populations for each protein differed by approximately 31-fold for Cry1Ac, 8-fold for Cry1Ab, 4-fold for Cry2Ab2.820 and 7-fold for Cry1A.105. The LC_{50} values for Cry1Ac for the five field populations (excluding V1) were the highest, indicating that Cry1Ac was the least lethal towards first instar FAW (Table 9). In contrast, the LC_{50} values of Cry1A.105 were the lowest compared to the other Cry proteins, across all six populations, including the V1 population (Table 9).

For second instar FAW larvae, the LC_{50} values for Cry1Ac could only be determined for K1F2, 4 952.1 (1 877.6 - 11 539.9) ng/cm^2, and BR1, 7 659.1 (5 774.8 - 10 824.8 ng/cm^2) (Table 10). Cry1Ac was not lethal towards second instar FAW larvae of the V1 and the field populations MH1, LE1 and K1F1 (Table 10). Similarly, Cry2Ab2.820 was only lethal to K1F2 second instar larvae (Table 10). Cry1Ab was not lethal to second instar FAW larvae of the V1 reference population, however, Cry1Ab was lethal to second instar FAW larvae of the field populations, where the LC_{50} values ranged between 668.6 - 7 477.4 ng/cm^2 (Table 10). The LC_{50} of Cry1A.105 against the second instar V1 population (858.5 ng/cm^2) was the highest (28-fold larger) compared to the LC_{50} value of the field populations which ranged between 30.4 and 68.6 ng/cm^2 (Table 10). Based on LC_{50} values, Cry1A.105 was the most toxic towards second instar larvae, just like first instar larvae, of both the V1 population and the five field populations.

Within each population (both V1 and field populations), statistical significance was found between the non-linear regression curves for the IC_{50} determination ($P < 0.001$), indicating that the concentration-response curves and the IC_{50} values for each Bt Cry protein were significantly different for each population (Tables 11 and 12). The IC_{50} values for all first

instar FAW larvae, including the V1 population, ranged between 17.58 - 742.4 ng/cm^2 for Cry1Ac, 21.25 - 190.1 ng/cm^2 for Cry1Ab, 1.9 - 30.47 ng/cm^2 for Cry2Ab2.820, and 0.7 - 13.98 ng/cm^2 for Cry1A.105 (Table 11). The IC_{50} values of first instar larvae of the V1 population were higher for Cry1Ab and Cry1A.105 but not for Cry1Ac and Cry2Ab2.820 (Table 11). The IC_{50} values all second instar FAW larvae, including the V1 population ranged from 116.8 to 5 015 ng/cm^2 for Cry1Ac, 10.09 to 1 190 ng/cm^2 for Cry1Ab, 2.42 to 89.28 ng/cm^2 for Cry2Ab2.820, and 2.47 to 37.18 ng/cm^2 for Cry1A.105 (Table 12). Thus, collectively across all populations and for both instars, the highest IC_{50} values were determined for Cry1Ac, followed by Cry1Ab, Cry2Ab2.820 and ending with the lowest IC_{50} value for Cry1A.105 (Tables 11 and 12).

4.5. Discussion

The LC_{50} values of first instar FAW larvae to Cry2Ab2 is highly variable and can range from 49.7 - 173.2 ng/cm^2 between five populations (Gutierrez-Moreno et al., 2020), 840 (530 - 1 580) ng/cm^2 (Soares Figueiredo et al., 2019), to cases where even >1350 ng/cm^2 could not result in mortality (Hernández-Rodríguez et al., 2013). Concentration of Cry2Ab at >31 000 ng/cm^2 have also been found to not cause mortality in *Spodoptera exigua* (Hernández-Martínez et al., 2008). However, the LC_{50} of the first instar V1 reference population to Cry2Ab2.820 (444.9 ng/cm^2) is similar to that reported as 460 - 890 ng/cm^2 by Gilreath et al., (2021). The range of LC_{50} values of first instar field populations to Cry2Ab2.820 (20.1- 80.9 ng/cm^2) of FAW fell somewhere in between the range of the above-mentioned studies. The range of IC_{50} values for Cry2Ab2.820 (1.9 - 8.4 ng/cm^2) against first instar MH1, K1F1, LE1 and BR1 populations was slightly higher than what was reported to be 1.76 ng/cm^2 by Bernardi et al., (2015). Interestingly, the 95% confidence interval of the LC_{50} value of Cry2Ab 2.820 to first instar V1 larvae (297.5 - 643 ng/cm^2) was in the range of the values reported for known resistant FAW populations where LC_{50} of neonates resistant to Cry2Ab2 have been reported at 146.96 (83.37–330.35) ng/cm^2 (Bernardi et al., 2015). It is therefore plausible that V1 and K1F2 FAW populations are resistant to Cry2 proteins. However, without a more in-depth investigation into resistant alleles or a comprehensive resistance ratio analysis, the resistance of the V1 and K1F2 populations cannot be postulated. The observation that Cry2Ab2.820 was not lethal towards second instar FAW larvae in 5/6 of the populations in the study does not directly infer resistance towards Cry2Ab2.820. The observation was more likely due to the low maximum concentration of Cry2Ab2.820 available during the study. The highest possible Cry2Ab2.820 concentration that was assessed was the neat undiluted Cry2Ab2.820 protein at >3 323 ng/cm2 compared to 17 381 ng/cm2 of Cry1Ab that was available. Thus, we could not evaluate whether higher concentrations of Cry2Ab2.820 would be lethal towards the FAW populations in the present study.

Overall, across both instars and all the populations, the highest LC_{50} and IC_{50} values were obtained for Cry1Ac. This observation was consistent with studies that found Cry1Ac to be the least toxic towards FAW larvae in comparison to other Cry1 and Cry2 proteins (Graser et al., 2017; Gutierrez-Moreno et al., 2020; Hernández-Rodríguez et al., 2013; Ríos-Díez et al., 2012; Soares Figueiredo et al., 2019). In particular, first instar V1 larvae were not killed despite being exposed to the maximum concentration of Cry1Ac (undiluted, neat Cry1Ac).

Similar findings have been observed where neonate FAW larvae did not succumb to Cry1Ac even at the highest concentration in the studies (Gutierrez-Moreno et al., 2020; Hernández-Rodríguez et al., 2013). Other studies have reported LC_{50} values of 47.55 (18.62 - 397.63) ng/cm^2 (Soares Figueiredo et al., 2019) and ranging between 15.3 -1815 ng/cm^2 in five populations (Gutierrez-Moreno et al., 2020) for Cry1Ac against FAW neonates. The LC_{50} values ranging between 235.5 - 7314.3 ng/cm^2 for first instar FAW field populations (MH1, K1F1, K1F2, LE1 and BR1) were thus in range with other studies (Gutierrez-Moreno et al., 2020; Soares Figueiredo et al., 2019).

The overall lowest LC_{50} and IC_{50} values across all populations of FAW were evaluated for Cry1A.105, thus Cry1A.105 appeared to be the most toxic toward FAW populations in South Africa. The range of LC_{50} values of Cry1A.105 (0.9 - 6.4 ng/cm^2) for first instar larvae across all populations in the present study was comparable with what has been previously reported to be 4.6 - 273.8 ng/cm^2 for five FAW populations across Latin America (Gutierrez-Moreno et al., 2020) and 4.75 (2.67–7.04) ng/cm^2 for populations with homozygous susceptibility traits, while lower than another study that reported LC_{50} values of 400 (261–652) ng/cm^2 for FAW neonates (Hernández-Rodríguez et al., 2013). The range of IC_{50} values (0.7 – 13.98 ng/cm^2) obtained for first instar larvae across all the populations was more similar to a study that reported IC_{50} values of 0.67 (0.53–0.84) ng/cm^2 (Bernardi et al., 2015) than a study that reported EC_{50} values of Cry2Ab2 (102.21–191.02 ng/cm^2) against resistant (Bernardi et al., 2015).

For Cry1Ab, first and second instar FAW larvae of the V1 population did not respond in terms of mortality even at the highest concentration of 13 559 ng/cm^2. However, for the field populations, the LC_{50} for first instar FAW larvae ranged between 25.5 to 196.9 ng/cm^2. These values were not an exact match with other studies of Cry1Ab against FAW but are in the range of values that have been reported for other studies where LC_{50} values were 3.76 (2.10–10.50) ng/cm^2 and 783 (394–2282) ng/cm^2 (Hernández-Rodríguez et al., 2013; Soares Figueiredo et al., 2019).

Comparing the susceptibility of FAW to Bt Cry proteins from other studies mentioned above, it is evident that LC_{50} and IC_{50} values can vary significantly within the same species even when the same diet-overlay technique is used across different laboratories. In the present study, the variation in the LC_{50} values of first instar FAW larvae of the V1 population for Cry1Ac and Cry1Ab, could not be observed between the V1 and the other populations

because LC_{50} values could not be obtained for Cry1Ac and Cry1Ab. However, the LC_{50} values of Cry1Ac and Cry1Ab for first instar FAW larvae, excluding the V1 population, differed by approximately 31-fold and 8-fold respectively. The variation of LC_{50} values of CryAb2.820 and Cry1A.105 for all populations, including the V1 population, differed by approximately 22-fold and 7-fold respectively. Similar variations in LC_{50} values have been reported before. A 30-fold difference in LC_{50} values has been observed between nine Bt strains and between 4-fold and at least 285-fold between Cry1B, Cry1C and Cry1D for three FAW populations (Mexico, Brazil and Colombia) (Monnerat et al., 2006). In a study that evaluated the baseline susceptibility of 28 populations of FAW from Mexico to Cry1F, the LC_{50}, LC_{95}, GI_{50} (growth inhibition) and GI_{95} values differed by approximately 11-fold, 6-fold, 15-fold and 16-fold respectively (Rivero-Borja et al., 2020). In a different study, the LC_{50} values of Cry1F, Cry1Ac, Cry1A.105 and Cry2Ab2 for first instar FAW larvae varied by approximately 1.6-fold, 12-fold, 3-fold and 3.7-fold respectively, across three FAW populations from Mexico (Gutierrez-Moreno et al., 2020). Thus, while the variation of mortality and growth inhibitory responses of FAW in the present study towards the four Bt proteins were high, it is not inconsistent with the variation observed in the populations described above.

The overall finding was that the field populations sampled 3 years after the V1 populations appeared to be more susceptible to Bt Cry proteins. The reduction in susceptibility of the field populations could be associated with inherent fitness costs as a result of resistant alleles that the populations carried over from the Western Hemisphere. Fitness costs are defined by a reduction in biological or life cycle parameters such as larval body weight, neonate-to-pupation rate, pupation time, pupal weight, sex ratio, and egg production (Gassmann et al., 2008). A FAW strain resistant to MON89034 x TC1507 x NK603 (conferring herbicide tolerance in addition to insect resistance through Cry1A.105 and Cry2Ab2) maize was found to exhibit a slight fitness cost that was recessive in inheritance (Bernardi et al., 2017). In two populations (Florida and Puerto Rico) of FAW resistant to Cry1F considerable fitness cost was found (Dangal and Huang, 2015). In three out of five FAW populations sampled from Bt maize in Brazil expressing a single Cry1Ab protein, no fitness costs were found (Sousa et al., 2016). Resistance of FAW to Cry2Ab2.820 is the only Bt Cry protein shown to not be associated with fitness costs. A study using FAW that is highly resistant to Cry2Ab2.820 was found to be the cause of a single autosomal, and recessive or incompletely recessive gene, which was not associated with significant fitness costs (Acharya et al., 2017). If there are

fitness costs, resistant homozygous individuals would be less likely to produce offspring thereby reducing the development of resistance in the population (Gassmann et al., 2008). It is possible that in the 3 years after the V1 population was sampled, the remaining field populations that carried Cry1 resistant alleles succumbed to fitness costs associated with Cry1 proteins. Thus, individuals with any recessive resistant traits for Cry1 proteins that they may have carried from the Western Hemisphere upon entry into South Africa may have not been able to reproduce, thereby contributing to a loss of recessive resistance alleles. The opposite may have been true for Cry2Ab2.820 resistance. Since Cry2Ab2 protein is not associated with fitness costs, field populations would have retained any resistant alleles. This would explain why the toxicity of Cry1 proteins increased for the field populations compared to the V1 population while the toxicity of Cry2Ab2.820 did not reduce between V1 and K1F2. On the other hand, because V1 has been lab-reared since 2018, it could only interbreed among itself thereby maintaining any resistant alleles. However, without further research into the presence of resistance alleles in the FAW populations, this observation can only be a possible scenario and not a definite one.

The variation in the susceptibility of the FAW populations to Bt Cry proteins in the present study does not necessarily have to be linked with resistance alleles and related fitness costs. Natural variation in the susceptibility of FAW populations from Mexico to Bt Cry proteins, reported by Rivero-Borja et al. (2020) and Gutierrez-Moreno et al. (2020), was observed in populations that have not been directly exposed to Bt Cry proteins since Bt maize is not cultivated in Mexico. Thus, the variation in the susceptibility of Mexican FAW populations to Bt Cry proteins should not have been associated with resistance alleles that might have evolved from selection pressure in the field by Bt maize. The natural variation between FAW populations that were not exposed to Bt maize portrays a scenario like that of the FAW populations in the present study because, to the best of our knowledge, the likelihood of exposure of the field populations in the present study to Bt maize before sampling was low. However, even if there were admixture between resistant FAW individuals that were exposed to Bt maize it still would not necessarily induce a difference in the susceptibility of the FAW populations in South Africa. A study investigating the gene flow between 24 FAW specimens, consisting of both corn strain (CS) and rice Strain (RS), sampled from nine locations between Brazil and Paraguay, found that gene flow was high between the populations, due to an average of 1 to 16 migrants per generation (Arias et al., 2019). It was also found that at least one FAW migrant was exchanged across all populations in a single

generation (Arias et al., 2019). However, the correlation between gene flow and reduced susceptibility to chemical insecticides such as flubendiamide and lufenuron was not observed (Arias et al., 2019). The study found that admixture between regions would assure the constant presence of resistance alleles, however, changes in susceptibility were not due to an increase in resistant alleles but rather depends on regional insecticide use. For example, in locations where the genetic structure was similar, a location with more insecticide use correlated with more insecticide resistance while the other location maintained susceptibility despite constant gene flow between the two locations (Arias et al., 2019).

Another possible reason for the difference in the susceptibility of the V1 and the other field populations could be due to strain-related genetic differences. In Chapter 2, it was observed that 73% of FAW specimens were hybrid CS and RS FAW larvae with additional genetic variation stemming from single nucleotide polymorphisms (SNPs) in the fourth exon and intron regions of the *triosephosphate isomerase* gene. FAW strains have been observed to differ in response to Bt Cry proteins, where CS FAW populations are the least susceptible to Bt Cry proteins (Ingber et al., 2021a, 2018). If this were to be true then the Bt Cry proteins in subsequent studies would be the least toxic to the LE1, K1F1 and K1F2 populations, which were the only populations where concordant *COI*-CS *Tpi*C haplotypes were observed. However, this was not the case. For Cry1Ab, the LC_{50} values of the LE1, K1F1 and K1F2 populations (25.5- 196.9 ng/cm^2) were very similar to what was determined for the field populations, MH1 and BR1 (91.9-116.1 ng/cm^2). Moreover, in terms of LC_{50} values for first instar FAW, Cry1Ac was more toxic to the LE1, K1F1 and K1F2 populations (488.8 to 813 ng/cm^2) compared to the MH1 and BR1 populations (235.5 to 7 314.3 ng/cm^2). However, it is not absolute that *COI*-CS does not exist in the other field populations, or that K1F1, K1F2 and LE1 harbour *COI*-CS haplotypes at higher proportions compared to the other populations as what was characterised in Chapter 2 because only three specimens were representatives of each field population. Larger studies have however shown that the majority of FAW in South Africa are hybrid CS and RS strains in South Africa (Nagoshi et al., 2019b). If FAW strain-specific haplotypes in South Africa are relatively homogeneous in terms of being dominated by hybrid CS and RS FAW as reported by Nagoshi et al. (2019b), then it makes sense that the toxicity of Bt Cry proteins did not vary as significantly as it would be between CS and RS among the different field populations. However, genetic characterisation of more specimens per population would need to be performed to confirm these observations.

In conclusion, the toxicity of the agronomically important Bt Cry proteins was variable between a reference and five field populations of FAW sampled 3 years apart. The susceptibility of the later-sampled field populations was also variable. The variability in susceptibility of FAW in South Africa may be due to a complex interaction between resistance alleles and fitness costs, regional selection pressures, or simply just natural variation between the populations that are not necessarily dependent on the FAW strain. Despite the variability in FAW susceptibility, Cry2Ab2.820 and Cry1A.105 were lethal to all field populations while Cry1Ab and Cry1Ac were lethal to all field populations except for V1. Thus, Bt maize expressing Cry1A.105 and Cry2Ab2.820 may be expected to be more effective than Bt maize expressing the Cry1Ab protein.

Table 9: Median lethal concentration (LC_{50}) of first instar larvae of a reference and field populations of FAW to agronomically important Bt Cry proteins as determined by diet-overlay bioassays

FAW population	LC_{50} (95% Fiducial limits) (ng/cm^2)[1]			
	Cry1Ac	Cry1Ab	Cry2Ab2.820	Cry1A.105
V1	>13 559	>17 381	444.9 (297.5- 643.9)	6.4 (2.9 – 12.5)
MH1	7314.3 (3252.3 – 33 0855)	116.1 (84.9 – 155.9)	55.1 (36.6 – 78)	1.1 (0.4 – 1.9)
K1F1	813.1 (611 – 1083)	196.9 (124.5 – 302.9)	39.6 (13.7 – 117)	0.9 (0.3 – 1.6)
K1F2	652.3 (279.1 – 1848.8)	25.5 (9.1 – 51.1)	80.9 (63.9 – 100.6)	1 (0.4 – 1.7)
LE1	488.8 (204.2 – 1611.2)	26.9 (11.6 – 49)	25.9 (18 – 34.7)	1.5 (0.9 – 2.1)
BR1	235.5 (121.6 – 449.3)	91.9 (57.4 – 139.9)	20.1 (15.4 – 27.5)	6.4 (1.9 – 7.7)

> indicates that the maximum dose (undiluted, neat Bt Cry protein) was used.

Table 10: Median lethal concentration (LC_{50}) of second instar larvae of a reference and field populations of FAW to agronomically important Bt Cry proteins as determined by diet-overlay bioassays

FAW population	LC_{50} (95% Fiducial limits) (ng/cm^2)[1]			
	Cry1Ac	Cry1Ab	Cry2Ab2.820	Cry1A.105
V1	>13 559	>17 381	>3 323	858.5 (560.2 – 1351.3)
MH1	>13 559	5 718.3 (3 012.4 – 13 655.2)	>3 323	64.7 (48.6 – 95.5)
K1F1	>13 559	7 477.4 (4 091 – 16 454.2)	>3 323	57.7 (37.7-116.1)
K1F2	4 952.1 (1 877.6 – 11 539.9)	4 283.8 (2 511.4 – 9 310.2)	2 144.7 (1 867.4 – 2 419.2)	68.6 (43.4 – 150.2)
LE1	>13 559	668.6 (473.2- 955.1)	>3 323	35.9 (24.2 – 74.8)
BR1	7 659.1 (5 774.8 – 10 824.8)	2 942.7 (2 165.3 – 3 798)	>3 323	30.4 (24.3 – 41.7)

> indicates that the maximum dose (undiluted, neat Bt Cry protein) was used.

Table 11: Median inhibitory concentration (IC_{50}) of first instar larvae of a reference and field populations of FAW to agronomically important Bt Cry proteins as determined by diet-overlay bioassays

FAW population[2]	IC_{50} (95% Confidence intervals) (ng/cm^2) per population[1]			
	Cry1Ac	Cry1Ab	Cry2Ab2.820	Cry1A.105
V1	176.9 (118.8 – 254.7)	190.1 (132.8 – 266.1)	27.15 (19.09– 37.34)	13.98 (7.13 – 23.95)
MH1	169.3 (58.17 – 395.6)	69.26 (26.60 – 203.0)	5.87 (0.60 – 13.32)*	4.2 (2.6 – 6.7)
K1F1	1 451 (588.4 – 4 406)	38.59 (24.59– 57.54)	8.4 (3.2 – 17.8)	3.8 (0.75 – 14.4)
K1F2	742.4 (330.2 – 1 645)	21.10 (5.92 – 51.58)	30.47 (8.82 – 97.75)	1.08 (0.41 – 2.21)
LE1	175.4 (84.2 – 356.4)	21.25 (10.86 – 38.35)	7.83 (4.1 – 12.6)	0.7 (0.2 – 1.5)
BR1	17.58 (14.3 – 21.2)	129.1 (48.70 – 436.4)	1.9 (0.3 – 5.1)	3.4 (1.7 – 6.4)

[1] P value; F (DFn, DFd) for each population showing whether best-fit IC_{50} values for each Bt Cry protein, determined from GraphPad prism 9.3.1, are significantly different. V1: $p < 0.001$; 36.91 (3, 1029), MH1: $p < 0.001$; 12.39 (3, 1061), K1F1: $p < 0.001$; 42.29 (3, 1069), K1F2: $p < 0.001$; 22.44 (3, 981), LE1: $p < 0.001$; 34.43 (3, 1187); BR1: $p < 0.001$; 16.40 (3, 921).

*Best fit IC_{50} values could only be determined with 90% confidence intervals.

Table 12: Median inhibitory concentration (IC_{50}) of second instar larvae of a reference and field populations of FAW to agronomically important Bt Cry proteins as determined by diet-overlay bioassays

FAW population [2]	IC_{50} (95% Confidence intervals) (ng/cm^2) per population[1]			
	Cry1Ac	Cry1Ab	Cry2Ab2.820	Cry1A.105
V1	653.0 (465.1 – 917.5)	148.4 (108.7 – 197.5)	71.63 (51.32 – 99.09)	37.18 (28.80 – 47.92)
MH1	116.8 (80.63 – 170.1)	52.88 (34.06 - 90.33)	18.62 (12.89 – 26.96)	5.63 (3.86 – 8.05)
K1F1	1 231 (523.1 – 2 628)	10.09 (6.65 – 14.49)	66.08 (34.04 – 130.7)	3.18 (2.00 – 4.93)
K1F2	1 247 (685.4 – 1 890)	60.70 (45.26 – 82.47)	89.28 (61.6 – 130.7)	2.67 (1.31- 4.88)
LE1	2 002 (1 070 – 3 514)	42.0 (33.29 – 52.91)	61.7 (44.92 – 85.25)	2.42 (1.61 – 3.51)
BR1	5 015 (2 559 – 9 099)	1190 (602.2 – 2 387)	2.42 (1.61 – 3.51)	5.16 (3.10 – 8.30)

[1] P value; F (DFn, DFd) for each population showing whether best-fit IC_{50} values for each Bt Cry protein, determined from GraphPad prism 9.3.1, are significantly different. V1: $p < 0.001$; 64.25 (3, 1342), MH1: $p < 0.001$; 43.48 (3, 1512), K1F1: $p < 0.001$; 45.93 (3, 1399), K1F2: $p < 0.001$; 61.60 (3, 1179), LE1: $p < 0.001$; 109.3 (3, 1219), BR1: $P < 0.001$; 61.40 (3, 1098).

Chapter 5:

Evaluation of the interaction between Cry2Ab2.820 and Cry1A.105 in fall armyworm, *Spodoptera frugiperda* (J.E. Smith)

5.1. Abstract

The fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith), is a polyphagous pest that has recently established outside of its native regions of the Western Hemisphere. Control of FAW in the Western hemisphere is predominantly based on *Bacillus thuringiensis* (Bt) crops expressing insecticidal Bt Cry proteins. The MON89034 event, a pyramided event, enables the expression of Cry1A.105 and Cry2Ab2.820 in the same plant and has been reported to be more toxic toward FAW than single Cry1 Bt events. MON89034 maize is available in South Africa and studies have shown high toxicity of MON89034 maize to FAW in South Africa. However, to the best of our knowledge, the interaction of Cry2Ab2.820 and Cry1A.105 in combination has never been evaluated. Since the likelihood of synergism or antagonism is an important indicator of the long-term efficacy of the MON89034 event for the control of FAW in South Africa, the study evaluated the interaction between Cry2Ab2.820 and Cry1A.105, at five different ratios in first instar FAW larvae, by droplet-feeding bioassays. The expected median lethal dose (LD_{50}) and the observed LD_{50} were compared by the construction of an isobologram and the calculation of the synergy factor (SF). Both the isobolographic analysis and the SF values showed that Cry2Ab2.820 and Cry1A.105 interacted synergistically at four ratios (1:1, 1:2, 1:3 and 2:1) and antagonistically at one ratio (3:1). Since the MON89034 event in Bt maize expresses Cry2Ab2.820:Cry1A.105 at a ratio of 1:3 in leaves, which is the key larval feeding site, the expression ratio is synergistic in the leaves. Thus, if MON89034 maize expresses doses of Cry2Ab2.820 and Cry1A.105 at synergistic ratios that are sufficient to suppress FAW populations, then MON89034 maize will have high efficacy for the control of FAW in South Africa.

5.2. Introduction

Bacillus thuringiensis (Bt) is a gram-positive bacterium that produces parasporal crystal proteins, known as Cry proteins, that have insecticidal activities against several insect orders (Bravo et al., 2011; Frankenhuyzen, 2009; Herrero et al., 2016). Bt Cry proteins have revolutionised pest control and have dominated the pest control industry since 1938, as Bt topical pesticides in France, and later in 1995, as Bt transgenic crops in the USA and spread to the rest of the world thereafter (Kumar et al., 2021; Sanahuja et al., 2011).

In 2019, 24 years since the first commercialisation of transgenic crops, transgenic crops were planted on 190.4 million hectares worldwide, by 29 countries (ISAAA, 2019). The four major transgenic crops in 2018 were soybeans (91.9 million hectares), followed by maize (60.9 million hectares), cotton (25.7 million hectares), and canola (10.1 million hectares) (ISAAA, 2019). Of the global area cultivated with transgenic crops, 42% of transgenic crops cultivated worldwide contained multiple Bt Cry protein events within one cultivar, known as stacked traits (ISAAA, 2019).

In the Western Hemisphere, Bt maize is especially important for the control of fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith), a newly established agricultural pest in South Africa (Blanco et al., 2007; Herrero et al., 2016). Initially, Bt maize with single events encoding Cry1F was popular for the control of FAW until the resistance of FAW towards Cry1F was observed in North and South America (Blanco et al., 2016; Burtet et al., 2017; Carrière et al., 2016; Omoto et al., 2016; Tabashnik et al., 2013). Since then, pyramided events, which are transgenic events in Bt maize that enable the expression of more than one Bt Cry protein, have proven to have better efficacy for the control of FAW compared to Cry1F (Burtet et al., 2017; Drury et al., 2008; Rule et al., 2014). Of particular importance to FAW is the MON89034 event enabling the expression of both Cry2Ab.820 and Cry1A.105 (Burtet et al., 2017; Drury et al., 2008). Cry1A.105 is a chimeric protein that contains domains I and II from Cry1Ab and Cry1Ac, domain III from Cry1F and a portion of the C-terminal region of Cry1Ac (Andersson et al., 2008; C. Wang et al., 2018). Since Cry1 and Cry2 proteins differ in insect midgut receptor binding, the MON89034 event enables redundant killing (Carrière et al., 2016; Roush, 1998). Redundant killing occurs when insects that are resistant to one toxin produced by a pyramided Bt trait are killed by another toxin produced by the same pyramided trait (Carrière et al., 2016; Roush, 1998). Thus, FAW resistant to Cry1A.105 may still be killed by Cry2Ab2.820 when exposed to the MON89034

event. Due to the cross-resistance between Cry1F and Cry1A.105 and the high prevalence of Cry1F resistance, the resistance of FAW towards Cry1A.105 is always likely (Blanco et al., 2016; Dively et al., 2016; Tabashnik and Carrière, 2017). Thus, redundant killing by Cry2Ab2.820 in the MON89034 event is extremely important to delay resistance development in FAW.

Increased adoption of Bt maize with pyramided events such as the MON89034 event has prompted studies into the assessment of interactions between Bt Cry proteins in pyramided events (Chakrabarti et al., 1998; Ibargutxi et al., 2008; Lemes et al., 2014; Soares Figueiredo et al., 2019; Yang et al., 2018). From a product standpoint, Bt Cry proteins that are proven to interact synergistically with each other would mean higher levels of control for the Bt maize event (Walters et al., 2018). On the other hand, antagonism may have adverse consequences for the product efficacy which may lead to resistance development (Walters et al., 2018)

Theories and terminologies applied to the interaction between two pharmacological drugs can be applied to Bt toxins where a combination of Bt toxins can produce additive, synergistic or antagonistic effects (Lederer et al., 2019; Ritz and Streibig, 2014; Roell et al., 2017; Tabashnik, 1992; Tallarida, 2001; Tammes, 1964). Additivity is defined as a cooperative action of components of a mixture, such that the total effect is equal to the sum of the effects of the components taken independently (Roell et al., 2017; Tallarida, 2001). The additive effect can be considered as a baseline for defining synergy since it is the effect that is theoretically expected (Roell et al., 2017). Thus, determining the additive effect is the first step in detecting synergism.

To test for the additive effect between two components in a mixture, a null model that describes a non-interactive effect must first be chosen (Lederer et al., 2019; Tabashnik, 1992). Deviations from this null model or non-interactive effect can then be interpreted as an interactive effect described as either synergism or antagonism (Lederer et al., 2019). One of the most popular principles of non-interactivity is Loewe Additivity (Loewe and Muischnek, 1926), also known as the concentration addition (CA) or simple similar action model (Lederer et al., 2019; Walters et al., 2018). The CA model assumes that the components in a mixture have the same mode of action such that each component within a mixture can be substituted as a constant proportion of another and produce the same response (Lederer et al., 2019; Loewe and Muischnek, 1926; Roell et al., 2017; Walters et al., 2018).

The calculation of the synergy factor (SF), first described by Tabashnik (1992), is a method based on CA that has been very popular for the evaluation of Bt toxins (Lemes et al., 2014; Li and Bouwer, 2014; Liao et al., 2002; Soares Figueiredo et al., 2019; Sreshty et al., 2011; Walters et al., 2018). The SF is obtained by dividing the theoretical or expected response, such as the LD_{50}, by the observed response (Tabashnik, 1992). The expected additive effect here is an SF of 1 because, at an SF of 1, the theoretical response matches the expected response without any deviation. When the SF is greater than 1, synergism is observed because a smaller response than the expected response is observed, indicating a deviation away from the expected additive effect. Similarly, an SF smaller than 1 indicates antagonism because a larger response was observed compared to the expected response (Li and Bouwer, 2014; Sreshty et al., 2011; Wirth et al., 1997).

Another popular method that is based on the CA model is the use of isobolograms (Grabovsky and Tallarida, 2004; Huang et al., 2019; Sørensen et al., 2007; Tallarida, 2011). The isobologram is a graphical method that enables the visualisation of the expected responses. An isobole is a set of all dose combinations in a mixture that reaches the same fixed effect, such as the LD_{50} (Lederer et al., 2019; Tallarida, 2012, 2006). In the isobologram method, the additive effect or null model is a linear isobole plotted on a two-coordinate plot where the fixed effect of one component, such as the LD_{50}, is plotted as the intercept of one axis and the fixed effect of the second component is plotted on the intercept of the other axis (Gessner, 1995; Grabovsky and Tallarida, 2004; Tallarida, 2012; Tammes, 1964). This linear isobole is also known as the line of additivity and represents dose pairs that, at any ratio, will fall on the line if the expected additive effect is observed (Grabovsky and Tallarida, 2004; Tallarida, 2012). Isoboles that fall outside the linear isobole or line of additivity therefore represent a deviation from the expected response thereby indicating either synergism or antagonism (Sørensen et al., 2007; Tallarida, 2012). If the isobole falls above the line of additivity, the interaction is antagonistic, while if the computed isobole falls below the line of additivity, the interaction is synergistic (Sørensen et al., 2007; Tallarida, 2012).

Bt Cry proteins have been reported to interact with other Bt insecticidal proteins, such as vegetative insecticidal proteins (Vip). Cry1Ca in combination with three Vip3A proteins interacted antagonistically in *Heliothis virescens* and FAW, but synergistically in *Diatraea saccharalis* (Lemes et al., 2014). Bt Cry proteins can also interact synergistically with Bt cytolytic (Cyt) proteins. Cyt1Aa and Cry11Aa have been reported to interact synergistically

in the yellow fever mosquito, *Aedes aegypti* with an SF ranging from 3.5 to 18 at three different ratios, indicating strong synergistic interactions (Pérez et al., 2005). Different combinations of *Bacillus sphaericus* and Bt have been found to interact additively or synergistically with SF values ranging from 1 to 18.5 when tested against *A. aegypti* and the southern house mosquito, *Culex quinquefasciatus* (Wirth et al., 2004). Similarly, isobolographic analysis and SF calculations of the interactions between *B. sphaericus* and Bt for *C. quinquefasciatus* indicated synergism and antagonism at different ratios (Sreshty et al., 2011).

The MON89034 event has been demonstrated to have higher efficacy in the control of FAW in South Africa compared to Bt maize expressing Cry1Ab (Botha et al., 2019). The evaluation of the interaction between Cry2Ab2.820 and Cry1A.105 is important because it is expressed in combination by Bt maize with the MON89034 event, which is cultivated in South Africa already and could be a viable solution for the control of FAW in South Africa. To the best of our knowledge, the interaction between Cry2Ab2.820 and Cry1A.105 in FAW larvae has never been evaluated. Thus, the present study evaluated the type of interaction between Cry2Ab2.820 and Cry1A.105 in FAW larvae through the construction of an isobologram and the calculation of SF values.

5.3. Methods and materials

5.3.1. Insects

The V1 population was used in this study. The V1 population was established and reared as per section 2.3.1.

5.3.2. Bt Cry proteins

The Cry2Ab2.820 (0.36 mg/ml) and Cry1A.105 (1.2 mg/ml) proteins were provided by the Monsanto Company, together with their respective solubilising buffers. Full details of the solubilising buffers are provided in section 3.3.2.

5.3.3. Bioassays

The LD_{50} of Cry2Ab and Cry1A.105 alone, and not in combination, was determined previously by droplet-feeding bioassays (section 3.4.3., Table 7). To evaluate the mortality response of first instar FAW to Cry2Ab2.820 and Cry1A.105 in combination at different ratios, droplet-feeding bioassays were performed. Eight doses of Cry2Ab2.820:Cry1A.105 at each ratio (1:1, 1:2, 1:3, 2:1 and 3:1) were evaluated. Since the volumes of Cry2Ab2.820 and Cry1A.105 solubilising buffer imbibed by first instar FAW larvae were determined to not differ statistically significantly (section 3.4.2., Table 6), the doses of each ratio were determined using the average volumes of Cry2Ab2.820 and Cry1A.105 imbibed by first instar FAW larvae, which is 6.33 nl. Buffer controls were also prepared corresponding to the ratios mentioned above. The experiment was repeated three times with 32 larvae per dose.

To enable the evaluation of the LD_{50} of each ratio, mortality was scored after 7 days. The scoring of mortality included functional mortality, where larvae that failed to moult to the next instar were considered dead (MarÇon et al., 2000). From our observations of FAW larvae, first instar larvae that failed to moult to the next instar weighed less than 0.5 mg, while second instar larvae that failed to moult to the next instar weighed less than 1 mg. Thus, larvae that weighed less than or equal to 0.5 mg for first instar larvae and less than or equal to 1 mg for second instar larvae were considered dead. The LD_{50} was determined by probit regression analysis (Finney, 1952).

5.3.4. Evaluation of the interaction between Cry2Ab2.820 and Cry1A.105

The interaction between Cry2Ab2.820 and Cry1A.105 was evaluated by the construction of an isobologram and the calculation of SF values.

The isobologram was constructed according to the CA model as described by the formula (Sørensen et al., 2007):

$$\left(\frac{d_a}{\delta_a}\right)^{1/\lambda} + \left(\frac{d_b}{\delta_b}\right)^{1/\lambda} = 1 \quad (1)$$

Where δ_a is the LD_{50} of Cry2Ab2.820 alone and δ_b is the LD_{50} of Cry1A.105 alone. d_a and d_b are theoretical concentrations of the individual observed LD_{50} values for each Cry protein at a range of theoretical proportions. The value of λ indicates the degree of deviation from the CA model. A line of best fit for each isobole, or dose pair, was then drawn where the x-axis intercept corresponded to the LD_{50} of Cry2Ab2.820 alone and the y-axis intercept corresponded to the LD_{50} of Cry1A.105 alone. The line of best fit, also known as the line of additivity, describes an interaction between two components conforming to the CA model and is thus constructed using equation 1 with λ=1. Values of λ smaller than 1 describe a degree of deviation away from the CA model corresponding to an antagonistic interaction, while a λ value larger than 1 describes a degree of deviation away from the CA model corresponding to a synergistic interaction. For the study, a λ of 0.5 and a λ of 1.8 were chosen to represent the lines of antagonism and synergism, respectively.

The synergistic factor (SF) is the ratio of the theoretical or expected LD_{50} to the observed LD_{50} determined by the droplet-feeding bioassay. The theoretical LD_{50} ($LD_{50(m)}$) of each Cry2Ab2.820:Cry1A.105 ratio was calculated using the Tabashnik (1992) formula:

$$LD_{50(m)} = \left[\frac{ra}{LD_{50}\,(a)} + \frac{rb}{LD_{50}\,(b)}\right]^{-1} \quad (2)$$

Where ra and rb are the relative proportions of Cry2Ab2.820 and Cry1A.105 in the mixture, respectively. $LD_{50(a)}$ is the LD_{50} of Cry2Ab2.820 alone, while $LD_{50(b)}$ is the LD_{50} of Cry1A.105 alone. If the SF=1 and the expected $LD_{50(m)}$ are within the range of the 95% fiducial limit of the observed LD_{50}, the interaction is considered additive, however, if the SF of the statistically determined additive observation is greater than 1.5, the interaction is considered weakly synergistic. If SF > 1 and the expected $LD_{50(m)}$ is greater than the upper

limit of the 95% fiducial limit of the observed, the interaction is considered synergistic. If SF < 1 and the expected $LD_{50(m)}$ is lower than the lower limit of the 95% fiducial limit of the observed LD, the interaction is considered antagonistic (Ibargutxi et al., 2008; Li and Bouwer, 2014; Liao et al., 2002; Sreshty et al., 2011).

5.4. Results

In isobolographic analysis, deviations from the line of additivity are represented by either $\lambda > 1$, which corresponds with a line of synergism, or $\lambda < 1$, which corresponds with a line of antagonism. The position of an isobole or dose pair of a ratio relative to one of the three lines indicates which interaction best describes that isobole. At 1:1, 1:2, 1:3 and 2:1 the isoboles of each ratio were best described by the line of synergism compared to the line of additivity or the line of antagonism, thus at 1:1, 1:2, 1:3 and 2:1, Cry2Ab2.820 and Cry1A.105 interacted synergistically (Figure 10). However, the isobole at the 1:2 ratio was closer to the line of synergism compared to the 1:1, 1:2 and 1:3 ratios, indicating a smaller deviation away from the line of additivity compared to the other three ratios, but a deviation that is still considered to represent a synergistic interaction (Figure 10). Thus, compared to the 1:1, 1:2 and 1:3 ratios, the interaction between Cry2Ab2.820 and Cry1A.105 is weakly synergistic at a 1:2 ratio. In contrast, the isobole of the 3:1 ratio was close to but just below the line of antagonism indicating that at the 3:1 ratio, the interaction of Cry2Ab2.820 and Cry1A.105 is weakly antagonistic.

The SF values for Cry2Ab2.820:Cry1A.105 ratios 1:1, 1:3 and 2:1 were greater than 1.5 and the $LD_{50(m)}$ was greater than the upper limit of the 95% fiducial limits of the observed LD_{50} of the respective ratio, thus at ratios 1:1, 1:3 and 2:1, Cry2Ab2.820 interacted synergistically with Cry1A.105 (Table 13). For the ratio of 1:2, the $LD_{50(m)}$ fell outside the 95% fiducial limits of the observed LD_{50} for the 1:2 ratio and the SF was greater than 1.5, thus, the interaction between Cry2Ab2.820 and Cry1A.105 at a 1:2 was weakly synergistic (Table 13). In contrast, at the 3:1 ratio, the $LD_{50(m)}$ fell within the 95% fiducial limits of the observed LD_{50}, however, the SF was less than 1, thus at a 3:1 ratio, the interaction between Cry2Ab.820 and Cry1A.105 was weakly antagonistic (Table 13).

Results from both the isobolographic analysis and the calculation of SF values indicated that the interaction of Cry2Ab2.820 and Cry1A.105 was synergistic at the 1:1, 2:1 and 1:3 ratios, weakly synergistic at the 1:2 ratio and weakly antagonistic at the 3:1 ratio.

5.5. Discussion

Out of five different ratios evaluated, Cry2Ab2.820 interacted with Cry1A.105 synergistically at four ratios and antagonistically at one ratio in FAW larvae. This observation indicates that the interaction between two Bt Cry proteins when in combination can deviate from a theoretical additive effect. Similar findings have been reported for other lepidopteran insects. In *Helicoverpa armigera*, synergism between Cry1Ac + Cry1F and Cry1Ac + Cry1Ca have been observed (Chakrabarti et al., 1998; Li and Bouwer, 2014). Similarly, in *Earias insulana*, Cry1Ac + Cry1Fa interacted additively across different combinations (Ibargutxi et al., 2008). While antagonism has also been reported for different combinations of Cry1Ac + Cry1Ab, Cry1Ac + Cry2Aa do not prove to interact in any significant way in *H. armigera* (Chakrabarti et al., 1998). Synergism between Cry1Ab + Cry1Ac has been observed for *Chilo partellus*, where the SF of nine different combinations between Cry1Ab and Cry1Ac ranged between 2.04 to 5.24 (Sharma et al., 2010).

A synergistic interaction was observed at ratios 1:1, 1:2, 1:3, and 2:1 of Cry2Ab2.820:Cry1A.105. To the best of our knowledge, interaction studies between Cry1A.105 and Cry2Ab2.820 proteins have not been reported. However, interaction studies between Cry1Ab and Cry2Ab proteins against FAW larvae have been reported and can be used as a comparison with our study. This is because Cry1A.105 is a chimeric protein composed of domains I and II of Cry1Ab (C. Wang et al., 2018). Domain I is responsible for membrane insertion and pore-formation, while domain II is responsible for insect midgut receptor-related interactions (Bravo et al., 2007; Sanahuja et al., 2011; Soberón et al., 2009; C. Wang et al., 2018). Thus, interaction of Cry1A.105 with FAW midgut receptors is very similar to that of Cry1Ab (Hernández-Rodríguez et al., 2013). A synergistic interaction between Cry1Ab:Cry2Ab at a 1:1 ratio has been reported in first instar FAW larvae where SF values of 2.2 and 10.10 were determined using expected and observed LC_{50} and LC_{90} values (Soares Figueiredo et al., 2019). Competition binding assays between Cry1Ab and Cry2Ab showed that the two proteins do not compete for the same FAW midgut receptors (Soares Figueiredo et al., 2019). The authors attributed the observation of synergism and the lack of antagonism to the lack of competition in binding receptors between Cry2Ab and Cry1Ab (Soares Figueiredo et al., 2019).

Several other theories explain the observation of synergism between Bt Cry proteins. The most straightforward explanation is that the two Cry proteins together are capable of inducing

larger pores, with higher osmotic potential in larval midguts thereby increasing the rate of septicaemia (Lee et al., 1996). Other theories are based more on the mode of action of Bt proteins. The interaction between Bt Cry proteins depends on the interaction of the Bt Cry proteins with the midgut of the host for successful pore formation. The formation of the pore is made possible by oligomeric structures, a complex facilitated by conformational changes of the protoxin into its active form (Bravo et al., 2004; Gómez et al., 2002). Thus, a hetero-oligomer structure formed by Cry2Ab2.820 and Cry1A.105 could have formed in such a way to enhance membrane insertion resulting in increased toxicity.

Cry2Ab2.820 and Cry1A.105 interacted antagonistically at a ratio of 3:1 despite the two Cry proteins having shown not to compete for midgut binding sites thereby proving that competition for binding sites is not the only cause of antagonistic interactions between Bt Cry proteins (Gilreath et al., 2021; Soares Figueiredo et al., 2019). The ability of Bt Cry proteins to form hetero-oligomers to induce synergistic interactions between Bt Cry proteins can also explain antagonism between toxins. A hetero-oligomeric structure could have formed between Cry2Ab2.820 and Cry1A.105 activated proteins which blocked one or more active sites of binding essential to pore formation (Lee et al., 1996). Alternatively, irrespective of receptor binding, the two proteins may compete for space on the midgut cell surface (Carmona et al., 2011; Del Rincón-Castro et al., 1999; Lemes et al., 2014; Rodríguez-Almazán et al., 2009). Thus, the antagonistic interaction between Cry2Ab2.820 and Cry1A.105 could have resulted from an indirect barrier between active toxins and receptors instead of a direct competition of binding sites between the Cry2Ab2.820 and Cry1A.105 proteins.

Overall, at the majority of the ratios evaluated in the study, Cry2Ab2.820 administered in combination with Cry1A.105 greatly enhanced the toxicity of both Cry proteins against first instar FAW compared to when they were administered as single Bt Cry proteins since they appeared to interact synergistically at four of the five ratios tested. However, the expression of Cry2Ab and Cry1A.105 when in combination in Bt maize should still be carefully monitored. For example, if a dose 25 to 50 times the LD_{99} for Cry2Ab2.820 or Cry1A.105 (the high dose required for the high dose/refuge method to delay resistance), is expected to be expressed by Bt maize with the MON89034 event but the dose of Cry2Ab2.820 and Cry1A.105 is actually expressed at a ratio that enables an antagonistic interaction between Cry2Ab2.820 and Cry1A.105, then FAW larvae in the field would actually be exposed to

sublethal concentrations of Cry2Ab2.820 and Cry1A.105 thereby being more at risk of developing resistance towards MON89034 maize.

Currently, in South Africa, 42 events are approved for transgenic crops conferring either single or stacked events for herbicide tolerance and/or insect resistance through Bt Cry proteins and Vip3A20 (ISAAA, 2022). Three Bt maize products containing the MON89034 event are approved for cultivation in South Africa: MON89034 alone or stacked MON89034 x NK603 and MON89034 x TC1507 x NK603 (ISAAA, 2022). Thus, the evaluation of the interaction between Cry2Ab2.820 and Cry1A.105 is crucial for assessing the efficacy of Bt maize expressing the MON89034 event in South Africa for the control of FAW.

The MON89034 event, developed under the Monsanto Company is trademarked under the name YieldGard VT Pro^{TM1}. Before the application by the Monsanto Company for the authorisation of YiledGard VT Pro^{TM1} in the European Union, field trials were conducted during the 2005 growing season in the United States and Argentina (Andersson et al., 2008; Monsanto, 2007). Expression levels across various tissues of MON89034 have been evaluated by the Monsanto Company, however, the expression levels of Cry2Ab2.820 and Cry1A.105 in leaves are the most important since that is primarily where FAW feeds (Chimweta et al., 2020; Matova et al., 2020). Silk, tassel and the grain of maize have also been found to be eaten by FAW, but to a lesser extent than leaves (Chimweta et al., 2020; Harrison, 1984; Kasoma et al., 2021; Overton et al., 2021).

Expression levels for Cry2Ab2.820 were lower than that of Cry1A.105 in most Bt maize tissue (Andersson et al., 2008; Monsanto, 2007). The mean expression level of Cry2Ab in MON89034 maize was 180 µg/g dry weight in leaf, 71 µg/g dwt in the silk and 1.3 µg/g dwt in grain, while the mean expression levels of Cry1A.105 in MON89034 maize was 520 µg/g dwt in leaf, 26 µg/g dwt in silk and 5.9 µg/g dwt in grain (Andersson et al., 2008; Monsanto, 2007). This translates to approximate ratios of Cry2Ab2.820:Cry1A.105 as 1:3 in leaf, 3:1 in silk and 1:5 in the grain. From the findings that Cry2Ab2.820 and Cry1A.105 interact synergistically at the 1:3 ratio, it can be expected that the expression of Cry2Ab2.820 and Cry1A.105 in leaves of MON89034 maize at the 1:3 ratio would cause high mortality in FAW larvae if they are expressed at the LC_{50} or LC_{90} of the MON89034 event. However, Cry2Ab and Cry1A.105 are expressed at a 3:1 ratio in the silk of maize plants which is the ratio that induces an antagonistic interaction between Cry2Ab2.820 and Cry1A.105. This is especially concerning since a higher average percentage of damage to silk compared to leaves

from FAW have been observed across four maize field in Zimbabwe (Chimweta et al., 2020). However, a study evaluating the efficacy of Bt maize with the MON89034 event using MON89034 leaf tissue bioassays found that only 1 larva out of 100 could survive on MON89034 Bt maize leaf after 10 days of feeding thereby indicating that the expression of Cry2Ab2 and Cry1A.105 from Bt maize in South Africa is high enough (kills >99% of FAW) for the control of FAW in South Africa if FAW feeds primarily on leaves (Botha et al., 2019).

In conclusion, outcomes of the evaluation of the interaction between Cry2Ab2.820 and Cry1A.105 by isobolographic analysis and the calculation of the SF were in agreement that 4 out of 5 ratios of Cry2Ab2.820 and Cry1A.105 evaluated were synergistic while 1 ratio was weakly antagonistic. Most importantly, Cry2Ab2.820 and Cry1A.105 interacted synergistically at the 1:3 and 1:1 ratio that is expressed by singe and stacked event MON89034 maize respectively, thereby showing Bt maize with the MON89034 event will be more toxic towards FAW than Bt maize containing the Cry2Ab2.820 or Cry1A.105 proteins alone at the same expression levels.

Table 13: Median lethal dose (LD_{50}) and synergistic factors against first instar FAW larvae at different ratios of Cry2Ab2.820:Cry1A.105

Ratio (Cry2Ab2.820:Cry1A.105)	LD_{50}[1] (ng)	95% Fiducial limits		χ^2	$LD_{50(m)}$ (ng)[2]	SF ($LD_{50(m)}/LD_{50}$)[3]
		Lower	Upper			
1:1	0.37	0.29	0.47	11.23	1.53	4.14
1:2	0.51	0.41	0.64	6.92	0.84	1.63
1:3	0.19	0.15	0.23	10.98	0.57	3.02
2:1	0.72	0.58	0.93	9.01	2.63	3.55
3:1	4.98	2.94	11.31	9.26	3.45	0.72

[1] LD_{50} values of Cry2Ab2.820 and Cry1A.105 at different ratios as determined by droplet-feeding bioassays
[2] Expected LD_{50} values of each Cry2Ab2.820:Cry1A.105 ratio as determined by the Tabashnik (1992) formula.
[3] Synergy factor determined by dividing the $LD_{50(m)}$ by the observed LD_{50} for each Cry2Ab2:820:Cry1A.105 ratio.

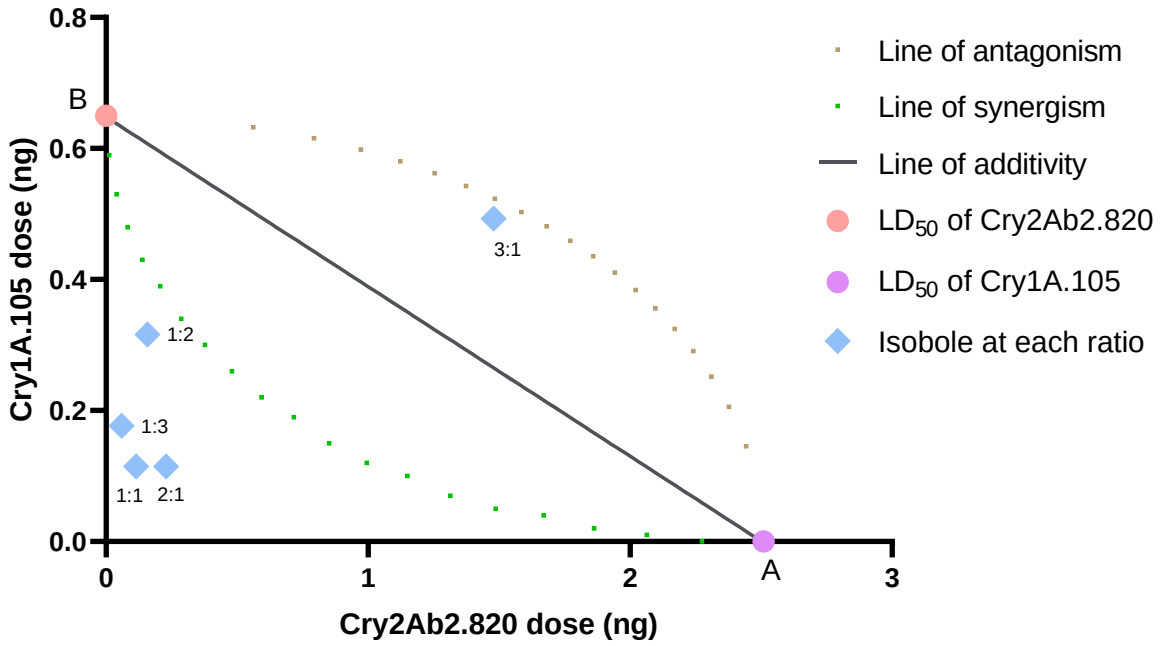


Figure 10: Isobologram demonstrating the interactions between Cry2Ab2.820 and Cry1A.105 in first instar FAW larvae. The solid straight line indicates the line of additivity**,** where the **X**-intercept at point A corresponds to the LD_{50} of Cry2Ab2.820 alone and the **Y**-intercept at point B indicates the LD_{50} of Cry1A.105 alone as previously determined through droplet-feeding bioassays. The curved isoboles represent the line of antagonism ($\lambda = 0.5$) and the line of synergism ($\lambda = 1.8$). The LD_{50} of each Cry2Ab2.820:Cry1A.105 ratio, determined by droplet-feeding bioassays**,** is plotted as isoboles (dose pairs) on the same cartesian plane.

Chapter 6:

General Discussion and Conclusion

6.1. General discussion

In the Western Hemisphere, the agronomically important fall armyworm (FAW*), Spodoptera frugiperda* (J.E. Smith), is controlled by Bt maize expressing either a single or multiple insecticidal *Bacillus thuringiensis* (Bt) Cry proteins. With the arrival and establishment of FAW in South Africa, the evaluation of the susceptibility of FAW populations to Bt Cry proteins would indicate whether Bt maize in South Africa would be effective for the control of FAW in South Africa. Thus, the overall aim of the study was to evaluate the toxicity of agronomically important Bt Cry proteins against reference and field populations of FAW, in South Africa.

The study focused exclusively on populations of FAW in South Africa collected during 2018 (reference population) and 2021 (field populations). The reference population is also a field population, however, since it was sampled three years before the other field populations, it served as a benchmark against which the susceptibility of FAW populations to Bt Cry proteins can be compared. In total, one reference population and five field populations were collected from FAW infested areas. Furthermore, although FAW is a polyphagous pest, reported outbreaks have primarily existed on maize crops, thus all FAW populations in the present study were collected from maize. While there are other Bt Cry proteins expressed in Bt crops in South Africa, the four most commercially important Bt Cry proteins were chosen to represent Bt Cry proteins in South Africa (ISAAA, 2022). The evaluation of the toxicity of Cry1Ab, Cry1A.105 and Cry2Ab.820 against FAW in South Africa was especially important because they are the most common Bt Cry proteins expressed by Bt maize in South Africa.

In Chapter 2, three FAW specimens from each population were genetically characterised because FAW strains have been shown to differ in response to Bt Cry proteins, where corn strain (CS) FAW larvae are less susceptible to Bt Cry proteins than rice strain (RS) FAW larvae (Ingber et al., 2021a, 2018). All specimens were confirmed to be the FAW species using the *mitochondrial cytochrome oxidase subunit I gene* (*COIA*) barcode region. Thereafter, strain-diagnostic SNPs in the *COIB* (segment downstream of *COIA*) and *triosephosphate isomerase* (*Tpi)* exon (TpiE4) and intron (TpiI4) regions were compared to

differentiate between the RS and the CS FAW. Overall, the majority (73%) of FAW specimens evaluated in the study were discordant *COI*-RS *Tpi*C, an observation that has been consistently seen across sub-Saharan Africa, India, Asia and Australia (Acharya et al., 2021; Nagoshi et al., 2020, 2019a, 2019b; Piggott et al., 2021). However, the most interesting observation was that, except for the V1 population, all populations were relatively heterogeneous, varying in the TpiE4 as well as the TpiI4 SNPs. Furthermore, the *COI*-CS haplotype, although found in only 4/15 specimens in the study, was identical to the Florida haplotype (FL-haplotype). Thus, in Chapter 2, it was concluded that the toxicity of Bt Cry proteins evaluated in the subsequent chapters may be expected to be more comparable to the toxicity of Bt Cry proteins towards CS, hybrid or FL-type FAW larvae in the Western Hemisphere. However, this was not the case since the susceptibilities of the LE1, K1F1 and K1F2 populations, which were the only populations where concordant *COI*-CS *Tpi*C haplotypes were observed, were not more similar to each other, in terms of their susceptibilities to Bt Cry proteins, than to the other populations that were predominately hybrids with no concordant *COI*-CS *Tpi*C haplotypes. Thus, it is more likely that strain differences or haplotype differences do not affect the susceptibility towards Bt Cry proteins significantly. Future studies could focus on genetically characterising more FAW samples from the same region in order to further investigate the genetic structure of each population.

To evaluate the toxicity of agronomically important Bt Cry proteins (Cry1Ac, Cry1Ab, Cry2Ab2.820 and Cry1A.105) against the V1 population, the early sampled and later-deemed reference population, droplet-feeding bioassays were performed. The droplet-feeding method was the first chosen method because it is an easier method for the determination of lethal doses (LDs) and growth inhibitory doses (IDs) compared to gravimetric and radioisotopic methods (Hughes et al., 1984; van Beek and Hughes, 1986). However, to the best of our knowledge, the compatibility of the droplet-feeding method with highly alkaline Cry protein solubilising buffers has never been evaluated. Thus, in Chapter 3, the aim was to evaluate the compatibility of the droplet-feeding method for the bioassay of agronomically important Bt Cry proteins in highly alkaline solubilising buffers against FAW. The findings showed that the droplet-feeding bioassay can be used with highly alkaline buffers for the assessment of the toxicity of Bt Cry proteins. Furthermore, the study showed that first and second instar FAW larvae imbibe the different Cry protein solubilising buffers at different volumes. This observation highlighted the importance of calculating doses based on the volumes of each feeding solution imbibed, which may change depending on the populations and components

of the feeding solution. Using the droplet-feeding method, the LD_{50} and ID_{50} values of each Bt Cry protein for first and second instars were determined. The order of least toxic to most toxic Bt Cry protein, in terms of both LD_{50} and ID_{50} values, were Cry1Ac < Cry1Ab < Cry2Ab2.820 < Cry1A.105.

Although the droplet-feeding method enabled accurate dose determination, the LD_{50} values for Cry1Ac and Cry1Ab could not be determined. We theorised that the mortality response could be improved if we increased the duration that FAW larvae are exposed to Bt Cry proteins. Thus, instead of droplet-feeding bioassays, the toxicity of Bt Cry proteins against FAW larvae was evaluated by the diet-overlay method in Chapter 4, where first and second instar FAW larvae fed on the diet, with Bt Cry protein on the surface, for 7 days instead of brief exposure to a droplet of Bt Cry protein. Although the diet-overlay method cannot be used to determine LDs and IDs, it is a common bioassay method for insect toxicity studies which would enable the easy comparison of the lethal concentrations (LCs) and growth inhibitory concentrations (ICs) with literature.

The aim of Chapter 4 was to evaluate the toxicity of Bt Cry proteins against a reference and field populations of FAW in South Africa. The findings of Chapter 4 showed that the LC_{50} and IC_{50} values for first and second instar FAW larvae were the highest for the V1 reference population compared to the five field populations. As discussed in Chapter 4, the lower susceptibility of the V1 populations and the variation in the susceptibility of the field populations could be related to fitness costs associated with resistance alleles, regional selection pressures or FAW strain-related genetic differences (Arias et al., 2019; Gassmann et al., 2008; Sousa et al., 2016). To confirm and expand on this observation, future studies could include more in-depth evaluations into the presence of Cry protein resistance alleles and the effects of regional selection pressures on the persistence of Cry protein resistant alleles in FAW.

The toxicity of Bt Cry proteins also varied among the different populations, where Bt Cry proteins lethal to one population were not lethal to others. The difference in susceptibility was more pronounced for Cry1Ab and Cry1Ac since the LC_{50} values obtained for Cry1A.105 and Cry2Ab2.820 in first instar and second instar FAW larvae were very similar between the FAW populations. For example, Cry1Ab was able to elicit a mortality response in second instar K1F2 and BR1 larvae but not in any other population. Moreover, excluding the V1 population, the largest LC_{50} for Cry1Ac in first instar FAW larvae was for MH1, while the

largest LC_{50} for Cry1Ab in first instar FAW larvae was for K1F1. These findings highlight the importance of establishing a baseline for the susceptibility of different FAW populations against Bt Cry proteins.

The findings from both the droplet-feeding and diet-overlay bioassays were in agreement that Cry2Ab2.820 and Cry1A.105 caused the highest mortality response in first and second instar FAW across all the FAW populations. However, in terms of growth inhibitory responses, Cry2Ab2.820 was not always more toxic than Cry1Ab. The ID_{50} for Cry1Ab was lower than that of Cry2Ab2.820 for second instar V1. Similarly, IC_{50} values of Cry1Ab were lower than that of Cry2Ab2.820 for second instar FAW larvae from the K1F1 and K1F2 populations. The significant growth inhibition observed in the present study caused by Cry1Ab to second instar FAW larvae has been observed before in Bt maize tissue bioassays (Botha et al., 2019; Omoto et al., 2016; Sousa et al., 2016). The growth-inhibition caused by Cry1Ab results in prolonged larval development which has been linked to resistance development because delayed larval development results in the desynchronised emergence of susceptible and resistant adult moths (Gould, 1998; Liu et al., 1999). This means that there are fewer chances for susceptible moths to breed with resistant moths, thereby increasing the frequency of homozygous recessive resistance alleles (Gould, 1998; Liu et al., 1999). While this may true for other noctuids, FAW in field settings has multiple overlapping generations year-round which means that the pool of susceptible moths available to mate with resistant moths is not diminished and heterozygous susceptible individuals are still produced (Sousa et al., 2016). In this case, prolonged larval development lessens the likelihood of resistance development in FAW towards Bt Cry proteins because it increases the likelihood of mortality by natural enemies (Gould, 1998; Uesugi, 2015). A reduction of adult moths with resistant alleles then results in fewer resistance alleles being passed onto the next generation (Gould, 1998; Uesugi, 2015). Thus in Brazil, despite MON810 (Cry1Ab) maize not expressing “high dose” Cry1Ab protein, the efficacy of MON810 maize in the field has not reduced for the control of FAW since 1999 (Fernandes et al., 2003; Frizzas et al., 2014; Omoto et al., 2016; Sousa et al., 2016; Waquil et al., 2013).

The two most toxic proteins to FAW larvae evaluated in the study were Cry2Ab2.820 and Cry1A.105 which are expressed together in the MON89034 event in Bt maize. To assess whether the ratio of Cry2Ab2.820 and Cry1A.105 that are expressed in combination by Bt maize, plays a role in the degree of toxicity, the interaction between Cry2Ab2.820 and

Cry1A.105 was evaluated. As seen in Chapter 5, at five different ratios, Cry2Ab2.820 and Cry1A.105 interacted synergistically at four ratios (1:1, 1:2, 1:3 and 2:1) and antagonistically at one ratio (3:1). This novel investigation demonstrated that Cry2Ab2.820 in combination with Cry1A.105 at certain ratios have improved toxicity towards first instar FAW larvae compared to each of the Cry proteins alone. Moreover, the protein content of Cry2Ab2.820 and Cry1A.105 in the MON89034 event is expressed at the synergistic ratio of 1:3 in MON89034 maize. Recently, the efficacy of MON89034 maize has been assessed which demonstrated that FAW larva exhibited high susceptibility to MON89034 maize leaves (< 1% survival) (Botha et al., 2019). Thus, MON89034 Bt maize should be expected to be effective at controlling FAW field populations.

Now that the susceptibility of multiple populations of FAW has been evaluated, it can serve as a benchmark of the susceptibility of FAW populations to Bt Cry proteins in South Africa. Future studies could include returning to the same locations where the field populations were sampled to assess whether there is a reduction in susceptibility of each population to Bt Cry proteins which could be indicative of Bt resistance development. Furthermore, a well characterised discriminating concentration or diagnostic concentration can now be chosen while taking into account the variation of the toxicity of Bt Cry proteins across different FAW populations (Halliday and Burnham, 1990; Huang, 2006). For example, diagnostic concentration for each Bt Cry protein can be chosen based on the upper 95% fiducial limit of the highest LC_{50} value for each Bt Cry protein obtained across all the FAW populations (Chandrasena et al., 2017; Farias et al., 2014b; Marçon et al., 1999). A well characterised discriminating or diagnostic concentration would enable efficient screening of resistance development of FAW across South Africa (Huang, 2006).

6.2. Conclusion

The study showed that FAW in South Africa is susceptible to current agronomically important Bt Cry proteins in South Africa, however, variation in susceptibility exists between different regional populations. This difference in variability could either be due to the fitness costs associated with resistance alleles, regional selection pressures, or genetic differences related to FAW strains. Despite the variation in susceptibility to Bt Cry proteins between different populations, there was a consistent observation that Cry2Ab2.820 and Cry1A.105 were highly toxic to all FAW populations. Furthermore, the study showed that in MON89034 Bt maize, Cry2Ab2.820 and Cry1A.105 are expressed at ratios that enable synergistic interactions. In conclusion, the study evaluated the susceptibility of FAW populations to Cry1Ab, Cry1Ac, Cry2Ab2.820, Cry1A.105 as well Cry2Ab2.820 in combination with Cry1A.105, using two different bioassay methods. The baseline susceptibility of six field populations of FAW against agronomically important Bt Cry proteins will serve as an important foundation for future resistance screening programs in South Africa.

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