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Comparison of different bioassay methods for the assessment of dose-response relationships
of entomopathogens and toxins against *Helicoverpa armigera* (Hübner, 1809) (Lepidoptera:
Noctuidae)

by

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Declaration

I declare that this dissertation is my own, unaided work. It is being submitted for the Degree Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

A handwritten signature in black ink, consisting of a stylized 'A' or 'R' shape with a horizontal line extending to the right.

1st day of November 2024 at Johannesburg

Abstract

Bioassays are an important tool for developing bioinsecticides against agricultural pests. The aim of this study was to compare two bioassay methods – diet overlay and droplet feeding – to identify the most suitable method for assessing dose-response relationships of entomopathogens and toxins against second instar larvae of *Helicoverpa armigera*. The toxins used were purified *Bacillus thuringiensis* Cry1A.105 and Cry2Ab2.820 proteins, the spore-crystal complex (SCC) of *B. thuringiensis* subspecies *kurstaki* strain HD-73, and the entomopathogen *Helicoverpa armigera* nucleopolyhedrovirus (HearNPV). Based on the heterogeneity factor, coefficient of variance (CV) and relative precision, the diet overlay bioassay was determined to be the best fit for use with HD-73 SCC and HearNPV. Suitable bioassay methods could not be determined for the purified *B. thuringiensis* proteins due to a poor probit model fit and low precision of estimated LC₅₀s and LD₅₀s. Validation of CV and relative precision across bioassays will ensure the most suitable methods are used for sustainable integrated pest management.

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List of Abbreviations

BSA	bovine serum albumin
Bt	<i>Bacillus thuringiensis</i>
Btk	<i>Bacillus thuringiensis</i> subspecies <i>kurstaki</i>
BV	budded virion
CAPS	N-cyclohexyl-3-aminopropanesulfonic acid
CBB	Coomassie brilliant blue
CI	confidence interval
Cry	crystal
CV	coefficient of variance
Cyt	cytolytic
DDT	dichlorophenyltrichloroethane
DNA	deoxyribonucleic acid
dsDNA	double-stranded deoxyribonucleic acid
dsRNA	double-stranded ribonucleic acid
DTT	dithiotreitol
FAO	United Nations Food and Agriculture Organization
FL	fiducial limits
GV	granulovirus
HearNPV	<i>Helicoverpa armigera</i> nucleopolyhedrovirus
HearSNPV	single nucleocapsid <i>Helicoverpa armigera</i> nucleopolyhedrovirus
HearMNPV	multiple nucleocapsid <i>Helicoverpa armigera</i> nucleopolyhedrovirus
IC ₅₀	median inhibitory concentration
ID ₅₀	median inhibitory dose
IPM	integrated pest management
LC ₅₀	median lethal concentration
LD ₅₀	median lethal dose
L:D	light: dark
MWM	molecular weight marker

NA	nutrient agar
NPV	nucleopolyhedrovirus
OB	occlusion body
ODV	occlusion-derived virion
RH	relative humidity
RNA	ribonucleic acid
SCC	spore-crystal complex
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
ssDNA	single-stranded deoxyribonucleic acid
ssRNA	single-stranded ribonucleic acid
subsp.	subspecies
USD	United States dollar
US EPA	United States Environmental Protection Agency
UV	ultraviolet
Vip	vegetative insecticidal protein

Chapter One – Introduction

1.1. *Helicoverpa armigera*

1.1.1. Global spread and impact

Helicoverpa armigera (Hübner, 1809) (Lepidoptera: Noctuidae), or the African bollworm, is known as one of the world's most destructive and economically important agricultural pests (Haile *et al.*, 2021). Until recently, this pest was absent from the New World, with distribution of the Old World insect seen mainly in Africa, Australia, Asia and Europe (Hardwick, 1965; Tembrock *et al.*, 2019). In 2013, *H. armigera* was reported in soybean and cotton crops in Brazil (Czepak *et al.*, 2013), marking the first known migration of the pest to the New World (Tay *et al.*, 2014; Arnemann *et al.*, 2019). However, some records have suggested that the pest established itself in Brazil as early as 2006 (Sosa-Gomez *et al.*, 2016; Tembrock *et al.*, 2019). The insect rapidly spread to North and South America, with confirmed establishment in Argentina, Uruguay, Bolivia, and Paraguay (Murúa *et al.*, 2014; Arneodo *et al.*, 2015; Arnemann *et al.*, 2016; Haile *et al.*, 2021). The pest is believed to have migrated to the Americas through international trade (Tay *et al.*, 2014; Arnemann *et al.*, 2019) and has replaced *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) as the primary agricultural pest in the region (Leite *et al.*, 2014; Tay *et al.*, 2014). Global economic losses caused by *H. armigera* are currently estimated upwards of USD \$3 billion annually (Haile *et al.*, 2021; Riaz *et al.*, 2021).

H. armigera is known to infect more than 150 plant families, and affects many cash and staple crops in Africa, including wheat, maize, cotton, soybean, and citrus crops (Tsafack *et al.*, 2015; Riaz *et al.*, 2021). The pest's polyphagous nature, alongside its high fecundity, diapause and high migratory abilities contribute considerably to its success (Fitt, 1989). The insect can also maintain significant egg and larval populations through differing climates and seasons (Fitt, 1989; Ravi *et al.*, 2005).

Due to the insect's persistence in agriculture, global distribution and resistance to several pesticides, and economic damage caused, *H. armigera* is commonly used for research purposes. Mass rearing of *H. armigera* is relatively easy and cost effective given the pest's short life cycle (Yücel & Genç, 2018; Allahyari *et al.*, 2022).

1.1.2. Biology and life cycle

H. armigera develops through life stages of egg, larva, pupa, and adult. Adult moths feed on flowers and nectar, and are most active at night, where female moths can lay up to 3000 eggs during oviposition (Cunningham & Zalucki, 2014). Hatched larvae develop through six instars, with larvae size increasing through each instar. Late instars of *H. armigera* cause the most damage by feeding and boring into the reproductive structures of the host plant, resulting in crop yield losses (Hardwick, 1965; Rodrigues *et al.*, 2019). Reproductive structures of the plant provide the pest with all its nutritional needs due to their high protein and nitrogen content (Queiroz-Santos *et al.*, 2018). Larvae are also protected from pesticide sprays when boring into the plant structures (Malinga & Laing, 2022).

1.1.3. Pest control and resistance in agriculture

The commercialisation and indiscriminate use of synthetic and chemical pesticides from the late 1940s to the late 1990s, for the control pests in agriculture has resulted in the rapid development of insect resistance to a wide range of traditional pesticides, such as organophosphates, deltamethrin, pyrethroids, and dichlorodiphenyltrichloroethane (DDT) (Gunning *et al.*, 1990; Gunning *et al.*, 2005; Walsh *et al.*, 2022). The failures of these broad-spectrum insecticides alongside the detrimental environmental effects have driven the development of more sustainable biopesticides (Downes *et al.*, 2016).

Given that insects are the most diverse group of living organisms on earth (Del Rincón-Castro and Ibarra, 2011; Deka *et al.*, 2021), it is expected that a large diversity of entomopathogenic microorganisms exist, especially given the evolutionary bond of pathogens and their hosts (Kumar *et al.*, 2011; Deka *et al.*, 2021). Although the development of Bt transgenic crops has reduced the use of and reliance on chemical pesticides, large-scale cultivation of Bt crops may accelerate pest resistance to Bt proteins (Wan *et al.*, 2012; Jin *et al.*, 2014). A 2019 study by Liu and Luo in China found that consecutive cultivation of Bt cotton in the same region decreased the effectiveness of the transgenic crops against *Helicoverpa armigera* (Liu & Luo, 2019). Researchers are making use of the ‘natural refuge strategy’ where Bt toxins are co-expressed in high concentrations in Bt crops, and Bt crops are cultivated in rotation or with non-Bt crops that act as the refuge (Li *et al.*, 2017), to delay and offset insect resistance (Jin *et al.*, 2014; Liu & Luo, 2019). This growing resistance of agricultural pests, once again, highlights the importance continuous research and development of sustainable IPM strategies.

1.2. *Bacillus thuringiensis*

1.2.1. Characteristics

Bacillus thuringiensis (Bt) is a Gram-positive, rod-shaped, entomopathogenic bacterium that produces parasporal crystal (Cry) proteins and cytolytic (Cyt) proteins during its sporulation and stationary growth phases (Höfte & Whiteley, 1989; Schnepf *et al.*, 1998). These proteins are water-soluble, pore-forming toxins that undergo conformational changes to be inserted into the cell membrane of the insect host (Bravo *et al.*, 2007). Cry proteins are insecticidal to the Lepidoptera, Coleoptera, Diptera and Hymenoptera orders of insects, as well as nematodes, while Cyt proteins are active against dipteran insects and coleopteran larvae. Vegetative insecticidal proteins (Vip), another form of Bt toxins, are lethal to lepidopteran and coleopteran insects, and are secreted during the vegetative growth stage of the Bt life cycle. Vips bare no sequence similarities to Cry and Cyt proteins (de Maagd *et al.*, 2003; Koch *et al.*, 2015).

1.2.2. Cry proteins

1.2.2.1. Nomenclature

Cry proteins constitute the largest group of insecticidal proteins produced by *Bacillus* species (Schnepf *et al.*, 1998; Das *et al.*, 2021). Initially, *cry* genes were named arbitrarily according to the source of their discovery. However, since the first cloning of an insecticidal crystal protein from Bt by Schnepf and Whiteley in 1981, over 700 *cry* genes have been discovered, thus rendering the arbitrary naming system unusable (Schnepf & Whiteley, 1981; Höfte & Whiteley, 1989; Bravo *et al.*, 2013). In 1989, Höfte and Whiteley named Cry proteins, in Roman numerals, according to their insecticidal activity only, where Cry I proteins were toxic to lepidopteran insects, and Cry II proteins were toxic to both dipteran and lepidopteran insects. Cry III and Cry IV proteins were toxic to coleopteran and dipteran insects, respectively (Höfte & Whiteley, 1989). Current nomenclature follows the revised structure of Crickmore *et al.* (1998). Here, Cry proteins are classified according to their sequence similarities at amino acid level, through four ranks (Crickmore *et al.*, 1998). The primary rank denotes Cry proteins that share less than 45% sequence similarity, i.e. toxins of each Cry group, (e.g. Cry1, Cry2). Secondary rank is allocated to toxins sharing less than 78% sequence similarity within each group (e.g. Cry1A and Cry1B), while tertiary rank is assigned to proteins sharing less than 95% sequence similarity (e.g. Cry1Aa and Cry1Ab). Finally, the quaternary represents proteins within the same tertiary level and share more than 95% sequence similarity (e.g. Cry1Aa1 and Cry1Aa2) (Crickmore *et al.*, 1998; van Frankenhuyzen, 2009; Bravo *et al.*, 2012; Crickmore

et al., 2021; Das *et al.*, 2021). While new insecticidal proteins are always being discovered and some are renamed, the current naming system remains widely accepted (Crickmore *et al.*, 2021).

1.2.2.2. Three-domain structure and mode of action

The insecticidal Bt Cry proteins are characterised by their three-domain, globular molecule structure. The N-terminal of domain I consists of a bundle of seven α -helices with a central, hydrophobic α -5 helix encircled by six aliphatic helices. Domain I functions in membrane insertion, oligomerisation of the protein, and pore formation (Bravo *et al.*, 2007; Mendoza-Almanza *et al.*, 2020). Domain II is made of three anti-parallel β -sheets packed around a hydrophobic core, and exposed loop regions. This region is responsible for the Cry protein's specificity (Bravo *et al.*, 2007; Palma *et al.*, 2014). Lastly, domain III consists of a two antiparallel β -sheet sandwich which is involved in receptor binding and pore formation. This is also known as the galactose-binding domain (Xu *et al.*, 2014; Palma *et al.*, 2014). Galactose binding is crucial for the toxin to exert its maximum insecticidal action against the insect (Xu *et al.*, 2014; Palma *et al.*, 2014).

Currently, two modes of action – the pore forming model and the signalling pathway model – of Bt Cry proteins against insect larvae are recognised (Mendoza-Almanza *et al.*, 2020; Liu *et al.*, 2021). Both models are explained using Cry1A proteins, e.g. Cry1Aa, Cry1Ab, or Cry1Ac (Gómez *et al.*, 2006; Mendoza-Almanza *et al.*, 2020).

The pore forming model states that when an insect ingests a Cry protein, usually through contaminated foliage, the protoxin, ranging from 72 to 135 kDa, is solubilised by the alkaline environment of the midgut and activated by gut proteases. The activated toxin, which ranges from 60 to 68 kDa, then interacts, sequentially, with high affinity receptors (Ibrahim *et al.*, 2010; Liu *et al.*, 2018). The first receptor, Bt-R₁, is a cadherin receptor (CADR) identified in the brush border membrane vesicles (BBMV) of the tobacco hornworm *Manudca sexta* (Vadlamudi *et al.*, 1995; Zhang *et al.*, 2005; Bravo *et al.*, 2007), and is functionally important to the toxicity of Cry1A proteins. Bt-R₁ functions in cleaving the α -1 helix (N-terminal) of the activated toxin, thus mediating the formation of the pre-pore oligomeric structure, which is crucial for membrane insertion (Gómez *et al.*, 2006; Mendoza-Almanza *et al.*, 2020). The formation of the pre-pore oligomer increases its affinity to the glycosylphosphatidylinositol (GPI)-anchored aminopeptidase-N (APN) and alkaline phosphatase (ALP) receptors, which

occur in rigid lipid rafts for stabilisation (Bravo *et al.*, 2007; Arenas *et al.*, 2010; Mendoza-Almanza *et al.*, 2020). Lipid rafts are composed of insoluble microdomains enriched in sphingolipids, cholesterol, and GPI-anchored proteins. The rafts are known to function in membrane and protein sorting, as well as receptor signalling and signal transduction on the plasma membrane (Simons & Toomre, 2000; Bravo *et al.*, 2007; Ouweneel *et al.*, 2020). Binding to APN and ALP results in oligomer insertion into the apical membrane and pore formation (Pigott & Ellar, 2007; Pardo-López *et al.*, 2013). A 1995 study by Masson *et al.*, shows that binding of the Cry1Ac toxin to the APN receptor is mediated by N-acetylgalactosamine (GalNac) moieties that specifically bind to domain III of the proteolysed toxin (Masson *et al.*, 1995; Bravo *et al.*, 2007; Pacheco *et al.*, 2009; Mendoza-Almanza *et al.*, 2020). Interaction with GalNac with the oligomeric Cry1Ac structure plays a significant role in the toxin's conformational change that facilitates insertion into the midgut membrane (Bravo *et al.*, 2004; Pardo-López *et al.*, 2006).

Pore formation causes an increase in selective permeability of the membrane, resulting in an influx of water, cations, and other small molecules into the cell, further resulting in cell lysis and killing of the insect (de Maagd *et al.*, 2001; Soberón *et al.*, 2009; Pardo-López *et al.*, 2013; Jurat-Fuentes *et al.*, 2021).

Finally, a 270 kDa glycoconjugate (BTR-270) isolated from BBMV of the gypsy moth *Lymantria dispar*, is shown to bind to Cry1A proteins with high affinity. Although the exact function of this receptor remains unknown, the highly glycosylated nature of the protein, primarily through N-linked glycans/sugar molecules, offers specific binding sites for the Cry toxins through recognition of aspartic-asparagine residues (Valaitis *et al.*, 2001; Valaitis, 2011; Best *et al.*, 2023). These residues create an anionic environment to which positively charged Cry proteins can specifically bind, thus forming a stable complex for downstream signal transduction. The glycans are also known to play a role in stabilising BTR-270 and preventing protein degradation in the BBMV of the insect midgut (Valaitis *et al.*, 2001; Valaitis, 2011; Best *et al.*, 2023). The proteolysis of the Cry toxin by midgut proteases and pore formation are the major events that characterise the pore forming model (Pacheco *et al.*, 2009; Jurat-Fuentes & Crickmore, 2017; Mendoza-Almanza *et al.*, 2020).

The signalling pathway model starts similarly to the pore forming model where the Cry protein is solubilised in the alkaline insect midgut, activated and proteolyzed by midgut proteases (Mendoza-Almanza *et al.*, 2020; Liu *et al.*, 2021). This model, however, completely omits pore

formation (Xu *et al.*, 2014; Liu *et al.*, 2021). Here, the Cry protein binds specifically to a cadherin receptor, activating the Mg^{2+} -dependent signalling cascade and G-protein coupled receptors. Adenylyl cyclase is also activated, triggering an increase in the level of the intracellular second messenger cyclic adenosine monophosphate (cAMP), further activating protein kinases A (Mendoza-Almanza *et al.*, 2020; Liu *et al.*, 2021). This triggers a signalling cascade resulting in the disruption of ion channels and cytoskeletons on the insect's membrane (Pigott & Ellar, 2007; Xu *et al.*, 2014). This results in programmed cell death, and, ultimately, insect death (Mendoza-Almanza *et al.*, 2020; Liu *et al.*, 2021).

The high affinity receptors seen in both modes of action are crucial to the viability of Bt Cry proteins as insecticides and their role in IPM strategies (Liu *et al.*, 2021). The specificity of the receptors to Cry proteins determines the range of Bt toxins, thus minimising the development of insect resistance and impact on the environment (Bravo *et al.*, 2007).

1.2.3. Commercial Bt products

1.2.3.1. Pyramided Bt Cry proteins – Cry1A.105 and Cry2Ab2.820

Transgenic plants were first introduced into commercial agriculture in 1996 (Storer *et al.*, 2012). Cry1Ab and Cry1Ac were the first Bt Cry proteins to be used in cotton and maize crops worldwide (Pilcher *et al.*, 1997; Tabashnik *et al.*, 2013; Grimi *et al.*, 2018). However, like chemical pesticides, widespread use of biopesticides lead to a rapid increase in insect resistance, particularly in lepidopteran insects, as the two Cry proteins are closely related in their mode of action (Storer *et al.*, 2012). To combat this development of resistance, pyramided proteins have been developed (Storer *et al.*, 2012; Carrière *et al.*, 2015).

Pyramided proteins are produced when two or more Bt toxins that are toxic to the same insect species are expressed in a transgenic crop (Roush, 1998; Carrière *et al.*, 2015). Bt toxins are only pyramided when they target different receptors in the insect midgut. This allows both toxins to be active in the insect, midgut at approximately the same time. This is expected to increase the efficacy of the toxins against pests and slow the development of resistance in an insect population (Storer *et al.*, 2012; Carrière *et al.*, 2015; Chen *et al.*, 2021).

Transgenic crop events refer to the process undertaken to introduce genetic traits or modifications into a particular plant species (Rule *et al.*, 2014; Aziz *et al.*, 2022). These events rely on recombinant DNA technology and *Agrobacterium*-mediated transformation (usually *Agrobacterium tumefaciens*) (Klee *et al.*, 1987; Ishida *et al.*, 2007; Yu *et al.*, 2022).

Monsanto Company event MON89034 is a maize product that expresses pyramided Cry proteins in the form of Cry1A.105 and Cry2Ab2 (US EPA 2007; Drury *et al.*, 2008). Cry1A.105 is a chimeric, 133 kDa protein consisting of domain I and II from Cry1Ab or Cry1Ac, domain II from Cry1F, and the C-terminal domain from Cry1Ac (US EPA 2007; Drury *et al.*, 2008; Hernández-Rodríguez *et al.*, 2013; Wang *et al.*, 2018). The 63 kDa Cry2Ab2 protein was originally produced for expression in Monsanto's Bollgard II cotton, through event MON15985, where Cry2Ab2 was pyramided with Cry1Ac (US EPA 2007; Hagenbucher *et al.*, 2016). Cry1A.105 has demonstrated activity against lepidopteran insects *Helicoverpa zea* and *Spodoptera frugiperda* (US EPA 2007; Drury *et al.*, 2008), while Cry2Ab2 demonstrated effectivity against lepidopteran bollworms *Helicoverpa armigera*, *Pectinophora gossypiella*, and *Earias insulana* (Dhanaraj *et al.*, 2018).

Transgenic events such as MON89034 and MON15985 are important for keeping up with the continuous development of insect resistance to Bt Cry proteins. Cry1 and Cry2 proteins are ideal for pyramiding because they bind to different receptors in the insect midgut (Welch *et al.*, 2015; Tabashnik & Carrière, 2020). This receptor specificity eliminates the threat for potential cross-resistance between the two Cry proteins, thus making their combined use highly effective in IPM (Tabashnik *et al.*, 2014; Tabashnik & Carrière, 2017; Kaur *et al.*, 2019).

1.2.3.2. *Bacillus* species and Bt subspecies

The *Bacillus* genus has several species, other than *B. thuringiensis*, which have commercial value in various industries, including agriculture and healthcare (Schallmeyer *et al.*, 2004). The ubiquitous nature of *Bacillus* species and their physiologically diverse characteristics make them attractive options for industrial use, such as their ability to produce enzymes (Rao *et al.*, 1998), antibiotics (Mohapatra *et al.*, 2021), and bioactive, secondary metabolites (Ongena & Jacques, 2008; Keswani *et al.*, 2020; Xiao *et al.*, 2022). The *Bacillus* genus accounts for majority of the Gram-positive bacteria in soil and the rhizosphere (Prashar *et al.*, 2014; Etesami *et al.*, 2023), alongside being the predominant microbial flora in marine ecosystems (Mawlankar *et al.*, 2016; Sharma *et al.*, 2020; Xiao *et al.*, 2022).

B. subtilis, *B. amyloliquefaciens*, *B. mycoides*, and *B. megaterium* are widely used for biostimulation and biofertilisation in modern cropping systems to enhance crop yield, sustainable agriculture and support global food security (Radhakrishnan *et al.*, 2017; Backer *et al.*, 2018; Kurniawan & Chuang, 2022; Etesami *et al.*, 2023). Similarly, *B. pumilus*, and *B.*

cereus are routinely used in fermentation as industrial enzyme producers (Küppers *et al.*, 2014; Chen *et al.*, 2020; Li *et al.*, 2023).

B. thuringiensis boasts several subspecies with insecticidal properties, making them highly useful tools in commercial agriculture for pest control. Subspecies such as Bt subsp. *berliner*, Bt subsp. *aizawai*, Bt subsp. *galleriae*, Bt subsp. *israelensis*, Bt subsp. *tenebrionis*, and Bt subsp. *kurstaki* have been widely studied for their efficacy (Meenatchi & Negi, 2021). However, it is important to note that although these subspecies designations are commonly employed to distinguish between groups with different insecticidal activities, none of the subspecies have been formally validated or named according to official taxonomic standards (Fiuza *et al.*, 2017; Crickmore *et al.*, 2021)

Bt subsp. *kurstaki* was first discovered by Edouard Kurstak in 1962 and remains the most widely used Bt subspecies in agriculture (Kurstaki, 1962; Meenatchi & Negi, 2021). Two prominent strains of Btk, Btk HD-73 and Btk HD-1 are responsible for the production of Cry1Ac and Cry2Ab2, respectively.

1.3. Entomopathogenic viruses

1.3.1. General characteristics

Like Bt-produced Cry proteins, viral entomopathogens play a crucial role in advanced IPM strategies. Viral entomopathogens occur naturally in the environment and contribute significantly to the regulation of pests and other microorganisms in an ecosystem (Alramadan & Mamay, 2019; Deka *et al.*, 2021). Entomopathogenic viruses are obligate microorganisms comprised of a nucleic acid that is responsible for directing replication within the host, and a proteinaceous capsid essential for the infection process (Deka *et al.*, 2021). dsDNA viruses are the most used in agricultural insecticide development as they are typically comprised of a large genome which can be manipulated, are host-specific, and harmless to other microorganisms, humans, and the environment (Deka *et al.*, 2021; Harish *et al.*, 2021). RNA viruses present with a smaller genome size, structural simplicity, and high replication rates, which make them unfavourable for genetic manipulation (Chen *et al.*, 2012; Sosa-Gómez *et al.*, 2020; Gelaye & Negash, 2023).

Entomopathogenic viruses are further classified as occluded and non-occluded viruses. Occluded viruses comprise of mature virions that are enclosed within a protein matrix, forming

para-crystalline bodies known as occlusion bodies (OBs) or polyhedra; while non-occluded viruses comprise of virions that occur freely and are characterised by the absence of OB protein (Del Rincón-Castro & Ibarra, 2011; Alramadan & Mamay, 2019; Keown *et al.*, 2023). Unlike non-occluded viruses belonging to families such as *Iridoviridae* and *Polydnaviridae*, occluded viruses from families like *Baculoviridae*, *Poxviridae*, and *Reoviridae* (Alramadan & Mamay, 2019; Sosa-Gómez *et al.*, 2020), are known to persist in natural environments due to the stability and protection from extreme temperatures and ultraviolet (UV) light provided by OBs (Sajjan & Hinchigeri, 2016; Deka *et al.*, 2019; Rohrmann, 2019).

1.3.2. *Baculoviridae*

1.3.2.1. General characteristics

Baculoviridae, commonly known as baculoviruses, are a large family of entomopathogenic viruses (Afolami & Oladunmoye, 2017). These viruses are well known as biological control agents, and are pathogenic to invertebrates, especially crustaceans and insects (Clem & Passarelli, 2013; Bateman and Stentiford, 2017). Most of these viruses infect orders Lepidoptera, Diptera and Hymenoptera (Deka *et al.*, 2021).

Baculoviruses are occluded, dsDNA viruses with a covalently closed circular genome. The viruses are large and genome size can range between 80 and 180 kilobase pairs (kbp), coding for approximately 180 genes (Funk *et al.*, 1997; Okano *et al.*, 2006; Ibarra & Del-Rincón-Castro, 2008; Miele *et al.*, 2011; Clem & Passarelli, 2013; Schroeder & Kevill, 2021). These viruses are genetically different from other entomopathogenic virus families, with more than 30 core genes conserved across all baculoviruses (Okano *et al.*, 2006; Miele *et al.*, 2011). These genes are conserved across different strains of virus to maintain the viral life cycle and function. Approximately half of these genes code for proteins associated with the virion structure such as the capsid or envelope of the virus, which are essential components for initial viral infectivity. The remainder of the proteins are involved in genome stability, replication, and proofreading (Faulkner *et al.*, 1997; Burke *et al.*, 2013; Wang *et al.*, 2017; Rohrmann, 2019).

The current classification of *Baculoviridae* comprises two groups – granuloviruses (GVs) and nucleopolyhedroviruses (NPVs) (Volkman, 1997). These are also further classified into four genera – *Alphabaculovirus* which constitutes the largest and most important group of NPVs as they are lepidopteran specific; *Betabaculovirus* which are lepidopteran specific GV; *Gamma*

Gammabaculovirus which are hymenopteran specific viruses; and *Deltabaculovirus* which are dipteran specific viruses (Miele *et al.*, 2011; Afolami & Oladunmoye, 2017). GV's are characterised by a single nucleocapsid within a virion, whereas NPVs can have single (HearSNPV) or multiple (HearMNPV) nucleocapsids within a virion (Fuxa, 1991; Rohrmann, 1992; Williams *et al.*, 2017; Rohrmann, 2019; Gelaye & Negash, 2023).

1.3.2.2. Mode of action

Baculovirus replication is uniquely biphasic and is characterised by the production of two virion phenotypes: occlusion-derived virions (ODVs), which occur inside OBs and initiate primary infection of the host, and budded virions (BVs) which are responsible for spreading infection from the insect midgut to other cells within the host (Szewczyk *et al.*, 2006; Battu *et al.*, 2023).

Infection almost always occurs through ingestion by the insect host (Fabre *et al.*, 2020; Gelaye & Negash, 2023). Although the OBs of the virus can resist digestive enzymes and other host barriers of the insect, the OBs will readily dissolve in the alkaline conditions (pH 9.5 – 11.5) of the insect larval midgut (Granados, 1980). Once dissolved, enveloped virions are released into the epithelium lining of the midgut. Primary infection takes place in the nucleus of the infected cell, with new progeny nucleocapsids being produced. (Fabre *et al.*, 2020). These progeny nucleocapsids bud through the nuclear membrane and acquire an envelope. Once in the cytoplasm, the nucleocapsids lose their envelope, and subsequently bud through the plasma membrane with a new envelope. The virions of the new budded virus are responsible for spreading infection to other cells in the host and produce a secondary round of replication (Gelaye & Negash, 2023). Upon cell lysis and overall death of the host, OBs are released into the environment, and can be ingested by other larvae with contaminated foliage (Rohrmann, 1986; Blissard and Rohrmann, 1990; Schroeder and Kevill, 2021; Keown *et al.*, 2023). The infection cycle is summarised in Figure 1 below (Battu *et al.*, 2023).

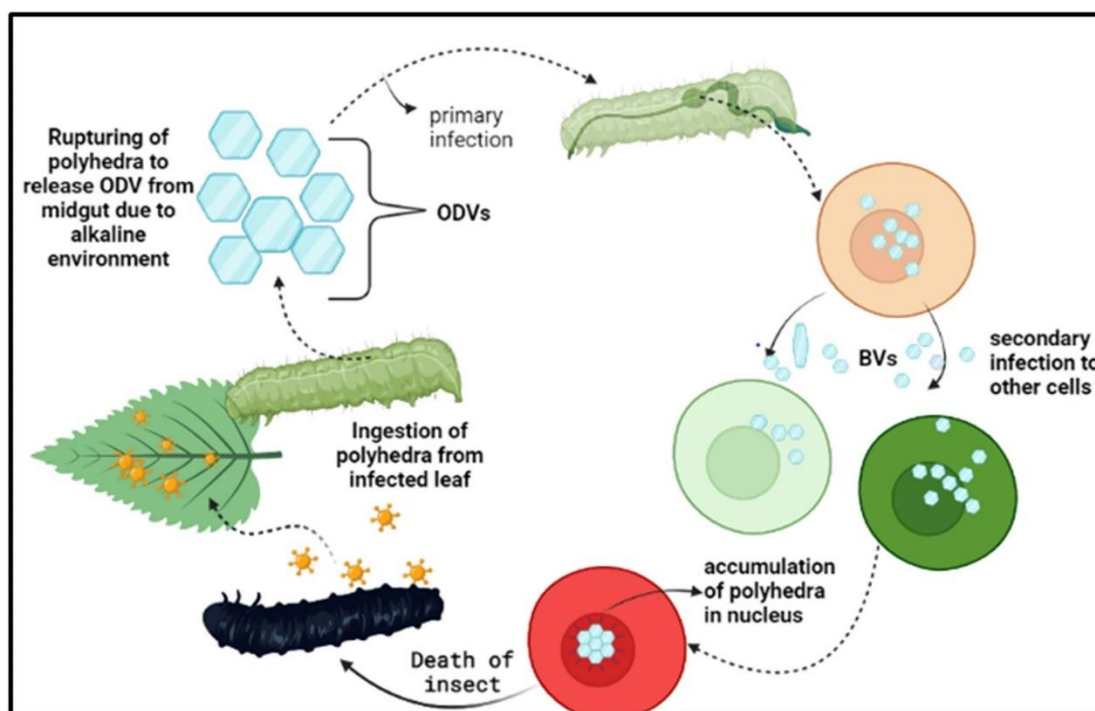


Figure 1. Summary of the infection cycle of baculoviruses. The biphasic replication cycle starts when an insect ingests viral particles, and results in the production of two virion phenotypes – occlusion derived virions (ODVs) that initiate primary infection of the host, and budded virions (BVs) that function in spreading infection to other cells and organs of the insect host. This image, obtained from Battu et al. (2023), is licensed under a Creative Commons Attribution 4.0. International License (<http://creativecommons.org/licenses/by/4.0/>). It has not been modified.

Infected larvae can lack external symptoms for up to 5 days post-infection. However, common symptoms include gradual changes in the colour of the integument, particularly paleness and opaqueness followed by darkening of the skin (Goulson & Gory, 1995; Williams *et al.*, 2017). Some larvae will demonstrate a lack of activity and appetite, while liquefaction and flattening of larvae are also observed (Rohrmann, 2019; Harrison and Hoover, 2012; Harish *et al.*, 2021).

1.3.3. HearNPV and bioinsecticide development

Helicoverpa armigera nucleopolyhedrovirus (HearNPV) is a viral entomopathogen in the *Alphabaculovirus* genus (Miele *et al.*, 2011; Beperet *et al.*, 2021). Several factors, including high pathogenicity and replication rates, a narrow target host range, and the wide range of proteins produced by the virus to assist in initiating and enhancing infection, have positioned the virus as a vital bioinsecticide in global agriculture (Peng *et al.*, 1999; Moore *et al.*, 2004; Harrison & Hoover, 2012). HearNPV formulations are common in agriculture, however, they are known to subdue instead of eliminating the pest population (Williams *et al.*, 2017; Black *et al.*, 2022). The virus also takes longer than other bioinsecticides to induce mortality (Black

et al., 2022). But, because the virus is present in the insect for an extended period, horizontal transmission is likely, thus, increasing the possibility of future infestations (Young and Yearian, 1986; Young and McNew, 1994; Black *et al.*, 2019; Black *et al.*, 2022).

A 2022 study by Arrizubieta *et al.*, sought to provide a solution to the slow infectivity of HearNPV through infection with a cooccluded HearSNPV and HearMNPV. Results of this study indicated that both viruses can coinfect host cells, but only within a limited period (approximately 24 hours). The increase in infectivity of cells was prevalent when hosts were infected with HearMNPV before the homologous HearSNPV was introduced (Arrizubieta *et al.*, 2022). Although promising, this method of prolonged and increased infectivity is yet to undergo field testing (Beperet *et al.*, 2021; Arrizubieta *et al.*, 2022).

HearNPV has emerged as a viable alternative to traditional pesticides and remains crucial in developing IPM strategies (Windus *et al.*, 2021). The virus' limited host range and prolonged infectivity presents scientists with an opportunity to develop novel HearNPV bioinsecticides (Beperet *et al.*, 2021; Arrizubieta *et al.*, 2022).

1.4. Biological assays

1.4.1. Bioassays and probit analysis

A laboratory bioassay is a method used to determine the toxicity of a substance to a biological organism (Finney, 1947; Karunarathne *et al.*, 2022). Bioassays are essential for identifying insect resistance mechanisms, studying modes of action, and determining correct dosage ranges for a particular pesticide and organism (Robertson & Worner, 1990). Traditionally, dose-response bioassays are used in insecticide development. Dose-response bioassays form the foundation of commercial biopesticide development (Karunarathne *et al.*, 2022).

The median lethal concentration (LC₅₀) and the median lethal dose (LD₅₀) are values used to describe the concentration and dose that kill 50% of the test population, respectively (Hamilton *et al.*, 1977; Wheeler *et al.*, 2005; Pillai *et al.*, 2021). Determination of these values explores the relationship between a toxin's concentration or dose to the ultimate response – death (Raj *et al.*, 2013). Death indicates a complete breakdown of the insect's defence systems and provides researchers with an unambiguous outcome of the experiment (Zhu, 2008).

Similarly, sublethal effects of a toxin can be estimated using the median inhibitory concentration (IC₅₀) and median inhibitory dose (ID₅₀). These values indicate the concentration

or dose of a toxin required to inhibit a pest's biological or physiological function by 50% (Li & Bouwer, 2012). Recognising sublethal effects and the point at which they occur in an insect population is important for identifying changes in insect behaviour after treatment with a biopesticide. Also, sublethal become critical once an insect has developed resistance towards a particular insecticide (Guedes *et al.*, 2015; de França *et al.*, 2017).

Bioassays and dose-response relationships are often analysed using probit analysis. Probit analysis is a regression method used to analyse a quantal response to increasing doses or concentrations of a stimulus (Postelnicu, 2014; Karunaratne *et al.*, 2022). Here, probits and logarithmic (log) doses or concentrations are used to estimate doses or concentrations of a specific biopesticide to the test population (Chandra *et al.*, 2015). Probit analysis assumes normally distributed data, otherwise log concentrations and doses are used. Most probit analyses assume 95% or 99% confidence intervals (CI) or fiducial limits (Chandra *et al.*, 2015).

Corrected percentage mortality is crucial to probit analysis. The corrected mortality value is used to represent the mortality that does not occur due to the given pesticide. The most common correction method used is Abbott's formula (Abbott, 1925), which accounts for the natural mortality of the insects (Kandil *et al.*, 2020).

1.4.2. Artificial diets in bioassays

Artificial diets play an important role in IPM strategies and provide a rapid and standardised medium on which bioassays can be performed. These diets are designed with the aim of mimicking the natural environment of the insect and ensuring that the insect population receives all the necessary nutrients for growth (Navon and Ascher, 2000). Also, the diet allows a way for the insects to directly feed on the toxin of interest (Romeis *et al.*, 2010; Li *et al.*, 2014). Standardising and maintaining constant rearing conditions reduces variability when performing bioassays, thus maintaining the reliability and quality of bioassay performed (Ren and Frymier, 2004). Bioassays should aim to mimic field conditions as accurately as possible to ensure that susceptibility and resistance data remain reliable when applied in the field (Kranthi, 2005). However, results obtained from bioassays on laboratory populations may not always translate similarly in the field populations (Grant and Bouwer, 2009).

1.5. Justification of the study

Although a complex and multifaceted issue, agriculture forms the backbone of global food security (Pawlak and Kołodziejczak, 2020). The rapid growth of the world's population, alongside the effects of climate change place a major burden on the agricultural industry. With at least 691 million people globally experiencing food insecurity in 2022 (FAO, 2023), managing agricultural practices and agricultural pests has become a crucial factor of maintaining and improving crop yields (Anderson *et al.*, 2019; Shah & Wu, 2019).

The recent spread of *H. armigera* into the New World has solidified the pest's global distribution and has presented an opportunity for the polyphagous pest to further widen its host range (Arnemann *et al.*, 2019; Haile *et al.*, 2021). In Africa, *H. armigera* is known to infect many staple and cash crops, including cotton, wheat, maize, sorghum, tomato, (Tsafack *et al.*, 2015; Riaz *et al.*, 2021) soya beans, chickpea, sunflower and citrus crops (Truzzi *et al.*, 2017; Edosa, 2019; Fite *et al.*, 2018). Current global economic losses caused by the pest are estimated at USD \$3 billion annually (Haile *et al.*, 2021; Riaz *et al.*, 2021). The pest's ability to survive in many climates across the world and its polyphagous nature make it suitable for exploring the broad spectrum of pest-host interactions using bioassays, and developing sustainable control methods (Tsafack *et al.*, 2015; Riaz *et al.*, 2021)

Bioassays are a crucial part of biopesticide development. Biopesticides are crucial to modern, agricultural IPM (Kumar *et al.*, 2021). However, the commercialisation of biopesticides faces several challenges, with the most significant being the lack of standardisation when evaluating biopesticide toxicity and efficacy using bioassays (Chandler *et al.*, 2011; Mills *et al.*, 2016; Kumar *et al.*, 2021). Bioassays are also used to evaluate the mode of action of a biopesticide on the host, therefore, comparing results from multiple bioassay methods provides insight into how the pest is affected by the biopesticide (Mills *et al.*, 2016). Through standardisation, the consistency and reproducibility of bioassay methods can be improved. This allows for more direct comparisons and similar quality control measures between studies (Blouquy *et al.*, 2021; Matope *et al.*, 2023).

This study seeks to contribute to the advancement of biopesticide development through the critical analysis of two common bioassay methods – the droplet feeding bioassay and the diet overlay bioassay. By identifying and comparing the effectiveness of each method to different entomopathogens and toxins used against *H. armigera*, this study aims to contribute to the

establishment of standardised protocols for comparable, reliable and sustainable pest management strategies.

1.6. Aim and objectives

1.6.1. Aim

To compare and identify the suitability of two bioassay methods used for the assessment of dose-response relationships of entomopathogens and toxins against *H. armigera*, by identifying the most effective method for each.

1.6.2. Objectives

1. To rear second instar larvae of *H. armigera* for use in bioassays, alongside maintaining multiple generations of the pest, under laboratory conditions.
2. To determine the LD₅₀ of Cry1A.105, Cry2Ab2.820, Btk HD-73 SCC and HearNPV OBs of second instar larvae of *H. armigera* using the droplet feeding bioassays.
3. To determine the LC₅₀ of Cry1A.105, Cry2Ab2.820, Btk HD-73 SCC and HearNPV OBs of second instar larvae of *H. armigera* using the diet overlay bioassays.
4. To compare the relative toxicity of Cry1A.105, Cry2Ab2.820, Btk HD-73 SCC and HearNPV OBs used against second instar larvae of *H. armigera*, using differences in their lethal doses and concentrations, and associated metrics.

Chapter Two – Methodology

2.1. Insect rearing

Laboratory populations of *H. armigera* were reared on a semi-synthetic, wheat-germ diet (Bot, 1966), with one larva placed into each well to avoid cannibalism (Bot, 1966; Fitt, 1989). Plates were incubated at 25 °C, 60 ± 10% relative humidity (RH), and a 12:12 light: dark (L:D) cycle. Pupae and eggs were sterilised in 0.25% (v/v) and 0.2% (v/v) sodium hypochlorite solution, respectively (Enkerlin & Pereira, 2022). Up to 34 pupae were transferred into glass jars, with the tops covered in mesh nets to allow for oviposition and aeration. Once emerged, adult moths were fed a 5% (w/v) sucrose solution presented on cotton wool rolls (Bot, 1966).

2.2. Btk HD-73

2.2.1. Spore-crystal complex (SCC) production

A 300 µl sample of Btk HD-73 cryostock was inoculated in 50 ml of nutrient broth, and incubated at 30 °C, 200 rpm overnight. The Btk HD-73 sample used here is *B. thuringiensis* subspecies *kurstaki*. The HD-73 refers to the specific strain being used. After observing turbidity in the nutrient broth, 5 ml of the overnight sample was inoculated into 50 ml of T3 medium for sporulation (Dulmage, 1970). This was incubated at 30 °C, 200 rpm, for 96 hours (Travers *et. al.*, 1987). After the 96-hour incubation period, the T3 media containing the SCC were transferred to six 50 ml Falcon tubes and subjected to centrifugation at 5000 *g* for 15 minutes at 4 °C. The supernatant of each tube was discarded, and the pellet resuspended in 20 ml of sterile distilled water. The samples were subjected to centrifugation for a total of three washes in sterile distilled water (5000 *g* for 15 minutes at 4 °C), with the supernatant discarded between each wash. All pellets were resuspended in 20 ml of sterile distilled water and stored at -80 °C until use.

2.2.2. Colony morphology

Nutrient agar plates were prepared and streaked with the turbid overnight Btk HD-73 sample that was grown in the nutrient broth. The plates were incubated at 30 °C for 24 hours. Colony characteristics were observed and noted (Finegold & Sweeney, 1961; Sanders, 2012).

2.2.3. SCC characterisation

Brightfield light microscopy and Coomassie brilliant blue (CBB) staining were used to confirm the presence of spores and crystals in the Btk HD-73 sample. A loopful of the sample was diluted in 200 µl of sterile distilled water and flooded with 0.13% (w/v) CBB G-250 in 50% (v/v) acetic acid. The sample was rinsed with sterile distilled water, before viewing under the 100× objective of the light microscope (Rampersad & Ammons, 2005; Suvathi *et al.*, 2019; Siallagan *et al.*, 2020).

2.3. SDS-PAGE densitometry

To confirm Cry1Ac production in Btk HD-73 SCC and screen for potential visible protein degradation in the purified Bt Cry proteins (Monsanto Company, USA), all three samples were subjected to electrophoresis. A sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was set up using the 7.5% TGX Stain-Free™ FastCast™ Acrylamide Starter Kit, (Bio-Rad Laboratories, Inc., USA). The purified Cry1A.105 and Cry2Ab2.820 proteins were expected to produce bands at approximately 131 kDa and 61 kDa (Hernández-Rodríguez, *et al.*, 2013; Hagenbucher *et al.*, 2017), respectively. As Btk HD-73 SCC contains the Cry1Ac protein, a band was expected at approximately 130 kDa (Wei *et al.*, 2016).

A 1 ml sample of the Btk HD-73 SCC was aliquoted into a microcentrifuge tube and centrifuged at 12 044.88g for 10 minutes, and the supernatant discarded. The pellet was resuspended in 100 µL of sterile distilled water before being subject to the SDS-PAGE.

Half-fold dilutions of bovine albumin serum (BSA), ranging from 1.0 mg/ml to 0.05 mg/ml, were included on the gel to assist with protein quantification. Both Cry proteins, the Btk HD-73 SCC sample, and the BSA samples were added to a 2× sample buffer and dithiotreitol (DTT) solution, before being placed into boiling water for 15 minutes (Rehbein and Schwalbe, 2015). Unstained Protein Standard, Broad Range #P7717S (New England Biolabs, USA), was used as the molecular weight marker (MWM).

The protein and BSA samples, alongside the MWM, were loaded onto the gel and subject to SDS-PAGE at 100V for 10 minutes, followed by 80V for 30 minutes. Electrophoresis was followed by gel staining, for 45 minutes, using a 0.1% (w/v) Coomassie Brilliant Blue (CBB) G-250 solution (Rehbein & Schwalbe, 2015). The gel was destained in destaining solution for 15 minutes (Rehbein & Schwalbe, 2015), then placed in sterile distilled water overnight.

To set up a standard curve of known BSA concentrations versus band volume, and to estimate the protein concentration of the SCC sample (Alonso Villela *et al.*, 2020), the gel was visualised using the Bio-Rad ImageLab software (Bio-Rad Laboratories Inc., USA).

2.4. Bradford assay

To determine the amount of protein produced in the Btk HD-73 SCC sample, a Bradford assay was performed according to Bradford (1976). Briefly, a pellet of the HD-73 SCC sample was resuspended in 2 ml of sterile distilled water. To generate a standard curve, BSA dilutions of 1.0 mg/ml, 0.8 mg/ml, 0.6 mg/ml, 0.4 mg/ml, 0.2 mg/ml, 0.1 mg/ml, and 0.05 mg/ml were prepared. All samples were diluted in sterile distilled water (20 µl sample to 980 µl sterile distilled water), before 1 ml of Bradford reagent was added to each sample. Samples were then incubated at room temperature for 5 minutes and observed for a brown to blue colour change (Bradford, 1976). A spectrophotometer set at wavelength 595 nm was used to measure absorbance in each sample three times. A standard curve was generated using the absorbance readings of the BSA dilutions to determine the protein concentration.

2.5. HearNPV occlusion body (OB) enumeration

A reference sample of HearNPV was obtained from the Invertebrate Pathology and Biocontrol Laboratory of the University of the Witwatersrand. The sample was presented as OBs in sterile distilled water. Phase contrast microscopy and the Neubauer Improved Bright Line haemocytometer (Hennenberg Sander GmbH, Germany), were used for the enumeration of HearNPV OBs (Evans and Shapiro, 1997).

2.6. Toxicity bioassays

Both purified Bt Cry proteins were provided with their corresponding N-cyclohexyl-3-aminopropanesulfonic acid (CAPS) buffers, i.e. Cry1A.105 solubilising buffer and Cry2Ab2.820 solubilising buffer (Monsanto Company, USA). These are referred to as corresponding buffers throughout.

2.6.1. Diet overlay bioassay

The diet overlay bioassays were performed using the modified method of Storer *et. al.*, (2010). Semi-synthetic wheat-germ diet was prepared as stipulated by Bot (1966), and equal amounts poured into 32-well plates. The diet was dried at room temperature, overnight, before being used in the bioassays.

Feeding solutions were prepared with different doses of toxin or entomopathogen and their corresponding buffers, before 75 μ L of the solution was evenly aliquoted into each well of prepared diet. Plates were then covered in paper towel and allowed to dry at 25°C, 60 $\pm$ 10% RH, for 2 hours. A single second instar larva of *H. armigera* was then placed into each well, and plates sealed and perforated before incubating at 25°C, 60 $\pm$ 10% RH, at a 12:12 L:D photocycle. Mortality was scored after 7 days of incubation. Larvae that did not move or respond to gentle prodding with a paintbrush were recorded as dead. Surviving larvae were weighed in milligrams. Four replicates of 16 larvae per replicate (n = 64) were used for each concentration of each toxin and entomopathogen. Corresponding Bt Cry protein buffers and sterile distilled water were used as the controls in this bioassay. Sterile distilled water was used as the control for HD-73 SCC and HearNPV bioassays.

2.6.2. Determination of volumes imbibed by larvae of *H. armigera*

The exact volume of entomopathogen or toxin ingested by second instar larvae of *H. armigera* was determined using the modified fluorescent dye method of Bouwer and Aveyidi (2006). The method was modified from van Beek and Hughes (1986).

H. armigera larvae were removed from rearing plates and placed into empty 32-well plates. The larvae were starved for 2 hours prior to performing the bioassay to encourage feeding. A feeding solution of 0.5 mg/ml erioglaucine (blue dye) and 2.4 mg/ml sodium fluorescein (yellow, fluorescent dye) dissolved in 1 ml of corresponding buffer or sterile distilled water – for each toxin or entomopathogen – was prepared.

Sterile distilled water was used as the control for the HD-73 SCC and HearNPV. Individual feeding arenas, one for each dose, were set up in 90 $\times$ 15 mm Petri dishes, with twelve 5 μ l droplets of feeding solution arranged equidistant to each other, in two circles. The outer circle had eight droplets, with four droplets in the inner circle. Thirty-two second instar *H. armigera* larvae that were previously starved, were placed into each arena and allowed to imbibe on the 5 μ l droplets of feeding solution. Larvae that had ingested solution were identified by green

colouring in their foregut and midgut, and immediately transferred into individual microcentrifuge tubes. For each feeding solution, n = 16 for each replicate of controls. Any arena with fewer than eight larvae which had imbibed on the feeding solution was omitted from the experiment. The arena was discarded once droplets started drying up or after 30 minutes (whichever came first). Collected larvae were frozen at -20 °C for at least 24 hours before further use.

Frozen larvae were homogenised in 1.5 ml of 50 mM phosphate buffer, pH 7.7, and the absorbance of sodium fluorescein in each larval sample measured using the FP-8200 Spectrofluorometer (JASCO, Japan). Measurement conditions were set at an excitation wavelength of 481.5 nm and an emission wavelength of 511.5 nm (van Beek and Hughes, 1986). The volume imbibed by larvae was calculated from a standard emission calibration curve set up using absorbance measurements of sodium fluorescein serial dilutions of 160 ng/ml to 1.25 ng/ml (Bouwer and Aveyidi, 2006). Volume ingested was calculated according to van Beek and Hughes (1986), and Kunimi and Fuxa (1996):

$$\text{nl ingested} = \frac{\text{ng fluorescein in 1.5ml of larval homogenate}}{\text{ng per nl fluorescein in the feeding solution}}$$

2.6.3. Droplet feeding bioassay

The protocol as described by Bouwer and Aveyidi (2006) was used to perform the droplet feeding bioassays. Prior to experiments, second instar larvae of *H. armigera* were starved for two hours, to encourage feeding.

A feeding solution of appropriate toxin or entomopathogen and 0.5 mg/ml erioglaucine was prepared and twelve 5 µl droplets arranged in a feeding arena, as described in section 2.6.1. Each corresponding buffer or sterile distilled water was used as the control. Larvae that had ingested the feeding solution, indicated by blue colouring in the foregut and midgut, were immediately transferred onto 32-well plates containing the semi-synthetic wheat-germ diet of Bot (1966). Plates were sealed and perforated for aeration, and incubated at 25°C, 60 ± 10% RH at a 12:12 L:D photocycle. Only one larva was placed into each well. Mortality was scored after 7 days. Larvae that did not move or respond to gentle prodding with a paintbrush were recorded as dead. Surviving larvae were weighed in milligrams. Four replicates of 16 larvae per replicate (n = 64) were used for each dose of each toxin and entomopathogen.

2.7. Statistical analysis

2.7.1. Determination of volume imbibed

GraphPad Prism 9.5.0 (GraphPad Holdings LLC, USA) and Microsoft Excel version 2405 (Microsoft Corporation, USA) were used to determine the average dose of each toxin or entomopathogen ingested by second instar *H. armigera* larvae in the droplet feeding bioassay. Statistical significance between the volumes imbibed were determined through the ordinary one-way ANOVA ($p < 0.05$).

2.7.2. Toxicity bioassays

Mortality data from both bioassays underwent probit regression analyses (Finney, 1971), using LdP Line software (LdP Line, Egypt). Mortality data was used to estimate the LC_{50} for each toxin and entomopathogen used in the diet overlay bioassays. Both mortality data from the droplet feeding bioassays and volume imbibed data were used to estimate the LD_{50} for each toxin and entomopathogen

2.7.3. Sublethal assessments

Surviving larvae of both bioassays were used to determine the effect of sublethal concentrations and doses of each toxin and entomopathogen on the growth of *H. armigera* second instars. Concentration-weight and dose-weight responses were used to calculate and estimate IC_{50} and ID_{50} for each toxin and entomopathogen in the diet overlay and droplet feeding bioassays, respectively. All analysis was performed on GraphPad Prism 9.5.0 (GraphPad Holdings LLC, USA). Weight-response data of each concentration and dose were normalised (smallest mean in data set = 0%, largest mean in data set = 100%), before undergoing non-linear regression (curve fit) analysis using the function: log(inhibitor) vs. response – variable slope (four parameters).

Chapter Three – Results

3.1. Btk HD-73

3.1.1. Colony morphology

Colonies of Btk HD-73 were observed to be round, with an off-white colour. The colonies were opaque and slightly raised, with a slightly convex and shiny surface. The colonies varied between smooth and wrinkled edges. The colony morphology observed is consistent with morphology of Bt subsp. *kurstaki* (Mukhija & Khanna, 2018). However, colony morphology alone cannot be used to differentiate between Bt and other similar *Bacillus* species such as *B. cereus* and *B. anthracis*, hence the need for additional characterisation steps, such as CBB staining used in this study (Rampersad & Ammons, 2005; Suvathi *et. al.*, 2019; Siallagan *et. al.*, 2020).

3.1.2. SCC CBB staining

The stained SCC sample displayed scattered, free bipyramidal crystals. Some of the crystals appear singularly, while the spores present with a halo, and mostly occur in clusters or in pairs (Figure 2). The presence of spores and crystals indicates that the Btk HD-73 sample successfully sporulated (Mourin *et al.*, 2015). This result is expected as the SCC is a derivative of Btk HD-73, which carries genes responsible for producing the Cry1Ac protein. As a member of Cry1 proteins, we expect to see the bipyramidal crystals in the SCC sample (Höfte & Whiteley, 1989). While some Bt subspecies or strains produce mixed crystals, e.g. bipyramidal and cuboidal crystals, Btk HD-73 is not one of them (Mukhija & Khanna, 2018).

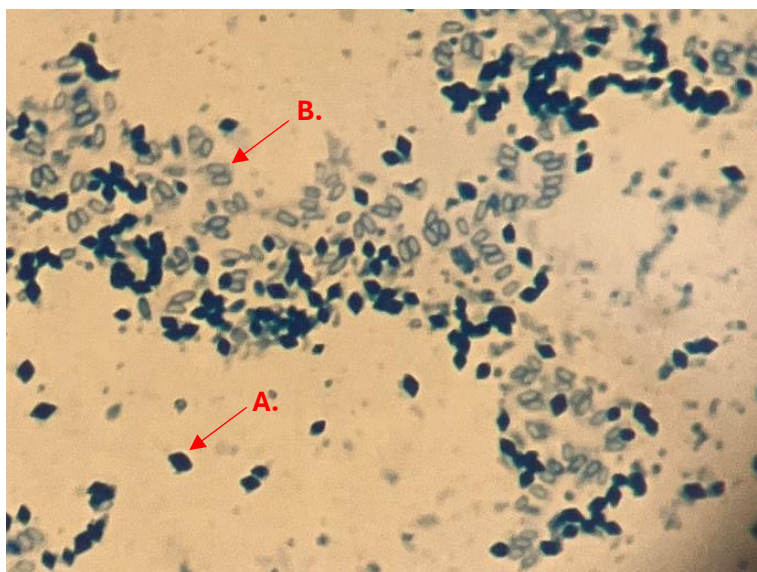


Figure 2. Morphology of the SCC after the 96-hour incubation of Btk HD-73 in T3 medium. Image shows spores and crystals CBB-stained (details in methods), under 100× oil immersion objective of a brightfield microscope. (A) Bipyramidal crystals dyed in the dark blue colour. (B) A pair of spores outlined in the blue halo.

3.2. SDS-PAGE densitometry

The purified Bt Cry proteins were provided by Monsanto (Monsanto Company, USA). Cry2Ab2.820 has a concentration of 0.36 mg/ml at 87% purity; Cry1A.105 has a concentration of 1.2 mg/ml at 80% purity.

Cry2Ab2.820 was identified with the thick band (band A) at approximately 61 kDa in lane 7, while Cry1A.105 was identified with the thick band (band B) at approximately 131 kDa in lane 8. The bands confirmed that none of the Cry proteins had visibly degraded in storage. The Cry1Ac protein in HD-73 SCC is seen at approximately 133 kDa in lane 9, at band C (Figure 3).

A standard curve of $y = 1.43 \times 10^{-5}x - 276$, with $R^2 = 0.9377$ was generated from the BSA concentrations. ImageLab software (Bio-Rad, Laboratories Inc., USA) was used to estimate the protein concentrations of Cry2Ab2.820, Cry1A.105 and Btk HD-73 SCC at 0.26 mg/ml, 0.10 mg/ml, and 0.79 mg/ml, respectively.

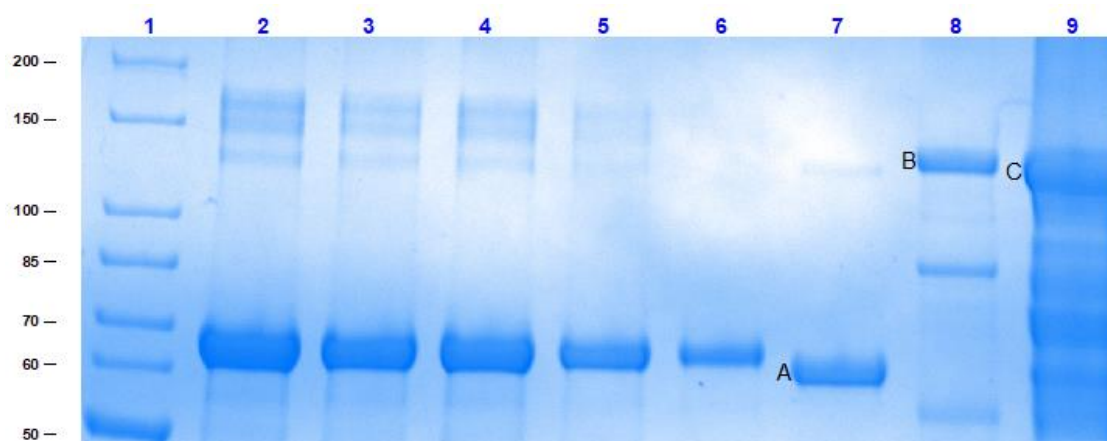


Figure 3. SDS-PAGE of purified Bt Cry proteins (Monsanto Company, USA) and the spore-crystal complex of Btk HD-73. Lane 1 - Unstained Protein Standard, Broad Range #P7717S (kDa) (New England Biolabs Inc., USA); Lanes 2 to 6 – BSA dilutions 1.0 mg/ml, 0.8 mg/ml, 0.6 mg/ml, 0.4 mg/ml, 0.2 mg/ml. Lane 7 – Cry2Ab2.820 at 61 kDa (band A). Lane 8 – Cry1A.105 at 131 kDa (band B). Lane 9 – Cry1Ac in Btk HD-73 spore-crystal complex at 133 kDa (band C). Gel was visualised using ImageLab software (Bio-Rad Laboratories Inc., USA).

3.3. Bradford assay

A standard curve of BSA concentration versus absorbance at 595 nm was generated, where $y = 0.5338x + 0.4104$; $R^2 = 0.9841$. Here protein concentrations were estimated: Cry2Ab2.820 at 0.21 mg/ml, Cry1A.105 at 0.64 mg/ml and, HD-73 SCC at 1.42 mg/ml. As Cry2Ab2.820 and Cry1A.105 are at 87% and 80% purity, and HD-73 SCC is a mixed sample, the Bradford assay cannot alone be used to estimate the total protein concentration.

Instead, the protein concentrations were estimated according to the following: band of interest percent in the corresponding SDS-PAGE lane $\times$ total protein concentration calculated in the Bradford assay. The protein concentrations were estimated to be: Cry2Ab2.820 at 0.15 mg/ml, Cry1A.105 at 0.1 mg/ml, and Cry1Ac in HD-73 SCC at 0.4 mg/ml. It is important to note that these estimates reflect a percentage of the total sample.

However, for consistency and ease of use, bioassay concentrations were based on the concentrations provided by Monsanto, while the total protein concentration estimated in the Bradford assay for HD-73 SCC (1.42 mg/ml) was used for both bioassay methods.

3.4. Toxicity bioassays

It is important to note that high control mortalities (> 15% for each replicate) were observed in the initial results of both bioassay methods. Thus, the estimates seen in this section should be interpreted with caution due to the potential impact of these high control mortalities on the data.

3.4.1. Diet overlay bioassays

The results of the diet overlay probit analyses are shown in Table 1. Cry1A.105 presents with a LC_{50} of 59.33 ng/cm². The concentration-response curve slope reported for Cry1A.105 is moderately shallow compared to the slope reported for HearNPV in the same bioassay. The differences between the observed and tabulated χ^2 are statistically significant (data not shown), indicating that the fit of the model to Cry1A.105 data could be improved. This result is reinforced by the h-value ($h = 2.95$), indicating variability or heterogeneity of individual larval responses to the same concentration of Cry1A.105. Although the CV reported is less than 100%, it is the highest reported of the toxins and entomopathogen used in the diet overlay bioassays. This again suggests, variability within the data set which may affect the LC_{50} estimate. The relative precision (1.40) is poorer than those observed for HD-73 SCC and HearNPV. Relative precision illustrates the degree of reproducibility of bioassay results.

LC_{50} and associated values could not be calculated for Cry2Ab2.820, as all concentrations tested in the bioassay resulted in mortality below 50%. Additionally, none of the concentrations tested in the bioassays, including the highest concentration of Cry2Ab2.820, demonstrated any concentration-response effect against second instars of *H. armigera*.

HD-73 SCC presents with a shallow slope, indicating low sensitivity of *H. armigera* second instars to the toxin. The h-value reported for HD-73 SCC is very close to 1, demonstrating that the observed variability closely matches the expected variability in the data set. This result is supported by the narrow 95% FLs seen with the estimated LC_{50} , alongside the CV and relative precision calculated for HD-73 SCC (Table 1). The CV and relative precision are the lowest for HD-73 SCC when compared to the toxins and entomopathogen used in the diet overlay bioassay. It is also important to note that the observed χ^2 is not statistically different from the value. Overall, this shows that the probit model is a good fit for HD-73 SCC.

HearNPV presents with a steep slope (slope = 1.03 ± 0.19), indicating high toxicity towards larvae per unit of concentration in the diet overlay bioassay (Table 1). The h-value ($h = 1.22$) reported indicates slightly less uniformity in insect response than expected from the bioassays. This demonstrated heterogeneity is supported by the 95% FLs reported with the LC₅₀. Although relatively narrow, the FLs are wider than those reported for HD-73 (Table 1). Ultimately, the probit model and HearNPV are a good fit. This result is confirmed by the CV and relative precision values.

Table 1. Median lethal concentrations (LC₅₀) of Cry1A.105 (ng/cm²), Cry2Ab2.820 (ng/cm²), Btk HD-73 SCC (ng/cm²), and HearNPV (OBs/cm²) against second instar larvae of *H. armigera*, as determined by diet overlay bioassays.

	LC ₅₀	95% Fiducial limits		Slope SE ^a	±	χ^2 ^b	h	CV (%)	Relative precision ^d
		Lower limits	Upper limits						
Cry1A.105	59.33 ng/cm ²	30.72	113.52	0.86 ± 0.10		20.68*	2.59	35.60	1.40
Cry2Ab2.820	-	-	-	-		-	-	-	-
HD-73 SCC	1.18 ng/cm ²	0.78	1.63	0.68 ± 0.09		8.94	0.99	18.38	0.72
HearNPV	3.90 OBs/cm ²	2.66	5.70	1.03 ± 0.19		6.12	1.22	19.88	0.78

- indicates values could not be calculated by probit analysis due to < 50% mortality observed at all concentrations

^a slope ± standard error

^b observed chi-square; *indicates that differences between the observed and calculated χ^2 are statistically significant

^c heterogeneity factor (chi-square value divided by degrees of freedom)

^d the degree of reproducibility of the bioassay

3.4.2. Determination of volume imbibed in droplet feeding bioassays

The volumes of different solubilising buffers imbibed by second instar larvae of *H. armigera* are presented in Table 2. Larvae imbibed the highest volume of Cry2Ab2.820 solubilising buffer, with the lowest volume imbibed being that of sdH₂O. The volumes of solubilising buffers imbibed were not statistically different ($F_{2, 63} = 0.5806$, $p = 0.5625$).

Table 2. Volumes of corresponding Cry protein solubilising buffers and sterile distilled water imbibed by second instar larvae of *H. armigera*, as determined by the droplet feeding method.

Solubilising buffer	Volume imbibed (nl)*	95% Confidence intervals (nl)	
		Lower limits	Upper limits
Cry1A.105	19.34	8.39	30.29
Cry2Ab2.820	26.40	3.134 ^a	58.54
Sterile dH ₂ O	16.73	7.458	25.98

*mean volume imbibed reported; means are not statistically different as determined by the ordinary one-way ANOVA, $F_{(2,63)} = 0.5806$, $p > 0.05$

^a positive value could only be calculated at 85% CI

3.4.3. Droplet feeding bioassays

The results of the probit analyses performed for the droplet feeding bioassays are presented in Table 3. Cry1A.105 is 1.27-fold more toxic than Cry2Ab2.820 against second instars of *H. armigera*. However, the absence of 95% FLs for the estimated Cry1A.105 LD₅₀ indicate that the model did not fit the data well enough to calculate the limits reliably ($g > 0.4$), thus suggesting some uncertainty around the LD₅₀. CV and relative precision could not be calculated due to the absence of the FLs. The low slope indicates a shallow dose-response relationship.

The differences between the observed and tabulated χ^2 reported for Cry1A.105 are, however, not statistically significant, suggesting that the probit model is a good fit for the data. This result is supported by the very low h-value ($h = 0.09$). Based on these two values, the probit model seems to fit the Cry1A.105 data well, however, the absence of FLs suggest low precision when estimating the LD₅₀.

Cry2Ab2.820 is observed to be less toxic than Cry1A.105. The LD₅₀ estimate presents with a very wide range of 95% FLs (Table 3), indicating differing sensitivities of larval *H. armigera* to Cry2Ab2.820 in the droplet feeding bioassays, i.e. no clear dose-response relationship or that the estimated LD₅₀ could vary widely. The relatively high standard error reported with the moderately steep slope (Table 3) supports the observation of high variability in the larval response to Cry2Ab2.820. This is further reiterated by the extremely high CV and high relative precision reported.

The χ^2 suggests that the model fits the Cry2Ab2.820 data moderately. This is supported by the lack of statistical difference between observed and tabulated χ^2 , and the moderate h-value (Table 3).

Overall, the probit model fits the Cry2Ab2.820 relatively well, however, the high variability and wide range of FLs indicate the precision of the LD₅₀ estimate is low.

The LD₅₀ reported for HD-73 SCC is relatively low, and the slope of the data indicates a relatively steep dose-response curve, suggesting good toxicity against second instars of *H. armigera*. The narrow 95% FLs and low h-value ($h < 1$) suggest limited heterogeneity in the larval response to HD-73 SCC. Furthermore, the low χ^2 and absence of statistical differences between the observed and calculated χ^2 suggest a good fit to the data. The high CV and high relative precision suggest low precision around the LD₅₀ estimate (Table 3).

The LD₅₀ reported for HearNPV is 3.90 OBs. The relative precision suggests a poor fit to the model, while the high CV indicates low precision around the LD₅₀ estimate. The differences observed between the calculated and tabulated χ^2 are not statistically significant.

Table 3. Median lethal doses (LD₅₀) of Cry1A.105 (ng), Cry2Ab2.820 (ng), Btk HD-73 SCC (ng), and HearNPV (OBs) against second instar larvae of *H. armigera*, as determined by droplet feeding bioassays.

	LD ₅₀	95% Fiducial limits		Slope ± SE ^a	χ^2 ^b	h ^c	CV (%)	Relative precision ^d
		Lower limits	Upper limits					
Cry1A.105	26.86 ng	-	-	0.33 ± 0.15	0.26	0.09	-	-
Cry2Ab2.820	34.10 ng	8.02	5851.43	0.73 ± 0.23	1.18	0.39	43.71	171.36
HD-73 SCC	0.56 ng	0.33	1.31	0.82 ± 0.19	1.44	0.24	44.64	1.75
HearNPV	0.72 OBs	0.32	1.622	0.53 ± 0.07	3.63	0.52	46.13	1.81

- indicates values could not be calculated by probit analysis; the probit model could not adequately capture the variability in the data ($g > 0.4$ demonstrates a poor fit of the model to the observed data)

^a slope ± standard error

^b observed chi-square

^c heterogeneity factor (chi-square value divided by degrees of freedom)

^d the degree of reproducibility of the bioassay

3.4.4. Sublethal assessments

The concentration-weight and dose-weight responses of surviving larvae from the diet overlay and droplet feeding bioassays could not be used to estimate IC₅₀ and ID₅₀. A poor probit model fit was observed for weight data across all toxins and entomopathogen for both bioassay methods.

Chapter Four – Discussion and conclusions

Defining appropriate methodology and parameters of toxicity bioassays is crucial to the evaluating the toxic efficiency of different insecticides and continuous development of sustainable IPM strategies (Paramasivam & Selvi, 2017; Blouquy *et al.*, 2021). Here, bioassay methods are compared using several metrics reported for Cry1A.105, Cry2Ab2.820, Btk HD-73 SCC and HearNPV used against second instar larvae of *H. armigera*. With the metrics utilised in this study, different data sets can be directly compared to each other (Salama & Salem, 1990; Macoris *et al.*, 2005; Matope *et al.*, 2023)

The results of Table 1 and Table 3 indicate that the diet overlay and droplet feeding bioassays are not suitable for all the toxins and the entomopathogen included in this study. The CVs reported for HD-73 SCC (18.38%) and HearNPV (19.88%) in the diet overlay bioassays (Table 1) were lower than those reported for the same toxin and entomopathogen in the droplet feeding bioassays, at 44.64% and 46.13%, respectively (Table 3). A CV estimate is used to measure the relative variability within data sets (Salama & Salem, 1990; Reed *et al.*, 2022). The lower CVs observed for HD-73 SCC and HearNPV suggest a more consistent response of larval *H. armigera* to the toxin and entomopathogen in the diet overlay bioassays when compared to the droplet feeding bioassays.

Similarly, relative precision analysis demonstrates that HD-73 SCC and HearNPV are better suited for use in the diet overlay bioassays. Relative precision, another crucial parameter of toxicity bioassays, demonstrates the reproducibility of a bioassay method. This parameter is typically used to detect small changes in toxin response (Gabbert *et al.*, 2022). Thus, a high relative precision value shows that the bioassays provide consistent responses, allowing for comparability across bioassay replicates and between different bioassay methods (Fay *et al.*, 2018; Gabbert *et al.*, 2022). A comparison of the h-values reported for the toxin and entomopathogen in both bioassays reinforce the CV and relative precision results (Cremonez *et al.*, 2023).

As previously mentioned, the control mortality of a toxicity bioassay is used to calculate for corrected percentage mortality responses to the toxins and entomopathogen. High control or untreated mortalities (> 15% mortality) were observed in both bioassay methods, suggesting potential variability in *H. armigera* responses to the toxins and entomopathogens used in the study (Phan *et al.*, 2020; Praulins *et al.*, 2022). This makes it challenging to achieve robust and statistically significant results that can be used to assess both the true efficacy and of the

tested toxins and entomopathogens, as well as the reliability of the bioassay methods used (Sparling, 2016; Paramasivam & Selvi, 2017). However, some of the parameters included in the bioassays (e.g. the heterogeneity factor, relative precision, and CV), allow for accurate comparison of the bioassay methods. These parameters are crucial for developing robust and reproducible results (Phan *et al.*, 2020).

Although Btk HD-73 SCC contains the *cry1Ac* gene, it is important to note that this study does not focus on or report the toxicity of the Cry1Ac protein, as HD-73 SCC is a mixed sample. Therefore, direct comparisons of HD-73 SCC to the purified Bt Cry proteins, i.e., Cry1A.105 and Cry2Ab2.820, are not applicable. Spore-crystal formulations of Bt are, however, common in commercial agricultural products (Gould & Anderson, 1991; Chattopadhyay & Banerjee, 2018; Kumar *et al.*, 2021).

The protein production observed in Btk HD-73 SCC is consistent with various studies, some of which used different media. Hasan *et al.*, (2010) demonstrated the growth, sporulation, and endotoxin production of Btk HD-73 on a mustard-seed based medium (Hasan *et al.*, 2010), while the 2015 study of Mourin *et al.* (2015), sought to optimise Cry protein synthesis by Btk HD-73 in a glucose-peptone medium and a soybean extract molasses medium (Mourin *et al.*, 2015). Both studies were able to identify protein production in HD-73 SCC at approximately 133 kDa on SDS-PAGE (Hasan *et al.*, 2010; Mourin *et al.*, 2015).

A LC₅₀ estimate could not be determined for Cry2Ab2.820 in the diet overlay bioassays, due to all concentrations producing a mortality below 50% in the test population. This result is not unexpected as resistance to the Cry2Ab2 protein has been observed in laboratory and field populations of *H. armigera* and *H. zea* (Bilbo *et al.*, 2019; Yu *et al.*, 2021; Calvin *et al.*, 2024). Resistance in these species is believed to be driven by natural mutations of the ATP-binding cassette (ABC) transporter (an insect midgut transmembrane protein) gene *ABCA2* (Wu *et al.*, 2019; Kaur *et al.*, 2019; Fabrick *et al.*, 2022). It is also important to remember that Cry2Ab2.820 is a pyramided protein consisting of Cry1Ac and Cry2Ab2 proteins which have already been utilised in early transgenic crops (Hagenbucher *et al.*, 2016). Thus, resistance already acquired to the individual proteins by *H. armigera* larvae may be affecting the functioning of the pyramided structure in the larval population.

The LC₅₀ reported for HearNPV in the diet overlay bioassays is 3.90 OBs/cm². Diseased larvae displayed symptomology consistent with HearNPV infection, including blackening of

the larvae and slow, stunted movement (Williams *et al.*, 2017). Also, some dead larvae displayed flattening and liquefaction (Rohrmann, 2019; Harish *et al.*, 2021).

Many previous studies utilising HearNPV in their bioassays typically report their LC₅₀ in OBs/ml, thus, making direct comparisons with our study challenging, due the difference in units. However, the toxicity of HearNPV in lepidopteran larvae is well-established.

Previous studies have demonstrated lepidopteran susceptibility to HearNPV, even in larvae with established resistance against Bt Cry proteins or Bt transgenic crops (Calvin *et al.*, 2024).

In this study, second instar larvae of *H. armigera* imbibed the lowest volume of sterile distilled water, at 16.73 nl, compared to the Bt Cry protein solubilising buffers. This result is considerably lower than those reported by Kunimi & Fuxa (1996). Overall, second instar larvae of *Trichoplusia ni*, *Photedes includens*, *Heliothis virescens*, and *Anticarsia gemmatalis* imbibed larger volumes, averaging 59.14 nl, 50.01 nl, 54.14 nl, and 78.37 nl respectively (Kunimi & Fuxa, 1996). The large difference between these volumes and second instars of *H. armigera* in our study can be attributed to the addition of sucrose as a phagostimulant, alongside the 20 to 24-hour larval starvation time (Kunimi & Fuxa, 1996).

This result differs from the findings of Chen and Bouwer (2019), where second instars of *Spodoptera frugiperda* ingested the largest volume of sterile distilled water, at 25.53 nl, compared to the solubilising buffers of Cry proteins (Chen & Bouwer, 2019). This difference in volume can be attributed to the dietary preferences of the insects. While both species are polyphagous, *H. armigera* has a wider host range than *Spodoptera* species. Based on this, it is suggested that *H. armigera* larvae possess the ability to distinguish feeding solutions of limited nutritional value

Second instar larvae ingested the highest volume of the Cry2Ab2.820 solubilising buffer compared to Cry1A.105 solubilising buffer and sterile distilled water (Table 2). However, the difference in the volumes imbibed were determined to not be statistically different (Table 2). Both Cry1A.105 and Cry2Ab2.820 were provided with their corresponding CAPS buffers (Monsanto Company, USA). Although the impact and underlying mechanisms of specific buffer components on the feeding behaviour and physiological changes in larval insects is not well-documented (Revadi *et al.*, 2021), Chen and Bouwer (2019) suggest that the CAPS buffers possess considerable antifeeding effects (Chen & Bouwer, 2019). In short, the

volumes imbibed by the second instar population are crucial to determining the exact doses used in the droplet feeding bioassays.

The LD₅₀ reported for HD-73 SCC at 0.5624 ng is comparable to that obtained for Cry1Ac at 0.505 ng, against second instar larvae of *H. armigera* (Li & Bouwer, 2012). The HD-73 SCC, however, presents with a steeper slope, at 0.82 ± 0.2 compared to 0.54 ± 0.05 of Cry1Ac (Li & Bouwer, 2012). This result suggests that HD-73 SCC displays higher toxicity per unit per unit dose. This result is expected as the mixed HD-73 SCC sample is likely to contain additional protein or peptide fragments that may assist in the delivery of the toxin to functional receptors in the insect midgut (Jurat-Fuentes & Adang, 2006; Chen *et al.*, 2007). The persistence and residual activity of spores and crystals within the SCC sample also have the potential to contribute to the toxin's lethality (Chen *et al.*, 2007). Furthermore, purification processes can potentially remove carrier proteins from purified Cry proteins, like Cry1A.105 and Cry2Ab2.820, resulting in a decreased overall toxicity when compared to the HD-73 SCC (Chen *et al.*, 2007).

Our data, although presenting with several instances of variability suggests that certain bioassay methods may be suited for use with specific toxins or entomopathogens. Ultimately, the results of the toxicity bioassays suggest that HD-73 SCC and HearNPV are best suited for use in diet overlay bioassays of second instars of *H. armigera*. The variability demonstrated in both bioassays relating to Cry1A.105 and Cry2Ab2.820 means we cannot conclusively state the more effective bioassay method for either purified Bt Cry protein.

To successfully achieve a standardised framework of toxicity bioassays used for insecticide development and observation of resistance development against lepidopteran pests, more than two bioassay methods would have to be tested. Determination of comparable parameters such as those seen in this study (χ^2 , heterogeneity factor, CV, and relative precision), in different development stages of the pest population would be crucial. Further experimentation would be required before a standardised framework could be implemented across different pest species.

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