Characterization and Evaluation of Potential Utilization of *Hear*NPV and *Plxy*GV Baculoviruses Isolates for Management of African Bollworm, *Helicoverpa armigera* and Diamondback Moth, *Plutella xylostella* in Kenya

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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DEDICATION

This work is dedicated to my parents Cornelius and Alice, my best friend and husband Moses Njire and my beloved siblings Edwin, Olive and Andrew for their love and support during the entire study period. Dad and Mum you have laid in me a good foundation that has seen me through to this level of education. May the Almighty God bless you.

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LIST OF ABBREVIATIONS

ABW	African Bollworm
ANOVA	Analysis of Variance
CRD	Completely Randomized design
DBM	Diamondback moth
DNA	Deoxyribonucleic Acid
GV	Granulovirus
***Hear*MNPV**	*Helicoverpa armigera* Multiple Nucleopolyhedrovirus
***Hear*NPV**	*Helicoverpa armigera* Nucleopolyhedrovirus
***Hear*SNPV**	*Helicoverpa armigera* Single Nucleopolyhedrovirus
ICIPE	International Centre of Insect Physiology and Ecology
IPM	Integrated Pest Management
KARI	Kenya Agricultural Research Institute
KES	Kenya Shillings
Kg	Kilograms
***Ld*MNPV**	*Lymantria dispar* Multiple Nucleopolyhedrovirus
MNPV	Multiple Nucleopolyhedrovirus
NPV	Nucleopolyhedrovirus
OB	Occlusion body
ODV	Occlusion Derived virions
***Plxy*GV**	*Plutella xylostella* Granulovirus
RCBD	Randomized Complete Block Design
REN	Restriction Endonuclease analysis
SNPV	Single nucleopolyhedrovirus

ABSTRACT

Diamondback moth and African bollworm are serious lepidopteran pests of the Cruciferae family of crops. They are a major challenge in the production of the crops they attack. Resistance by these pests to commonly used pesticides has occasioned the need for development of alternative methods of management including the use of biological control agents. Widespread use of biological control in developing countries has been hampered by lack of cheap and efficient systems of mass producing bio-control agents. This study was, therefore, carried out to develop a system for the production of baculoviruses and to evaluate the virulence of Kenyan isolates of baculoviruses for management of Diamondback moth and African bollworm. The baculovirus isolates that were evaluated, *Plutella xylostella* GV (PlxyGV) and *Helicoverpa armigera* NPV (HearNPV), were produced within the bodies of their natural host reared on artificial diets. Rearing insects on imported diet is expensive and therefore diets were developed from locally available materials and evaluated to determine their ability to support growth of Diamondback moth and African bollworm. Three Kenyan baculovirus isolates, PlxyGV F4, PlxyGV I0 and HearNPV F1 from wild Diamondback moth and African bollworm larvae exhibiting virus symptoms were characterized. Bioassays were done to determine the lethal dose and the lethal time of the three isolates. Further, the efficacy of the PlxyGV I0 isolate was evaluated in field experiments. The average survival rates of African bollworm and diamondback moth reared on the modified multi-species southland and modified pieris diets were 95% and 97% in respectively compared to 90% survival rates of both insects on the multi-species southland diet. This revealed superiority of the modified

diets over the standard. Morphology, biological activity and DNA make-up of the baculovirus isolated from Kenya was similar to isolates reported in literature. The size of occlusion bodies from light microscopy was approximately 0.15 x 0.35 µm for granulovirus isolates and 0.5-5 µm for nucleopolyhedrovirus indicating that they were true baculovirus isolates. The total estimated genome length of the PlxyGV isolate is 93.3 kb while there was similarity in overall pattern and size between the Kenyan HearNPV isolate and the main genotype of the Chinese isolate indicating that they were true baculovirus isolates. In field trials, the Kenyan isolates of PlxyGV caused up to 97% mortality in DBM population compared to 95% caused by a chemical standard (Tracer 480SC). The findings in this study indicate potential for utilization of the Kenyan isolates of baculoviruses in the management of Diamondback moth and African bollworm pests.

CHAPTER ONE

1.0 GENERAL INTRODUCTION

1.1 BACKGROUND

Insects comprise the most diverse group of animals on earth, with approximately 1.5 million species described (Kunimi, 2007)**.** Some species among these organisms cause serious damage to the crops, which can severely limit agricultural production. Insects which interfere with human health, activity or property, or reduce quality or yield of agricultural products, are termed insect pests. Among the major insect pests are Diamondback moth, *Plutella xylostella* (Linnaeus) and African bollworm *Helicoverpa armigera* (Hubner).

Diamondback moth (DBM) and African bollworm (ABW) are lepidopteran pests of many crops in the world. Both DBM and ABW undergo a complete metamorphosis; egg, larval, pupal and adult stages. The larval stage is the injurious stage of both insects (King, 1994). DBM is probably of European origin but is now found throughout the world in places where crucifers are grown. It is the single most destructive pest of crops in the brassica family such as broccoli, Brussels sprouts, cabbage, Chinese cabbage, cauliflower, collards, kale, kohlrabi, radish, turnip, and watercress, the mustard family and several leafy green house plants. It is also a pest of several cruciferous weeds such as wild radish, *Raphanus raphanistrum* L., wild turnip, *Brassica tournefortii* L., turnip weed, *Rapistrum rugosum* L., mustards, *Sisymbrium orientale* L., *S. officinale* L., *S. irio* L., *and S. thellungii* L., charlock, *Sinapis arvensis* L., muskweed, *Myagrum perfoliatum* L. and shepherd's purse,

Capsella bursapastoris L. (Michalik, 1994). In Kenya, up to 100% damage on cabbages by DBM has been observed, even with regular pesticide application.

African bollworm is found all over the world except in the American continents (Fitt, 1989). It is the most polyphagous and injurious pest of agriculture and home gardens in many countries and hence, is a pest of major importance in most areas where it occurs and damages a wide variety of food, fibre, oilseed, fodder and horticultural crops. It is a serious pest of cotton, beans, maize, and sorghum. Alternative hosts include: tobacco, tomato, many pulses, and wheat (Annecke and Moran, 1982). It is a major pest due to its high mobility, wide host range, high fecundity and reproductive rate, and capacity to develop resistance to pesticides. The habit of feeding inside the fruiting parts of the plant during most of its development makes bollworms less vulnerable to insecticides. Insecticides should be applied before the caterpillars bore into the fruits/pods.

ABW has a strong ability to develop resistance to insecticides. Currently, there is a widespread occurrence of resistance to popular synthetic pyrethroids in Africa and elsewhere (McCaffery, 1998; Torres-Vila *et al.*, 2002). Moreover, its preference for the harvestable flowering parts of high-value crops including cotton, tomato, sweet corn, and cut-flowers makes it responsible for huge economic losses and socio-economic costs. Crop loss at farm level in Kenya have been estimated at over 50% on cotton and pigeon pea, over 20% on sorghum and millet, 24% fruit damage on tomatoes and 100% stems loss on cut flowers (Kibata, 1997).

Biological control is one alternative for overcoming the problem of pest resistance. Biological control is preferred because it is considered relatively safe compared to the conventional pesticides. There are several biological control agents being employed in the control of DBM and ABW, such as *Trichogramma* spp. which is an egg parasitoid of ABW, *Bacillus thuringiensis* (Bt), *Diadegma semiclausum* (Hellen) against DBM and baculoviruses against both ABW and DBM (Muhammad *et al*., 2005).

Baculoviruses are DNA viruses that occur only in arthropods and belong to the family Baculoviridae. The Baculoviridae consists of two genera based on occlusion body (OB) morphology (Murphy *et al*., 1995) namely Nucleopolyhedrovirus (NPV) and Granulovirus (GV). Viruses of the genus NPV produce relatively larger (0.13-15µm) proteinaceous polyhedron shaped occlusion bodies (OB) that contain many virions within each OB as compared to granuloviruses. The viruses of the genus GV are characterized by smaller (0.3×0.5µm) ovicylindrical shaped occlusion bodies called granules containing a single virion (van Regenmortel *et al.*, 2002). Baculoviruses possess a large circular double stranded DNA genome that is packaged in a rod-shaped nucleocapsid surrounded by an envelope. The virions are embedded in a proteinaceous occlusion body (Blissard *et al*., 2000). Many Baculoviruses have been isolated from insect species of the order Lepidoptera and show a high degree of host specificity (Blissard *et al*., 2000), but exhibit variable tissue specificity patterns depending on the viral species (Federici, 1993). A number of NPVs and GVs have successfully been propagated *in vitro* (Tanada and Hess, 1991; Williams and Faukner, 1997).

Baculoviruses have been developed as microbial insecticides against a range of Lepidopteran pests of crops, vegetables, forests and pastures (Moscardi, 1999). For insect species such as the cotton bollworm and diamondback moth that have developed strong resistance to chemical and/or *Bacillus thuringiensis Berliner* insecticides, the application of baculoviruses is one of the few options left for effective control. Several baculoviruses have been reported to infect DBM populations in India (Rabindra *et al*., 1997), SouthEast Asia (Kadir *et al*., 1999a) and Far East (Asayama and Osaki, 1970; Yen and Kao, 1972) and other Lepidoptera species such as *Autographa californica* Multiple NPV (AcMNPV). The infection of DBM by granulovirus was first reported by Asayana and Asaki (1969). Since then several workers have reported that GVs show promise as control agents for DBM (Kao and Rose 1976; Nakahara *et al.,* 1986). Although some NPVs have shown pathogenicity to DBM (Kadir *et al*., 1999a; b), the only DBM specific baculoviruses have been granuloviruses.

The majority of baculoviruses used as biological control agents are in the genus *Nucleopolyhedrovirus:* for instance *Helicoverpa armigera* NPV against the boll worm, *Autographa californica* MNPV, *Lymantria dispar* NPV, *Spodoptera frugiperda* MNPV and *Heliothis armigera* NPV. While several baculoviruses are commercially used in many countries for control of lepidopteran pests, data on the relative pathogenicity of some viruses is often lacking, hence there is need for more research in order to be able to use them as effective biological control agents (Santharam and Balasubramanian, 1992). Granuloviruses offer potential for the control of DBM without deleterious effects on the environment (Falcon *et al*., 1968; Huber and

Dickler, 1977; Vail *et al.*, 1991; Jaques *et al.*, 1994; Lacey *et al*., 2000). A number of other NPVs, some uncharacterized (Padamvathamma and Veeresh, 1989), have been reported as infecting DBM, but a review of the potential of DBM pathogens concluded that only the GV showed promising levels of pathogenicity (Wilding, 1986).

The majority of baculoviruses used as biological control agents are in the genus *Nucleopolyhedrovirus.* These viruses are excellent candidates for species-specific, narrow spectrum insecticidal applications (Gröner, 1986). They have been shown to have no negative impacts on plants, mammals, birds, fish, or even non-target insects. This is especially desirable when beneficial insects are being conserved to aid in an overall IPM program, or when an ecologically sensitive area is being treated.

Baculoviruses, like all other viruses, are unable to reproduce without a host, that is, they are obligate parasites. Therefore, their production for use in pest control can either be in cultured cells or in live hosts. Rearing of these hosts is done either on artificial diets or on plants in the field. While production of the insects on live plants is much cheaper as compared to production on artificial diets, the later offer more homogenous conditions and promote consistent growth. Artificial diets are preferred for commercial rearing since they allow control of the production process. Rearing insects on artificial diets is challenging due to high costs, especially for developing countries (Ahmed *et al.*, 1998). Various artificial diets have been developed and proposed for maintenance and continuous rearing of economically important insects (Cohen, 2001), but they are quite expensive for use in mass production of insects. There is, therefore, a need for development of alternative inexpensive diets using

locally available materials, which can support growth of insects. This will reduce the overall cost of production of the baculoviruses.

1.2 STATEMENT OF THE PROBLEM

Control of pests such as Diamondback moth (DBM) and African bollworm (ABW) by conventional chemicals insecticides has so far been the most common practice in Kenya. This unilateral approach and over reliance on chemicals has resulted in development of insecticide resistance, pesticide poisoning of farmers, the presence of the insecticidal residues on marketable products and hazard to beneficial organisms (Sudderudden and Kok, 1978; Ooi, 1979; Lim, 1982). To prevent the situation from reaching a disaster phase, broader ecological-based approaches based on the integration of various pest management techniques should be used.

Largely, because biological control is a natural phenomenon and can potentially provide a harmonious, economical and permanent solution, the use of biocontrol agents such as baculoviruses is now being given priority. The increased use of baculoviruses as biological control agents depends on the discovery and characterization of new isolates active against pest species (Lasey *et al*., 2000). In Kenya there is no baculovirus being used as a biopesticide hence the need to evaluate and characterize Kenyan isolates for the management of specific pests.

Although biological control has great potential for Integrated Pest Management (IPM), their augmentation into existing programmes has been slow. Use of biological control on a large scale can succeed only if mass production is made technologically possible, for instance through automation (Cohen, 2001). Rearing of insects on

artificial diets is essential for the automation process. Despite accessible information on artificial diets and the fact that over a hundred insectaries market predators and parasites (Hunter, 1994), artificial diets are not used for commercial rearing of insects. Cohen and Smith (1998) explained that this discrepancy between research and commercial use of artificial diets result, in part, from failings in the diets in terms of expense and labor in diet production. At ICIPE in Kenya, Diamondback moth and African bollworm are reared on their natural hosts. This technique produces heterogenous insects and is uneconomical for large scale production of the insects. This is mainly because of the large space required and labor. Therefore, development of inexpensive diets in this study, for artificial rearing of ABW and DBM by use of locally available and cheap ingredients in the diet formulations would contribute to the enhancement of biological control.

1.3 JUSTIFICATION

The traditional control of both DBM and ABW has predominantly been based on use of synthetic pesticides. Concerns over the safety, environmental impact, and sustainability of synthetic pesticides have stimulated development and use of softer control methods within the integrated pest management (IPM) strategy. Natural enemies (entomopathogens, predators and parasitoids) and their use as biological control agents play key roles in IPM. ABW and DBM have been reported to develop resistance toward the synthetic pesticides. Notably, DBM has also shown resistance towards *Bacillus thuringiensis* var. *Kurstaki* in countries like USA (Hawaii and Florida), Japan, Philippines, Malaysia and China (Tabashnik *et al*., 1990; Mohan and Gujar, 2000) whereas *Diadegma* sp has been inefficient in controlling the pest.

Until recently, there was no significant resistance reported in the baculovirus strains already in use. However, some codling moth populations with a reduced susceptibility to CpGV were reported from Germany and France (Fritsch *et al*., 2006; Sauphanor *et al*., 2006). Baculoviruses therefore, are excellent candidates for species-specific, narrow spectrum insecticidal applications (Subramanian *et al*., 2008).

Baculoviruses have shown to have no negative impacts on plants, mammals, birds, fish, or even on non-target insects. This is especially desirable when beneficial insects are being conserved to aid in an overall IPM program, or when an ecologically sensitive area is being treated (Dent, 1991). It is widely acknowledged that baculoviruses can be very effective in controlling specific insect pest (Federici, 1997) based on selection for highly virulent strains (Munoz *et al*., 1998). Some viruses can be produced *in vitro* (within cell cultures in the laboratory, not requiring whole, living insects). These are more expensive than those that can only be produced *in vivo*, that is, inside living insects. The costs of rearing live hosts can be greatly reduced by use of local products in making host diets. It is hoped that insect cell culture systems currently being developed for other uses may ultimately make viral pesticides more cost-effective (Ahmed *et al.*, 1998)**.**

According to Butt and Goettel, (2001), recent rapid advances in biopesticides technology have concentrated on developed country markets and high-tech approaches. This technology can be effectively adopted to meet African needs and conditions through application of some innovative solutions. These include: characterisation of effective local pathogens for African pest, development of novel biopesticide formulations based on locally available agricultural by-products,

development of novel and appropriate technology for small scale production and commercial manufacturing of biopesticides and development of appropriate pathogen application technology.

In Kenya there is no baculovirus that has been produced or registered for use against any crop pest (Ogutu *et al.*, 2002). However, preliminary studies have been carried out on Kenyan isolates of DBM granuloviruses by Grzywacz *et al* (1998). This showed considerable potential as a biopesticide for controlling DBM. Further work to evaluate its potential for commercial use was deemed necessary.

1.4 Objectives of the study

1.4.1 Null Hypothesis

Kenyan baculovirus isolates, *Hear*NPV and *Plxy*GV are not effective as biopesticides against lepidoptera pests, African bollworm and Diamondback Moth.

1.4.2 General objective

To assess the feasibility of producing Kenyan isolates of *Hear*NPV and *Plxy*GV and evaluate their potential as biopesticides against ABW and DBM in Kenya.

1.4.3 Specific objectives

- To develop semi- synthetic diets for rearing *Helicoverpa armigera,* African bollworm (ABW) and *Plutela xylostella,* Diamondback Moth (DBM*).*
- To determine the virulence of Kenya isolates of *Hear*NPV and *Plxy*GV on African bollworm and Diamondback moth respectively

- To test the efficacy of baculovirus products, *Plxy*GV and *Hear*NPV against DBM and ABW respectively.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Pest status of Diamond Back Moth and African Boll Worm

DBM is a major pest of cruciferous crops worldwide (CAB International, 2000). In Kenya it is a major pest of brassica vegetables (Ouko, 1997). Its pest status has risen rapidly since the 1960s when large scale application of chemical insecticides was started in vegetables (Talekar and Shelton, 1993). With its ability to develop high level of resistance to chemical and biological insecticides, *P. xylostella* has become very difficult to manage (Roush, 1997). Georghiou (1981) reported DBM resistance to 36 insecticides in 14 countries.

In Kenya the most commonly adopted management strategy has been the use of synthetic pesticides (Gathui *et al*., 1994). Since the 1970s, there has been a strategy shift in the type of chemicals used, from organochlorides, carbamates and organophosphates to synthetic pyrethroids (Anonymous, 1999). The driving forces behind this changing pattern include the development of new, more effective insecticides and loss of effectiveness of older chemicals due to pest resistance. The increasing demand for quality agricultural products has encouraged extensive use of pesticides by farmers. Frequent application of larger doses reduces the profitability of the produce especially for the smallholders (Ouko, 1997) and is damaging to the environment.

ABW (*Helicoverpa armigera*) is one of the most serious pests around the world (Fitt, 1989). It is highly polyphagous, attacking a great variety of agricultural crops such as

cotton, pepper, tomato, tobacco, maize, sorghum, sun-flower, pigeon pea, chickpea, groundnut, soybean, and okra (King, 1994). *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus (*Ha*SNPV) is a highly infectious and selective pathogen of the cotton bollworm and has been developed as a commercial bio-insecticide with an annual production of 200,000–300,000 kg to treat approximately 100,000 ha of cotton each year in China (Zhang, 1994).

Bacillus thuringiensis var. Kurstaki (Berliner) has been used for over 20 years against *H. armigera*. In Australia, effectiveness of various products has been inconsistent thereby resulting in lack of confidence in their use as control options (Forrester *et al.*, 1993). Development of resistance to baculoviruses had not been reported anywhere in the world (Jones, 1994) until recently when some codling moth populations showed reduced susceptibility to Codling moth, *Cydia pomonella* granulovirus (CpGV) in Germany and France (Fritsch *et al.*, 2006). These populations were detected in organic apple plantations where codling moth control failed despite intensive CpGV application. Bioassays in the laboratory further demonstrated that the median lethal concentrations (LC_{50}) of these populations increased up to 1000-fold, suggesting a significant resistance to *Cp*GV. Although it is not clear whether these populations represent previously existing local variations in susceptibility or are signs of an emerging resistance as a consequence of repeated *Cp*GV application, it is of eminent importance for the development of resistance management measures to understand the molecular and genetic mechanisms behind this resistance. This is also the first example, where field resistance against commercially applied baculoviruses has been

documented. So far, resistance of target insects against baculoviruses has been identified only after strong selection constraints in the laboratory.

An experiment carried out to investigate this quality or viral insecticides revealed that colonies of *H. armigera* infected with NPV and GV continuously for 23 generations remained very susceptible to the viruses. The mortality of the first and the last generation did not vary significantly. Whitlock (1977), therefore, concluded that the development of resistance to NPVs and GVs in susceptible hosts takes longer than to the synthetic insecticides. Resistance to viral pesticides is a more complex phenomenon than to insecticides because in addition to the usual mechanisms an immune response may be involved.

2.2 Utilization of the baculovirus pesticides in the world

Baculoviruses have been developed as microbial insecticides against a range of lepidopteran pests of field crops, vegetables, forests and pastures (Moscardi, 1999). In 1975, a baculovirus was registered in the United States for use against the cotton bollworm, *Helicoverpa zea*. In 1982 baculoviruses were used for the management of Nun moth, *Lymantria monacha* in pine forests in Germany (Huber, 1986). One of the most successful examples of use of a wild type baculovirus is application of Velvet bean caterpillar *Anticarsia gemmatalis* multiple NPV for the management of the velvet bean caterpillar in soybean crops in Brazil where it is applied to more than 1 million hectares annually (Moscardi, 1999). Another successful example of a baculovirus insecticide is *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus (*Ha*SNPV) that has been developed as a commercial bio-pesticide for African bollworm, *H. armigera*, in China (Sun *et al*., 2002). Several *C.*

pomonella granulovirus, *Cp*GV based products are commercialized and widely used in biological control as well as in integrated apple production. It is estimated that these products are applied on more than 100,000 ha in Europe, with a steadily increasing market due to their superior efficacy, environmental safety, and the control failure of many chemical insecticides (Jehle and Eberle, 2006).

The largest producers of baculoviruses are found in China and Brazil. In China, 100 tons of bio-insecticides based on the *H. armigera* NPV are produced annually for use on cotton by several commercial companies. A number of baculoviruses are produced on a smaller scale, for instant *Spodoptera litura* NPV, *autographa californica* MNPV, *Plutella xylostella* GV and *Gynaephora ruoergensis* NPV. In Brazil, the baculovirus product is in use in approximately two million hectares of soybean and is also sold to neighboring countries. In Europe, as well as in the United states, the coldling moth, a major pest in orchards is treated with *Cydia pomonella* GV which is produced in several European countries, Russia and Canada (van Beek and Davis, 2007).

In Africa, there are several examples of promising baculovirus programs. Tanzania has waved registration requirements for *S. exempta* NPV, and efforts to produce the virus are ongoing in collaboration with a Brazillian producer. In South Africa, a GV against False Codling moth *Cryptophlebia leucotetra* on citrus has been commercialized. CIP (International Potato Center) in Peru produces a GV against *Phthorimaea operculella* on stored potatoes in Tunisia and Egypt. Thailand and Vietnam have active programs to promote the use of baculoviruses, and the governmental agencies have produced limited quantities of insecticides especially *S.*

exigua NPV against beet armyworm on grapes, soybeans, onions and cabbage (van Beek and Davis, 2007).

2.3 Baculoviruses as pest control agents

Nucleopolyhedroviruses are the most studied and used commercially in pest control. They infect over 400 species of insects and are well known to cause major lethal epizootics in important lepidopteran pest species. They are relatively fast acting, killing infected insects within 4-7 days. NPVs are rod shaped double stranded DNA viruses of the family Baculoviridae which infect a wide range of insect species, chiefly Lepidoptera but also some members of the Hymenoptera and Diptera. These viruses are the cause of highly infectious lethal disease in the larvae of susceptible species. The NPVs are highly specific hence infect only one insect species. However, some strains infect a few species of closely related insects. The viruses are named after the insect from which they were first isolated and identified (Kurstak and Moramorosch, 1978).

Granuloviruses differ from the Nucleopolyhedroviruses in terms of the number of virions. GVs have one virion (rarely two) in each protective inclusion crystal. The crystals are much smaller than the polyhedra and rod shaped with round heads. The single virion within each granule is surrounded by a crystalline protein matrix similar to that of the polyhedra of NPV. In GV, this protein is called granulin. About 150 species, mainly lepidoptera are known to be susceptible to GV. Generally, infection by GVs is much slower than that of NPV (Federici, 1997).

These viruses produce a large number of occlusion bodies in infected cells (polyhedra and granules) which allow the virus to survive in the environment and to transmit the disease from one insect to another (van Regenmortel *et al.*, 2002). During the replication cycle, baculoviruses produce two phenotypes; the extracellular or budded virus (ECV or BV), responsible for the spread of infection within the insect larvae and the occlusion derived virus (ODV), responsible for the infection from one insect to another. The BV is produced early in the infection and the ODV is produced late in the infection, inside the nucleus of infected cells (Harrap, 1972). The ODVs are found occluded inside a protein matrix made of a protein called polyhedrin forming occlusion bodies called polyhedra (Maruniak, 1986) which are responsible for protecting the virions in the environment before a new susceptible host is infected (Steinhaus, 1960).

2.4 Life cycle of baculoviruses

Infection occurs following ingestion of virus-contaminated foliage by a susceptible host insect. On ingestion of occlusion bodies, for instance polyhedra, the polyhedrin matrix dissolves in the alkaline mid-gut and the ODV are released. The ODVs pass through the peritrophic membranes and enter the columnar cells of the midgut epithelium. Virus replication takes place in the nucleus of the infected cells. Initial rounds of replication results in production of a second viral phenotype budded virus (BV). BVs circulate in the haemolymph and initiate sites of secondary infection throughout the host body. The midgut haemocytes, tracheal matrix and fat body cells are the most readily infected in susceptible hosts (Federici, 1997). A switch occurs during the late phase of infection from BV production to ODV and polyhedra

production. Polyhedra accumulation within the nuclei of epidermal and fat body tissues result in pale coloration at very late stages of infection and polyhedra are released into the haemolymph at advanced stages of disease development. Larvae appear swollen and become lethargic. They hung head down with flaccid body and liquefied tissues which may facilitate polyhedral dispersal (Mukhopadhyay and De, 2009). The cuticle of the cadavers rupture easily, thereby releasing polyhedra into the environment. Time taken for the virus to kill the host is dependent on the virus, host combination and environmental conditions. Time between ingestion and death of the host allows for extensive virus replication such that a single cadaver can yield as many as 10^{10} polyhedra (Entwistle and Evans, 1985).

2.5 Disadvantages of baculovirus as pest control options

Baculoviruses as crop protection agents in comparison with chemical pesticides are disadvantageous in that they are costly to produce, they have relatively low speed of action, narrow host range, and high inactivation rate (Moscardi, 1999). These drawbacks are overcome by improvements in the virus itself through selection or by genetic modification, virus formulation, the dosage and timing of applications or cheaper production systems.

Since baculovirus-induced cessation of feeding, especially in the case of vegetable pests is often too slow to prevent economic damage, attempts have been made to speed up the response. It is recognized that the baculovirus expression vector system could be used to derive insecticidal genes into the insect (van Beek and Davis, 2007).

2.6 Safety of baculoviruses as bio-pesticides

Attempts at controlling insect populations with baculoviruses; granuloviruses (GVs) and nucleopolyhedroviruses (NPVs) date to at least the early 1890s (Huber, 1986). Members of the virus family Baculoviridae are DNA containing viruses that are entirely restricted to arthropods. Most baculoviruses infect only small groups of closely related insects, although members of the nucleopolyhedrovirus genus, *Autographa californica* Multiple nucleopolyhedrovirus (*Ac*MNPV) has a host range encompassing over a dozen families of lepidoptera, including a number of important pest species (Granados and Williams, 1986). More recently, environmental safety due to their host range has been a major rationale for their use. Achievements in pest management have been associated with relatively long-term approaches to control (Fuxa, 1990; Moscardi, 1999).

Baculoviruses have been developed as microbial insecticides against a range of lepidopteran pests of field crops, vegetables, forests and pastures. These include false codling moth, African bollworm, Diamondback Moth, Tea tortrix and the Velvet bean caterpillar. They are safe and selective bio-insecticides that are restricted to invertebrates (Moscardi, 1999). They are generally very host-specific and not harmful to non-target insects or other taxa. They account for over 60% of all the viruses known to infect insects; they are not pathogenic to vertebrates and plants.

Direct effects of baculoviruses on other organisms in the environment are restricted to their hosts and most have been observed only in their original hosts or in the target species of bio-control applications. Baculoviruses infection interferes with other pathogens or parasitoids feeding on a shared host. Some combinations between

baculoviruses and other pathogens have been used in field experiments to improve the control of pests. The analysis of the interference with parasitoid developments is used to assess the possibility of adverse effects on populations providing additional natural control of pest species (Vail, 1981). A reduced level of a parasitoid population has been observed after aerial application of *Ld*MNPV (Mettenmeyer, 2002).

Baculoviruses can be combined with other pesticides in application. Virus particles are generally unaffected by pesticides, although some chlorine compounds should be expected to damage or destroy viruses if applied at the same time. Baculoviruses efficacy, however, can be altered in many ways by the effects of chemical pesticides on the host insect. A review by Jacques and Morris (1981) showed that of 10 pesticide-virus combinations, nine resulted in an additive effect on insect mortality.

2.7 Artificial rearing of Diamond Back Moth and African Bollworm

Diamondback moth and African bollworm were first reared on their host plants. This method has been abandoned because it required large quantities of host plants and a large space. Increased demand for large numbers of laboratory reared insects necessitated the development of more efficient and economical methods of production (Ahmed *et al.,* 1998). Rearing of Diamondback moth on artificial diet was thus initiated in 1998.

Microbial contamination is one of the major problems affecting the rearing of insects (Sikorowski and Lawerence, 1994). Wheat germ medium had been used to rear several species of plant feeding lepidopteran pests including African bollworm (Novon *et al.*, 1990) by modifying it. Although there is some success in efforts to rear

successive generations of economically important insects entirely on an artificial diet, in many cases there is loss of both fitness and reproductive potential which cause longer development times and lower fecundity (Coudron *et al*., 2002). As a result, the cost-saving ratio is diminished.

CHAPTER THREE

3.0 DIET DEVELOPMENT FOR REARING OF DIAMONDBACK MOTH (*PLUTELLA XYLOSTELLA*) AND AFRICAN BOLLWORM (*HELICOVERPA ARMIGERA*)

3.1 Introduction

Diamondback moth (DBM), *Plutella xylostella* and African bollworm (ABW), *Helicoverpa armigera* are serious pests in the order Lepidoptera. DBM is a destructive pest of cole crops and requires globally over US$1.0 billion in estimated annual management costs (Talekar and Shelton, 1993). ABW is a polyphagous pest. It is a major pest threat because the larva can feed on a wide range of economically important crops including cotton, corn, tomato, legumes and tobacco (King, 1994, Shanower and Romeis, 1999). Management of these pests has been primarily by use of synthetic pesticides. Over-reliance on synthetic pesticides has negative impact on the ecosystem and insects have also developed resistance to them. Efforts to reduce the use of conventional pesticides have increased pressure to seek alternative management methods including the use of bio-pesticides (Dale *et al.*, 2001).

Economical production of physiologically and behaviorally competent DBM and ABW is critical to most research on the development of bio-pesticides against these insects (Carpenter and Bloem, 2002). Colonies of insects for research and production of bio-pesticides have been maintained on their natural host plant, which is very costly and labor intensive (Carpenter and Bloem, 2002). To develop an artificial diet for rearing insects, various aspects such as the cost and labor in diet preparation have to be put in consideration (Cohen and Smith, 1998).

Various artificial diets have been developed and proposed for the maintenance, and continuous rearing of economically important insects such as ABW and DBM (Ahmed *et al*., 1998; Cohen, 2001; Castane and Zapata 2005). A few laboratory-adapted colonies exist in the United States and Europe although there has been a fair amount of difficulty establishing colonies of DBM on artificial diets. As a consequence, researchers have often opted for rearing DBM on live plants even though this practice requires more labor, additional space, and greatly increases the chance for microbial contamination. Examples of these include colonies of DBM maintained on mustard seedlings in the United States (Leibee, 1996), on cabbage leaves in Mauritius (Carpenter and Bloem, 2002), on sawi leaves in Malaysia (Omar and Mansor, 1993), on rapeseed seedlings and radish seedlings in Japan (Shirai,2000) and on *Tropaeolum majus,* a wild host of DBM, in Vietnam (Koshihara and Yamada, 1976). A complementary or alternative approach would be to maintain colonies on artificial diets.

Although there is some success in efforts to rear successive generations of economically important insects entirely on artificial diets, in many cases there is loss of both fitness and reproductive potential which cause longer development times and lower fecundity (Coudron *et al*., 2002). Modification of already existing artificial diets is therefore necessary to produce healthy insects at low costs (Domek *et al.*, 1997). This study sought to develop diets from locally available materials and test their ability to support the growth of African bollworm and Diamondback moth.

3.2 Materials and Methods

3.2.1 Diet development for African Bollworm (ABW)

Healthy African bollworm larvae collected from tomato plantation in Mwea area in Kenya were reared on a commercial semi-synthetic diet (Multi-species Southland diet) in the insectary. Each ABW larva was reared in a plastic sample cup of diameter 3.8 cm and volume 25 cm^3 filled half-way with the multi-species southland diet modified by adding florescent brightener. The temperatures were maintained at room temperature (25±2º C) and 55-65% Relative Humidity (R.H).

African bollworm larvae were observed throughout the larval period and all those that died before pupation were removed from the population. All the larvae that pupated were used to establish a clean laboratory colony. The pupae were sterilized using 2% hypochloride for two minutes and dried on soft tissue according to Kenya Biologics Limited (KBL) standard operating procedure. They were put in 2 litre plastic ice cream containers (50 pupae in an area of 200 cm^2) until moths emerged. Each ABW male moth was put into a container together with a female moth and covered with a black muslin cloth that acted as an ovipository site and a vented lid for aeration. The adults were fed on 10% honey solution and 10% sugar solution soaked in cotton wool in plastic sample cups and placed inside the containers. The ovipository materials (muslin clothes) were changed daily for five days to remove the eggs laid.

Eggs were sterilized by soaking the muslin clothe in 0.2 % hypochloride for two minutes and then rinsed with tap water three times. The clothes were drip-dried before being placed in 2 litre capacity plastic ice-cream containers until they hatched into neonates. Neonates were placed each in a sample cup containing Southland multi-

species diet to prevent entomopathogen contamination among larvae and cannibalism at later stages. Dead larvae were removed from the colony each day to maintain a healthy population. Pupae were removed from the diet daily and transferred to a Petri-dish lined with paper towel before sterilization.

3.2.1.1 Diet development

Table 3.1 Ingredients for the African bollworm artificial diets (Modified southland diet, MSL, Wheat germ diet, WGD, Wheat bran diet, WBD, Soybean diet, SBD, Wheat germ/Soybean diet, WG/S and Chick pea diet, CPD

Ingredients (g/1/2l)	**MSL**	**WGD**	**WBD**	**SBD**	**WG/S**	**CPD**
Wheat germ	17	35.5			17	
Soy flour	18.5			35.5	19.6	
Sucrose	17	17	17	17	17	
Active yeast						14.5
Wheat bran			35.5			
Chickpeas						100
Cellulose	2.5					
Wesson salt	5	5	5	5	5	
Vitamin mix	6	6	6	6	6	3
Ascorbic acid	2	2.3	2.3	2.3	2.3	1.4
Methyl paraben	2.5	2.5	2.5	2.5	2.5	1.5
Sorbic acid	0.5	0.5	0.5	0.5	0.5	0.9
Agar	8.5	8.5	8.5	8.5	8.5	5.2
Water (ml)	375	375	375	375	375	375

Six diets were made using locally obtained chick peas, wheat bran, wheat germ, soya bean flour, (Table 3.1). All dry ingredients were weighed out carefully and kept in separate containers. They were then mixed together thoroughly to get a homogenous mixture. 10% of the total volume of water was added and mixed thoroughly. Agar was boiled in the remaining 90% volume of water and cooled to 70º C. It was added and mixed thoroughly to make a smooth paste. The hot diet was dispensed using a squeeze bottle into clean sample cups placed on flat surface and left for 30 minutes to cool and solidify.

3.2.1.2 Rearing procedure

Modified glass capsule vial technique for individual larval development was applied. Instead of glass vials, plastic sample cups were used and covered with vented lids for ventilation. ABW neonates were placed in each sample cup and covered with a lid. The larvae were left to feed on the diet up to pupation. The pupae were sterilized and 20-30 pupae were put in each 2 litre capacity plastic containers until adults (moths) emerged. Adults were checked and removed daily from the emerging container and put into mating and ovipository cages.

3.2.1.3 Mating and Oviposition

Two litre capacity plastic tabs covered with muslin clothes and vented lids were fabricated into ovipository cages. The muslin clothes were used to allow aeration and also to act as ovipository surfaces. The adults were sexed and put into the plastic tabs, a pair in each tab. They were fed on 10% sucrose and 10% honey solutions. The muslin clothes were changed daily and the number of eggs laid per day counted. To

determine fecundity the total number of eggs per ovipository period were divided by the number of female adults in the cage.

Plate 3.1 African bollworm (ABW) rearing procedure. Plate 3.1 (a), diet in sample cups, (b) ABW larvae on the diet, (c) ABW pupae on a Petri dish, (d) ABW moths in laying cages.

3.2.1.4 Data collection and analysis

Data on larval period, percent larvae survival, percent pupation, pupal weight, number of eggs per female moth were recorded. The two best diets based on the above mentioned parameters were compared and the best diet for rearing *H armigera* larvae selected.

3.2.2 Diet development for Diamondback month, *Plutella xylostella* (DBM)

Healthy DBM Larvae collected from kale plantations in Nanyuki, Kenya were put in styrofoam boxes (15cm×25cm×5cm) containing a thin layer of a commercial semi-synthetic diet (Multi-species South-land diet) modified by adding linseed oil, kale powder and florescent brightener. The DBM Larvae were placed under room temperature (25±2º C) until the larvae pupated. After pupation the pupae were placed in a 30 cm^3 wire mesh cage for adults to emerge. Adults were fed on 10% honey and 10% sugar solutions. Six strips of creased wax papers, soaked in cabbage juice, made by blending fresh cabbage leave, were hung in the cages from the 3rd day after pupation to act as ovipository surfaces. The wax papers were changed daily to ensure homogeneity during hatching. Eggs were sterilized in 0.2% Sodium hypochlorite for two minutes and then dried. Dry strips of wax papers with eggs were attached to the lids of 2 litre capacity plastic ice cream containers containing a thin layer of DBM diet. The eggs hatched and the larvae fed on the diet until pupation.

3.2.2.1 Diet development

Table 3.2 Ingredients for the African bollworm artificial diets (Pieris diet, PD, Modified Pieris diet, MPD, Wheat germ Diet,WGD, Wheat bran diet, WBD, Soybean diet, SBD, and Wheat germ/Soybean diet, WG/S

Ingredient (g/ 1/2l)	**PD**	**MPD**	**WGD**	**WBD**	**SBD**	**WG/S**
Casein	18.9					
Milk powder		18.9	15	15	15	15
Wheat germ	16.2	16.2	36.5			17
Wheat bran				36.5		
Soybean					36.5	19.5
kale powder	7.5	8.1	7.5	7.5	7.5	7.5
Sucrose	18.9	18.9	17	17	17	17
Cellulose	2.6	2.6	2.5	2.5	2.5	2.5
Wesson Salt	5.4	5.4	5	5	5	5
Vitamin mix	6	1.8	6	6	6	6
Ascorbic Acid	2.3	4.3	2	2	2	2
Choline chloride	0.5	0.5	0.5	0.5	0.5	0.5
Paraben	2.5	0.8	2.5	2.5	2.5	2.5
Agar	13.5	13.5	8.5	8.5	8.5	8.5
Sorbic acid	0.5	0.5	1	1	1	1
Formaldehyde		0.5	0.5	0.5	0.5	0.5
10%	0.57					
Linseed oil (ml)	2.5	1.5	1.5	1.5	1.5	1.5
Water	445	445	445	445	445	445
4M KOH	2.7	2.7	2.7	2.7	2.7	2.7

Six diets were made using locally obtained wheat bran, wheat germ, and soya bean flour (Table3.2). All DBM diets were modified by adding kale powder and linseed oil. Linseed oil is important in the formation of wings in adults whereas kale powder makes the diet more likeable by DBM. All dry ingredients were weighed out carefully and kept in separate containers. They were then mixed together thoroughly to get a homogenous mixture. One tenth of the total volume of water was added to the mixture and stirred to make a fine mixture. Wet ingredients were also measured and added to the mixture. Agar, boiled in the remaining 90% water and cooled to 70º C was added to the mixture and mixed thoroughly to make a smooth paste. The hot diet was dispensed using a squeeze bottle into clean sample cups placed on flat surface and left for 30 minutes to cool and solidify.

3.2.2.2 Rearing procedure

Modified glass capsule vial technique for individual larval development was applied. Instead of glass vials, plastic sample cups were used and covered with vented lids for aeration. Twenty DBM neonates were placed in each sample cup and covered with a lid. The larvae were left to feed on the diet through the entire larval period. After pupation the pupae were counted, weighed and put in $30cm^3$ wire mesh cages until adults emerged.

3.2.2.3 Mating and oviposition

After adult emerged, strips of wax paper were hung inside the cages to act as ovipository sites. To mimic the natural ovipository sites (plant leaves) the wax papers were first creased, soaked in cabbage juice and dried. The papers were changed daily

and the total number of eggs laid per day counted. To determine the number of eggs per female DBM, the total numbers of eggs were divided by the number of female adults (Assumed to be half the population. Adult DBM were fed on 10% sucrose and honey solutions.

3.2.2.4 Data collection and analysis

All the diets that had low insect survival rates were eliminated. Two diets; modified Pieris diet and Southland multi-species diet were compared. Data on larval period, percent larvae survival, percent pupation and pupal weight were recorded statistically compared using ANOVA and the best diet for rearing *P. xylostella* larvae selected.

3.3 Results

3.3.1 Performance of *H. armigera* larvae on African bollworm Diets

The results on pupal weight and survival rate, larval mortality and development period obtained from the various diets developed for rearing *H. armigera* were used to eliminate diets that showed poor ability to support insect growth. Table 1 shows low percentages of pupae that survived from colonies reared on chickpeas, wheat bran, wheat germ, soybean, soybean/wheat germ diet as compared to modified southland diet.

A comparison between the modified Southland diet and the imported Southland diet showed that there were no significant differences ($F=58.0$; $df=1, 59$; $P>0.05$) between the various growth parameters but the modified Southland diet was the best for rearing *H. armigera* (Table 3.3).

Table 3.3 Larval period, larvae survival rate, pupal weight, % pupation, pupae survival rate and fecundity of *H. armigera* reared on various diets

Diet type	Larval period (Days)	Larvae survival (%) ±se	Pupal weight (g) ±se	Pupation (%) ±se	Pupal survival (%) ±se	Fecundity (eggs per female adult) ±se
Chickpea diet	24±2	50±6	0.30±0.02	47±3	45±6	_
Wheat bran	22±2	64±3	0.33±0.01	64±2	62±2	_
Wheat germ	22±3	67±4	0.32±0.03	65±3	62±3	_
Soybean	23±2	41±5	0.30±0.02	40±3	35±4	_
Soybean/wheat	22±1	77±3	0.35±0.03	77±5	75±3	300±9
Modified S/land diet	17±2	95±2	0.40±0.05	95±3	91±3	350±12
Southland diet	17±3	90±2	0.37±0.04	88±4	85±4	313±3

3.3.2 Performance of *P. xylostella* larvae on Diamondback moth diets

Results of *P. xylostella* reared on various diets are shown in Table. 3.4. The mean pupal weights of DBM insects reared on modified *Pieris* diet was significantly different from those reared on southland multi-species diet ($F=37.5$; df =2, 4, $P<0.01$). Percentage larvae survival rate was also significantly different ($F=51.57$; df =2, 4; $P<0.01$) with insect reared on modified Pieris diet being better than those reared on southland multi-species diet. There was no significant difference between their pupation ($F= 7.2$; df =1, 4; $P=0.055$) though the pupation level was higher for insects reared on modified pieris diet.

Table 3.4 Larval period, larvae survival rate, pupal weight, % pupation, pupae survival rate and fecundity of *P. xylostella* reared on various diet formulation.

Diet type	Larval period (Days) ±se	Larvae survival (%) ±se	Pupal weight (g) ±se	Pupation (%) ±se	Pupal survival (%) ±se
Wheat bran	14±2	10±1	0.0054±	10±2	10±2
Wheat germ	14±2	19±4	0.0054±	15±2	15±1
Soybean	14±2	7±2	0.0052±	5±3	5±2
Soybean/wheat	12±2	50±4	0.0055±	48±5	47±3
Modified Pieris diet	11±2	97±2	0.0060±	95±4	90±2
Southland diet	12±1	90±	0.0056±	88±2	85±2

Modified Pieris and modified multi-species southland diets were the most suitable for DBM and ABW rearing respectively. In addition to the basic ingredients in pieris diet, linseed oil and kale powder were incorporated. Linseed oil provided cholesterol which is essential for successful insect molting and wing formation (Chapman, 1991) while kale powder was used to mimic the flavor of DBM natural hosts; Cole crops. Sorbic acid in both diets controls microbial contamination (Carpenter and Bloem, 2002). DBM larval development period of DBM reared on modified pieris diet was not significantly different from those reared on multi-species southland diet ($P>0.05$). ABW larval development time of ABW larvae reared on the modified multi-species southland diet was not significantly different from those reared on multi-species southland diet ($P>0.05$). Therefore, DBM and ABW can be successfully reared on the

modified diets. Clean environment and sterile laboratory equipment, eggs and diets are however necessary to avoid microbial growth.

3.4 Discussions

An appropriate artificial diet is one that satisfies the chemical, physical and nutritional requirements of the insects (Friend, 1958). *H .armigera* rearing on Southland imported synthetic diet took approximately 17 days for the insect to grow through the entire larval period. Typically there are 5-7 instars and a larva period of 12-32 days before pupation occurs (Rajagopal and Channa Basavanna 1982, Reed, 1965). This is very highly dependent on the prevailing climatic conditions such as temperature and relative humidity. In the laboratory, the conditions are controlled and hence the insect grew more consistently than in the wild where there is fluctuation in the conditions. After pupation the pupae period lasts for a period of 10 days before emergence of moths (adults). The adults started laying eggs three days after emerging and the laying period lasted for 10 days. Each pair of moths laid approximately 3000 in the entire laying period (Shanower and Romeis 1999).

The life cycles of ABW reared on developed artificial diet were normal when compared to the wild insects, as reported by Shanower and Romeis, 1999. Their fertility ranged from 95-98%. Insect reared on Southland had a survival rate of 90%. On commercial diet survival rate is aimed at being as high as possible to reduce losses in the cost of its production vis-à-vis the number of insects produced on it.

Whereas in this study fecundity of an average of 350 eggs per female was recorded, Burton (1970) reported an average oviposition of 406 eggsfor mated females reared

on a corn-soybean meal-based diet and Burton and Perkins (1972) found 1901 eggs from a female *H. zea* reared on a wheat-soy blend diet. Egg production is affected by temperature and was adversely affected by higher temperature (25±2º C) in the insectary, which is probably due to inhibition of mating and oviposition (Ahmed *et al*. 1998; Kersting *et al.,* 1999; Urbaneja *et al.,* 2002)

P. xylostella larvae reared on the modified Pieris diet and Southland diet had a larval period of 9-13 days. This was in close relation to what had been recorded by Dunhawoor and Abeeluck (1997) on DBM reared five types of diets which ranged between 8- 13 days. DBM larval development period on modified Pieris diet and Southland diet was almost similar to that on cabbage (10-11 days). Dunhawoor and Abeeluck (1997) recorded DBM average pupal weights ranging from 0.0030-0.0056g which were lower but similar to the DBM pupal weight from this study, 0.0052-0.0060g.

CHAPTER FOUR

4.0 CHARACTERIZATION OF KENYA ISOLATES OF *PLXY*GV AND *HEAR*NPV ISOLATES

4.1 Introduction

Baculoviruses are insect viruses used as biological control agents (Moscardi, 1999). The *Baculoviridae* family is divided into two genera: *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GV) (Theilmann *et al.*, 2005). The two genera in the family Baculoviridae, are distinguished by their occlusion body (OB) morphology (Blissard *et al.*, 2000). In NPVs, numerous virions are occluded in an OB referred to as a polyhedra or polyhedral inclusion body (PIB). The virions consist of either one nucleocapsid embedded in a membranous envelop, singly embedded NPV (SNPV) or many nucleocapsids embedded in a membranous envelop, multiple embedded NPV (MNPV). In contrast, for GVs only a single virion is typically occluded in a smaller (0.25–1 μm) OB referred to as a granule. In both PIBs and granules, virion(s) are embedded in a proteinaceous matrix consisting primarily of polyhedrin in NPVs or granulin in GVs. There are several other proteins found in OBs either as structural proteins, calyx or polyhedron envelope protein, or associated with OBs for example, p10, alkaline protease and viral enhancing factor (VEF) (reviewed by Funk *et al.*, 1997).

Characterization of Kenyan baculovirus isolates in this study was done so as to compare them with other isolates being used in other countries and to evaluate them as biopesticides against Diamondback moth and African bollworm.

4.2 Materials and Methods

4.2.1 Observation and identification of occlusion bodies

4.2.1.1 Granuloviruses (GVs)

A total of 360 DBM larvae were collected from Ontulili farm Nanyuki Kenya, 0° 1' 0" N / 37° 4' 0" E, 319 live and 41 dead. Sixty live larvae were place in each Styrofoam box, which were partially filled with artificial diet (Multiple species, Southland diet,). The 41 dead DBM cadavers, were placed in individual microfuge tubes and collectively marked as Batch 1, then preserved at -20°C. In addition to the 41 cadavers collected from the field, 7 others died during incubation, bringing the total to 48, displaying symptoms of baculovirus.

The cadavers were homogenized in 400 µl, two per microfuge tube, resulting in 24 samples. The homogenates were used to infect healthy larvae and incubated at 25º±2 C. All the DBM larvae that died with the virus were harvested and added together to make one sample designated F1.P4. A volume of 10 µl of the suspension was pipetted and loaded on a Thoma Weber haemocytometer (0.02 mm chamber depth). The haemocytometer was covered with a cover slip and a drop of emulsion oil placed on it. The sample was viewed under a light microscope at 1000 × dark field magnification. This was repeated for three samples of the same suspension. Crystalline particles in Brownian motion were observed in the sample indicating presence of granuloviruses in the sample.

4.2.1.2 Polyhedroviruses (NPVs)

African bollworm *(H. armigera)* larvae were collected from a wheat farm in Naromoru, Kenya 0° 10' 0" S / 37° 1' 0" E. A total of 291 live *H. armigera* larvae were collected and placed each in sample cup partially filled with artificial diet (Multi-species Southland diet). Six *H. armigera* cadavers were also collected, three of which displayed baculovirus symptoms. The live larvae were incubated at an average temperature of 27°C. Four additional larvae died while displaying baculovirus symptoms such as increased larval size and liquefied tissues at later stages of infection, bringing the total candidate isolates to seven. The seven cadavers were stored in the freezer at -20º C.

The cadavers were homogenized individually with 400 µl water per larva in 1 ml microfuge tubes and used to re-infect healthy 3rd instar ABW larvae. All the larvae that died with virus symptoms were mixed and homogenized and added together to make one sample designated F1.P1. The sample was examined under a phase contrast light microscope to determine if baculoviruses were present. A cover slip was placed on an improved Neubauer type haemocytometer (0.1 mm chamber depth) and10 µl of the suspension were loaded on the well. Three samples of the same suspension were viewed under a light microscope at 400× dark field magnification (10 × eyepiece and 40 × objective). Crystalline round-shaped particles in Brownian motion were observed hence indicating presence of baculoviruses in the samples

4.2.2 Multiplication of viruses

Viruses were multiplied by infecting healthy larvae with the isolates. The details for each baculovirus are provided below:

4.2.2.1 PlxyGV

Second instar DBM larvae were infected by surface contaminating their diet with the virus suspension. Fine sprays of the virus suspension were dispensed on the diet using a hand sprayer, ensuring total coverage of entire surface. This was done on 2 litre capacity plastic ice scream containers containing approximately 400 larvae per container. The inoculated insects were incubated at 24-27ºC and 45-65% relative humidity for a period of 4-6 days. DBM cadavers were harvested by use of a modified suction pump and homogenized using a blender. The homogenate was sieved using a 75µm size sieve to remove insect body parts, diet particles and frass to make crude virus suspension. The suspension was stored at 4º C for field experiments or purified for subsequent experiments.

4.2.2.2 HearNPV

African boll worms were infected by applying *Hear*NPV suspension on the surface of the diet in sample cups for each 3rd instar larva. The cups were then closed with their lids and the insects incubated at a temperature of 23-27ºC and 45-65% relative humidity for a period of 6-9 days. Dead larvae were harvested using forceps and homogenized with 1 ml water per larvae. The homogenization was done using a blender and the homogenate sieved with a 75 µm size sieve to remove large insect body materials to obtain a crude virus suspension. The crude virus suspension was stored at 4 ºC for the field experiments or purified for subsequent experiments.

4.2.3 Purification of the viruses

4.2.3.1 PlxyGV

PlxyGV was purified by differential centrifugation method using KBL standard operating procedure (Appendix: 3). A 10^{th} volume of 10% SDS solution was added to the homogenate and incubated at room temperature for 1 hour. The mixture was spun at 3000 rpm for 15 minutes to remove insect body materials. The supernatant was retained and pellets discarded. The supernatant was spun at 11000 rpm for 30 minutes. The pellets were re-suspended in 100 mM NaCl to remove SDS sticking to the surface of the granule. The mixture was spun at 11,000 rpm for 30 minutes at 4-7º C. The supernatant was discarded and the pellets re-suspended in distilled water. The mixture was spun at 11,000 rpm for 30 minutes and the pellets obtained re-suspended in distilled water and supernatant discarded. This was repeated two times and the pellets finally re-suspended in a small amount of distilled water before storage. The concentration of the virus was determined by counting the number of granules under a phase contrast light microscope using a 0.02 mm depth hemocytometer. Granular counts were made on three samples of the same virus sample at the dilution.

4.2.3.2 HearSNPV

Purification of *Hear*NPV was done by differential centrifugation method using a high speed Sorvall centrifuge. An equal volume of 1% SDS solution was added to the homogenate (virus) and incubated at room temperature for one hour. The mixture was spun at 100 rpm for five minutes in a high speed sorvall centrifuge. The pellets were discarded and the supernatant retained. The supernatant was spun at a high speed of 5,000 rpm for 10 minutes at 4-7ºC, the supernatant was discarded and the pellets re-

suspended in 2 ml 100 mM sodium chloride for each gram of original larvae. This was spun for 10 minutes at 5,000 rpm, the supernatant discarded and the pellets re-suspended in approximately three times the volume of distilled water. The mixture was spun for 10 minutes at 5,000 rpm, the supernatant discarded and the pellets re-suspended in distilled water. This was repeated two times and finally the pellets were re-suspended in 10µl distilled water. The concentration of the virus was determined by counting the OBs under a phase contract light microscope with a 0.2 mm depth hemocytometer. Occlusion body counts were made on three samples of the same virus sample at the same dilution.

4.2.4 Characterization of the virus isolates

The GV and NPV virus isolates were characterized morphologically, biologically and genetically to verify that they resembled other virus isolates in the same group characterized elsewhere in the world and to determine their virulence.

4.2.4.1 Morphological characterization of virus Kenyan isolates of *Plxy*GV and *Hear*NPV

Samples of viruses purified by differential centrifugation were used for morphological characterization. Pellets of *Plxy*GV and *Hear*NPV were fixated separately in 3% glutaraldehyde and 2% paraformaldehyde, treated with osmiumtetroxide, followed by dehydration in increasing concentrations (50, 70, 80 and 95%) of alcohol, and finally embedded in London Resin (LR) White. Sectioning was done on a Leica Ultracut E microtome and the ultrathin sections of approximately 60 nm of the pellets were then stained with 2% uranylacetate, contrasted with lead citrate and photographed under a Jeol electron microscope (model JEM 1011). Two samples of each of the virus

isolate, HearNPV and PlxyGV were observed. Observations were made on the shape, structure and number of virions within an occlusion body. These morphological features were compared with other characterized baculovirus isolates to determine the type of baculovirus isolated.

4.2.4.2 Determination of the virulence of HearNPV and PlxyGV (Biological Characterization)

Virulence and speed of action, as related to dose, are important effectiveness-determining properties of insect-pathogenic bio-control agents. The virulence of Kenyan isolates of *Hear*NPV and *Plxy*GV was determined by use of laboratory bioassay methods. Surface contamination, droplet feeding and diet plug bioassay methods were used for diamondback Moth larvae whereas diet plug and droplet feeding bioassay methods were used for African Bollworm larvae. Bioassay methods used in these experiments were dependent on the insect species and age.

4.2.5 Bioassays

All bioassays were carried out in Kenya Biologics Laboratory (KBL) at 25±2ºC and approximately 55% R.H. The insects were obtained from KBL insectary. DBM and ABW semi-synthetic diets were prepared in KBL insectary following a diet preparation standard operating procedure.

4.2.5.1 Droplet feeding bioassay

Five concentrations of *Plxy*GV, designated as *Plxy*Gv F1.P4, were prepared by 1:4 serial dilutions from 1.0×10^7 to 1.0×10^5 with distilled water containing 1% blue food colour. Water with food color was assayed as control. A tray was carefully lined with

parafilm and using a micropipette, two concentric rings of small droplets (1-2µl per drop) of virus suspension were made. This was repeated twice for all five virus concentrations and the negative control. Twenty neonates were transferred to the middle of the concentric rings using a horse-tail brush and covered with sample cups. They were allowed to stand at room temperature for 15 minutes. Naturally neonates tend to move upwards. As they moved from the middle of the rings to climb the walls of the sample cups they passed through the treatment and ingested the suspension containing virus particles. All larvae with blue coloration were assumed to have ingested the virus and were transferred to sample cups containing diets, ten larvae in a sample cup. The sample cups were covered with lids and mortality recorded from the second day for a period of five days.

The same procedure was applied for African bollworm neonates. Four concentrations of *Hear*SNPV, designated *Hear*SNPV F1.P1, were prepared by 1:9 serial dilutions from 2.6×10^6 to 2.6×10^3 with distilled water with 1% blue food colour. Water with food color was assayed as control. This was repeated three times for every concentration.

4.2.5.2 Diet plug bioassays

Five concentrations of *Plxy*GV designated as *Plxy*GV F1.P4 were prepared by 1:4 serial dilutions from 1.0×10^7 to 1.0×10^5 with distilled water containing 1% blue food colour. Water with food color was assayed as control. Second instar DBM larvae were starved for six hours by removing them from the diet and putting them in empty containers. Using sterile surgical blades, tiny plugs (volume $1mm^3$) of DBM diet were made. Each plug was put into 1 ml eppendof tubes. Using a 20µl micropipette, 5µl of

the suspension was put on the plug and a larva put into each tube to feed on the infected plug. Forty larvae were inoculated with each virus concentration and the negative control. This was repeated two times for every virus concentration and the control. DBM larvae were transferred to sample cups containing diet only when they finished the treated plugs. Mortality was recorded daily from the 3rd day after infection for five days.

The procedure was repeated with 3rd instar ABW larvae. Four concentrations of *Hear*SNPV, designated *Hear*SNPV F1.P1, prepared by 1:9 serial dilutions from 2.6×10^6 to2.6×10^3 with distilled water with 1% blue food colour were used as inoculums. Water with food color was assayed as control. Each concentration was replicated twice. The number of larvae that died per concentration each day was recorded from the 4th day for 7 days and cumulative mortality on the 7th day was used for analysis.

4.2.5.3 Surface contamination Bioassay

Five concentrations of *Plxy*GV designated as *Plxy*Gv F1.P4 were prepared by 1:4 serial dilutions from 1.0×10^7 to 1.0×10^5 with distilled water containing 1% blue food colour. Water with food color was assayed as control. Ten sample cups containing DBM diet were treated with the five concentrations; each concentration had two cups and 20 insects placed in each sample cup to expose 40 larvae to each concentration. Mortality was recorded 24 hours after the larvae were exposed to the virus to remove any insects that die out of mechanical injury and to determine the total number of insects at risk of dying out of virus contamination. Cumulative mortality was recorded

daily from the 3rd day of the experiments to the 7th day. Cumulative mortality at day 7 was expressed in percentage.

The number of larvae that died per concentration each day were recorded from the 4th day for 7 days and percent mortality on the 7th day was used for analysis.

4.2.5.4 Data analysis

Dose-response data was analyzed by probits using DuPont program to determine LC_{50} and 95% confident limits for each concentration.

4.2.6 Genetic characterization of Kenya baculovirus isolates, PlxyGV and HearNPV

Genetic characterization of *Hear*NPV and *Plxy*GV was done by Restriction endonuclease analysis (REN) which is based on restriction enzyme generated banding of DNA fragments run out on a gel. Purified virus isolates were used for this experiment.

4.2.6.1 *Hear*NPV

4.2.6.1.1 Liberation of virion

Virus particles were liberated from the granula using the 9.5 pH of a 50 mM Na_2CO_3 solution. To approximately 2.5×10^{11} granula in 0.5 ml H_2O, 25µl of 10% SDS was added and incubated at 60^0 C for 15 minutes. The temperature of the suspension was allowed to decrease to 37^0 C. Protease K, 20mg /ml, was added and the suspension incubated for 1-2 hours at 37^0 C. An equal volume of 100 mM Sodium bicarbonate (Na_2Co_3) was added to the suspension and incubated until the suspension became

clear. The granula envelopes were pelleted in a microfuge at 5000 rpm for 2 minutes. The suspension with the liberated virion was kept for DNA isolation.

4.2.6.1.2 Isolation of DNA

Protein was removed from the virions and DNA isolated through repeated and gentle extractions with an equal volume of phenol then phenol: chloroform: isoamylacohol (ratio of 25:24:1), followed by chloroform. After each the extraction, water and solvent phases were separated by centrifugation at 12000 rpm for 5 minutes and the water phase transferred to a clean tube for the next extraction. Extractions were done until no visible protein interphase remained. DNA was precipitated overnight at -60^0C by addition of 10^{th} volume of 2.5 M sodium acetate, pH 5.2 and 2 volumes of isopropanol. DNA was pelleted by centrifugation at 12000 rpm for 15 minutes and then rinsed with 70% ice-cold ethanol. After centrifugation for five minutes, the supernatant was removed and the pellet was dried for approximately one minute in a laminar flow hood. The pellets were re-suspended in 60 µl distilled water and left overnight at 4^0C.

4.2.6.1.3 Determination of DNA concentration

The DNA concentration was determined using a Biospec-mini DNA/RNA/Protein Analyzer (Shimadzu). 1µg and 3µg DNA were digested with EcoRI, HindIII, and BamHI, according to manufacturer's specifications. After two hours of incubation at 37^oC, the reactions were stopped by addition of $1/6^{th}$ volume loading buffer.

4.2.6.1.4 Gel-electrophoresis

A 0.7% agarose gel, prepared in Tris-buffered EDTA (TBE), containing 10µg/ml ethidium bromide was prepared and set up in a 22 cm gel box with TBE as running buffer. The gel was loaded with different amounts of digested DNA per well for each enzyme (1 and 3 µg/well), run at 2.4 V/cm for 10 hrs, photographed on a UV-trans-illuminator, and digitally stored.

4.2.6.2 PlxyGV

4.2.6.2.1 Liberation of Virion

PlxyGV particles were liberated from the granula using pH 9.5 of a 50mM Sodium bicarbonate (Na_2CO_3) solution. A virus suspension of approximately 2.5×10^{11} granula in 0.5ml H_2O was taken and 50 µl proteinase K (20mg/ml) and 50µl 10% SDS added to it. The suspension was incubated at 37ºC for 1-2 hours. An equal volume of 100 mM Na_2CO_3 was added to the suspension and incubated until it was clear. Granular envelopes were pelleted in microfuge by spinning the mixture at 5000 rpm for 2 min.

4.2.6.2.2 Isolation of DNA

DNA was isolated by repeated phenol/ chloroform (5×) and Chloroform (1×) extractions, until the interphase was free of visible protein residues. The DNA was precipitated out of the water phase by adding two volumes ice-cold 100% ethanol and incubated in the freezer at -60ºC overnight. The DNA was then pelleted in a microfuge by spinning the sample at 5000 rpm for 10 minutes, washed with 70% ethanol, dried and re-suspended in distilled water.

4.2.6.2.3 Digestion of DNA

After determining the concentration of the DNA in a spectrophotometer, the DNA was digested with restriction enzymes EcoRI, HindIII and BamH I, according to manufacturer's specifications. After two hours incubation at 37ºC the reaction was stopped by addition of 1/6th volume of loading buffer.

4.2.6.2.4 Gel electrophoresis

A 0.7% agarose gel in Tris-buffered EDTA (TBE), containing 10µg/ml ethidium bromide, was prepared and set up in a gel box with TBE as a running buffer. The gel was run at 2.4V/cm for eight hours, and photographed on a transilluminator and digitally stored.

4.3 Results

4.3.1 Morphological characterization

4.3.1.1 Morphological characterization of *Plxy*GV isolates

The two PlxyGV samples when photographed under the electron microscope appear to consist of uniform particles which show all the characteristics of a granulovirus (plate 1). Typically, granulovirus have virions consisting of dark stained nucleocapsids which are surrounded by a membrane. The nucleocapsids are singly embedded in a paracrystalline protein matrix, which in turn is surrounded by an envelope. The size of the virus particles was approximately (0.15×0.20µm) to (0.15×0.30µm) and is typical of granulovirus. The diameter and length of the granules vary among different viruses (Tanada and Hess, 1991).

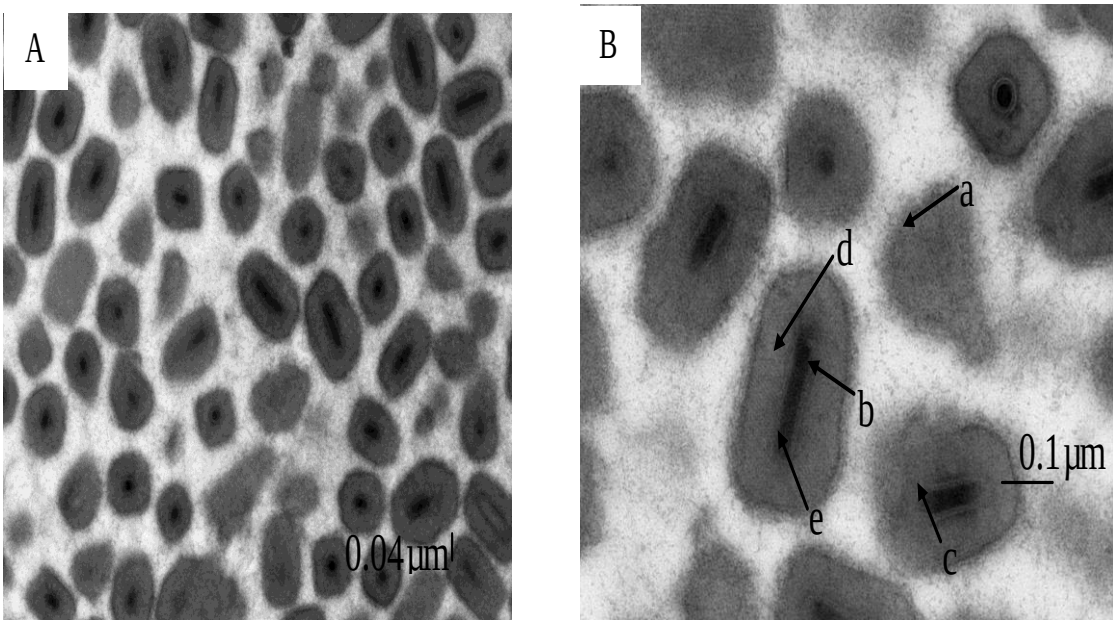

Plate 4.1 Electromicrographs of PlxyGV F1.F4 at a magnification of 10 KV (A: left panel) and 25KV (B: right panel). Arrows indicate: (a) particle section outside the plane of the virus particle; (b) granular envelope; (c) virion envelope; (d) nucleocapsid; (e) paracrystal protein matrix (granulin)

In conclusion, both sample 1 and 2 show particles of a morphological appearance fully consistent with and typical of that of members of the family *Baculoviridae*, genus granulovirus. This is an indication that both samples represented granulovirus suspensions.

4.3.1.2 Morphological characterization of *Hear*NPV isolates

Alkaline digested I0 sample showed that it was contaminated with a granulovirus. Alkaline digested HearNPV (F0) sample when viewed under the electron microscope

appeared to consist of nucleopolyhedrovirus of the single nucleocapsid subgroup (Plate 4.2).

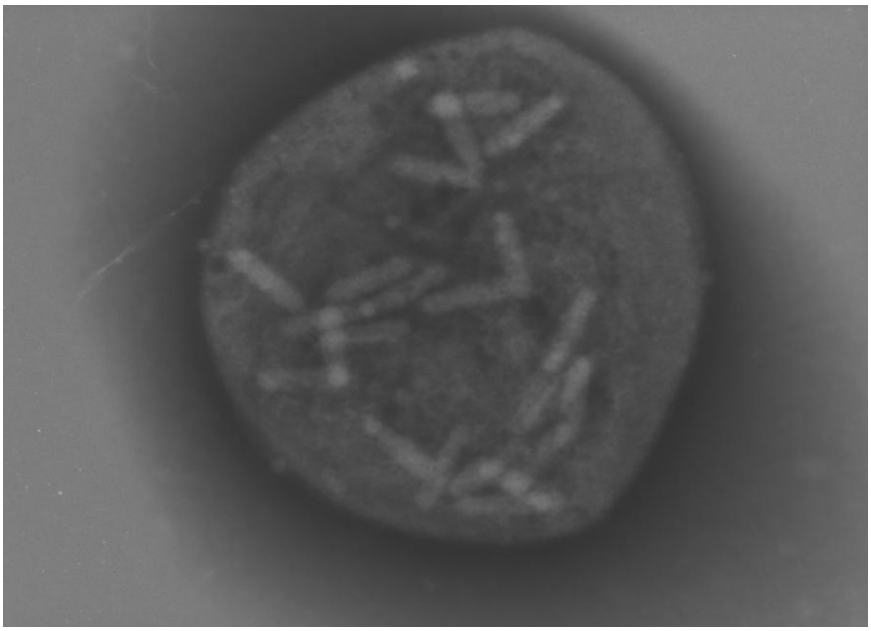

Plate 4.2 Alkaline digested HearNPV (F0) showing a partly hydrolyzed occlusion body. The unstained (light) nucleocapsids appear as single entities (single nucleocapsids per envelope) rather than as bundles surrounded by a membrane.

Ultra thin sections of field sample, F0, under the transmission electron microscope, showed particles with characteristics of a nucleopolyhedrovirus of the single capsid subgroup (Plate 4.2). Typically, nucleopolyhedrosis viruses have large (0.5-5 µm) occlusion bodies consisting of a paracrystalline protein matrix which in turn is surrounded by an envelope. The occlusion bodies contain dark-stained nucleocapsids which are surrounded by a membrane either individually or in groups. When a single nucleocapsid is surrounded by a membrane the virus is of the single capsid subgroup

(SNPV) and when more than one nucleocapsids are surrounded by a membrane the virus belongs to the multiple capsid sub-group (MNPV).

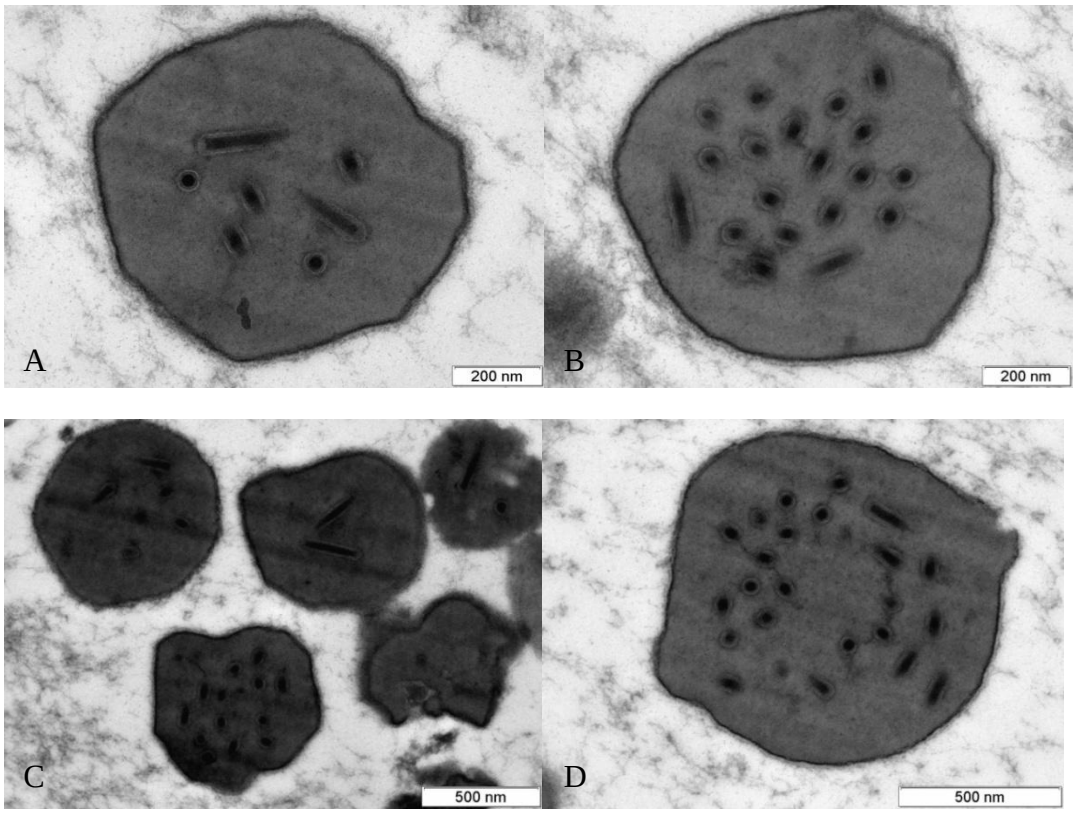

Plate 4.3 Electron micrographs of sections through a nuclear polyhedrovirus. The crystalline nature of the polyhedron protein can be seen, together with the random orientation of virus particles within the polyhedron. Transverse sections of the virus particles show a variable number of nucleocapsids per envelope, 7 in A, 19 in B and 25 in D. C shows five different polyhedra particles.

4.3.2 Biological characterization of PlxyGV and HearNPV isolates

The surface contamination method was the most appropriate way to assay DBM. Bioassay results gave a clear dose-response relation for both field and insectary isolates (Fig.4.1).

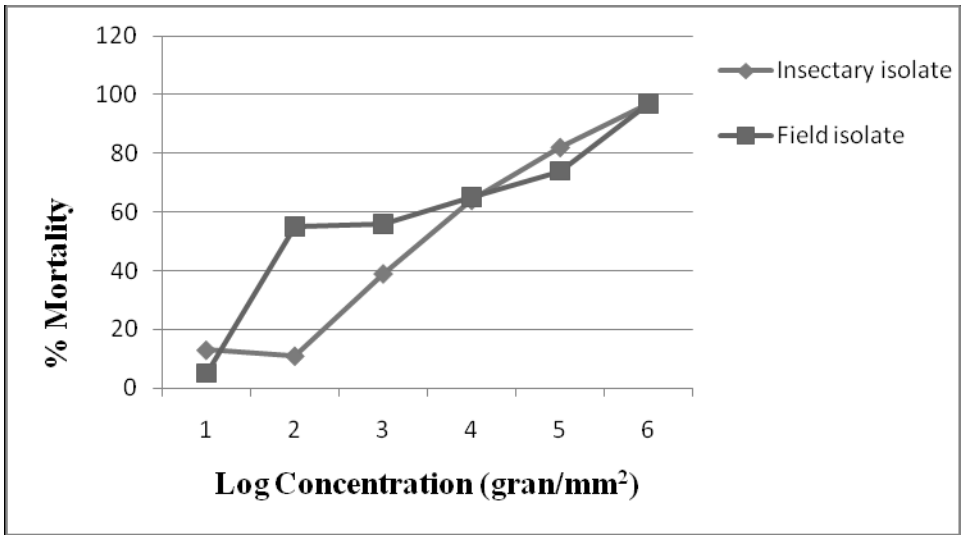

Figure 4.1 Larval mortality scored at different application rates of granulovirus field and insectary isolates on the diet surface.

LD_{50} estimates from probit analysis were 7,109 and 7,649 granules/mm^2 for the field and insectary isolates, respectively (Table 3).

Table 4.1 Probit analyses of dose-mortality for PlxyGV in the field (F4) and insectary (I5) using the surface contamination bioassay method

Virus isolate	LD_{50} (granula/mm^2)	Standard error	95% Confidence limits
PlxyGV-F4	7,109	1,535	4,085 – 10,133
PlxyGV-I5	7,649	2,358	3,012 – 12,286

Graphs of the survival time of 2nd instar larvae inoculated at different doses and kept at an average temperature of 25°C (range 23.2-27.1) are presented in Fig. 5 and Fig. 6. The LT_{50} of those larvae that died was estimated to range from 5.5 to 7.5 days for the F4 and 6.3 to 7 days for the I5 sample, respectively.

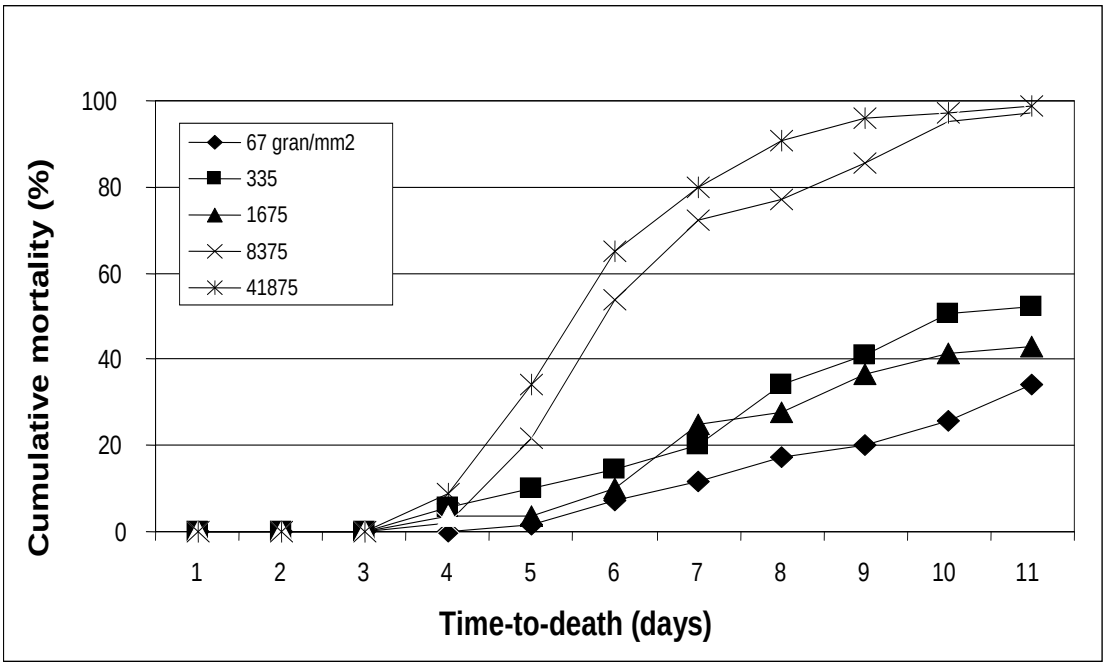

Figure 4.2 (a) Survival time of 2nd instar *P. xylostella* exposed to five different concentrations of PlxyGV field isolate (F4)

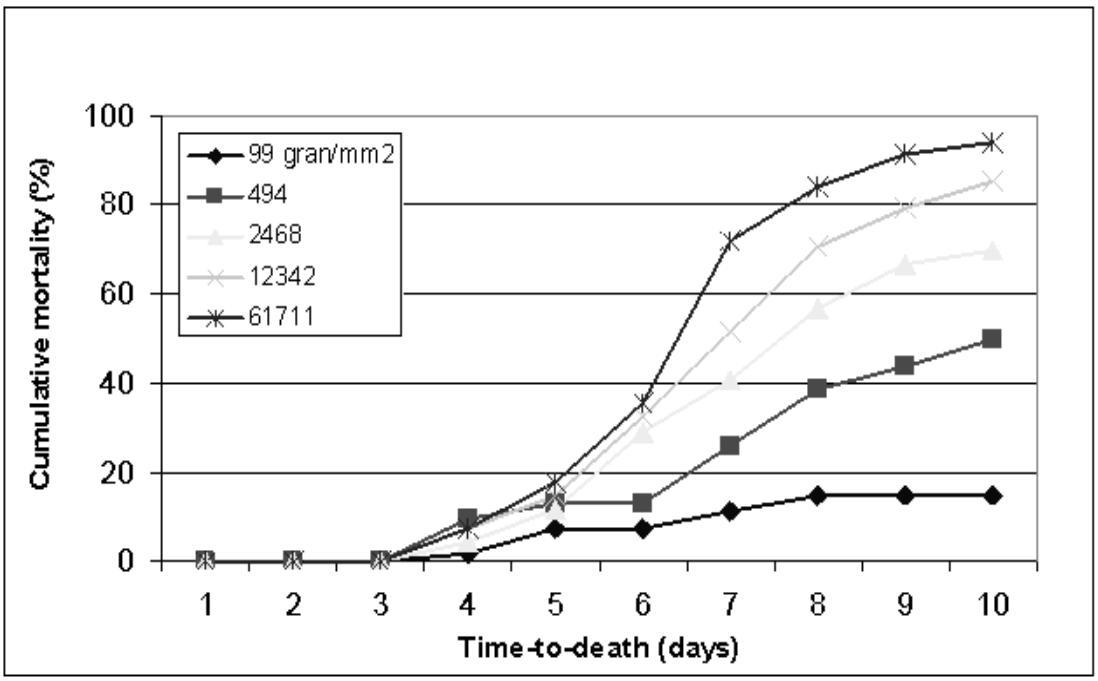

Fig. 4.2 (b) Survival time of 2nd instar P. xylostella exposed to five different concentrations of PlxyGV insectary sample (I)

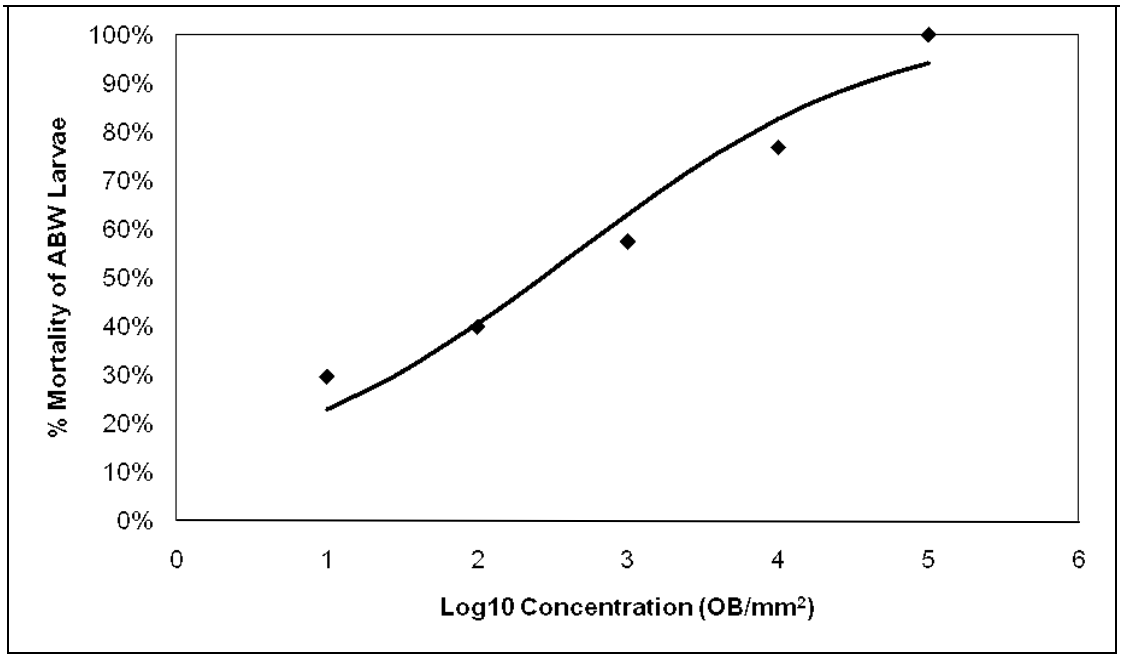

Figure 4.3 Mortality (%) of ABW larvae inoculated with different concentrations of *Hear*NPV (F0). The nearly straght line obtained from probit transformed response data enables estimation of Lethal Dose (LD50)

Table 4.2 Dose-mortality responses for third-instar *Helicoverpa armigera* inoculated with *Helicoverpa armigera* nucleopolyhedrovirus (*Hear*NPV)

Sample	LD_{50}	Slope ±SD	Heterogeneity Factor
1	**257**	**0.37 ± 0.12**	**0.58**
2	**831**	**0.29 ± 0.23**	**0.07**
3	**154**	**0.56 ± 0.12**	**0.58**
4	**426**	**0.66 ± 0.17**	**0.06**

The relatively low slope values (0.29 - 0.66) for all the four HearNPV replicates indicate variation in larval susceptibility implying that high doses of the Kenya isolate

*Hear*NPV are required to obtain higher mortalities. There was an increase in mortality as the concentration of the virus assayed increased (Fig.4.3)

The LD_{50} are low, 1.54×10^2 to 8.3×10^2, indicating high virulence of the HearNPV isolate. A relatively high mortality (> 50%) was obtained with a dose of 13 OB/larvae indicating a high virulence of this virus isolate to the 3rd instars larvae of *H. armigera*. A lower dose of one OB/ larvae caused 35% mortality whereas a high dose of 1300 OB/ larvae caused 100% mortality of 3rd instar larvae. Diet plug bioassay method is a relatively precise method as it allows accurate estimation of the number of occlusion bodies that are ingested by a larva.

4.3.3 Genetic characterization of Kenyan isolates of PlxyGV and HearNPV

Lane 1: EcoRI: The estimates for the fragment lengths after establishing a standard regression line by plotting the log molecular weight of the marker fragments against the migration distance revealed for EcoRI (Plate 4.4, lane 1, left panel) the following fragment length estimates, from top to bottom: 20.3 (two top bands are not resolved), 16.3, 15.1, 13.6 and 7.7, respectively. The total estimated genome length is 93.3 kb. The total molecular weight of the PlxyGV genome is 100.999 kbp. It is likely that there are smaller bands not visible on the gel, accounting for the difference of 7.7 kb. There is a submolar band at approximately 8.5 kb, indicating some heterogeneity in the sample, which is characteristic for field isolates.

Lane2: HindIII: Using the same regression line the fragment lengths of the HindIII digest (Plate 4.4, left panel, lane 2) were estimated at 14.5 (doublet), 11.0 (triplet),

10.5, 8.9 (doublet), 3.8, 3.6 1.1, 0.9, and 0.1, kbp, respectively. The total estimated genome length is 99.8 kbp, only 800 base pairs removed from the literature.

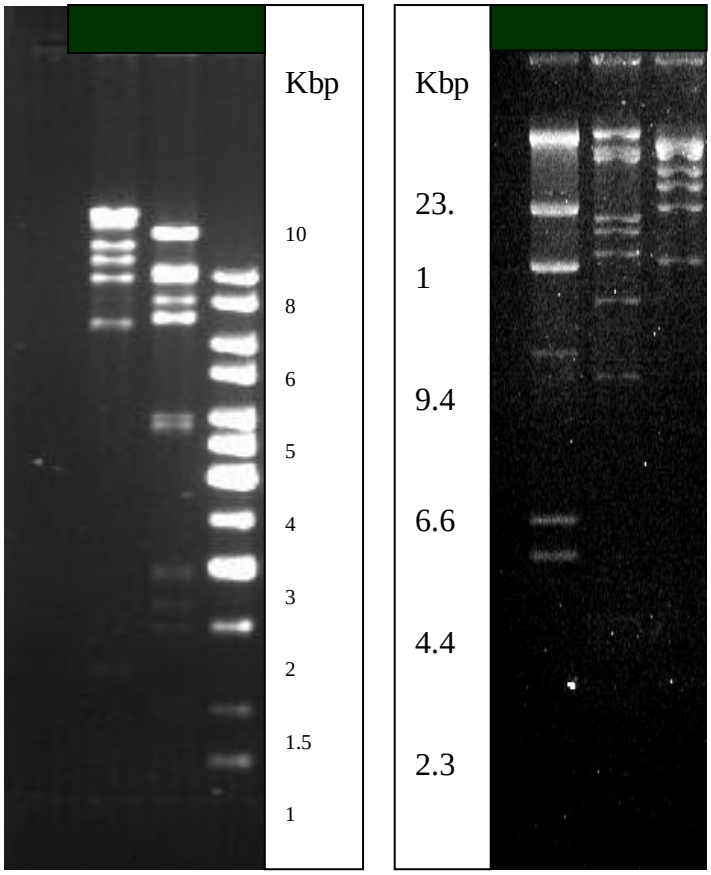

Plate 4.4 Gel-electrophoresis patterns of PlxyGV digested with EcoRI, HindIII, and BamHI . Lane 1, EcoRI; lane 2, HindIII; lane 3, 1 kb ladder. Right panel: lane 1, high molecular weight markers; lane 2, BamHi; lane 3, EcoRI. The 0.7% agarose gel in the left panel was run at 2.4 V/cm for 16 hours, while the 1% agarose gel in the right panel was run at 1.8 V/cm for 18 hours. Kbp, kilobase pairs.

Lane 3: BamHI: Based on the regression line established for the high molecular weight markers and their migration distances, the fragment sizes from the BamHI (Plate 4.4, right panel, lane 2) were estimated as follows: 20.0, 17.7, 17.4, 12.3, 11.2,

19.8, 7.4, and 4.6 kbp from top to bottom, respectively. The total genome length estimated from the fragments amounts to 110.4 kbp, in close agreement with the sequence count of 100.999.

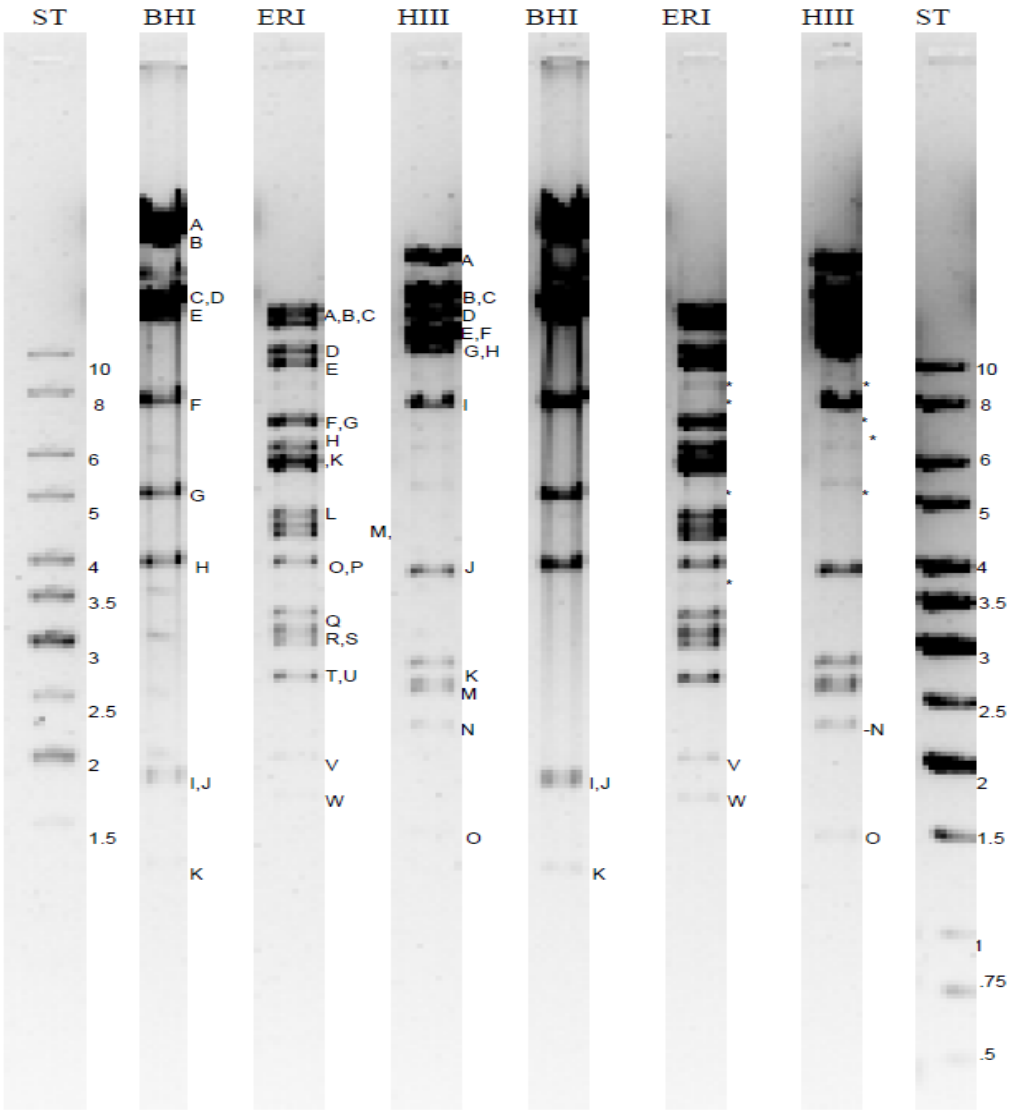

Plate 4.5 Restriction endonuclease profile of HearSNPV-F1 for three enzymes. Marking of the lanes: ST, 1 kb ladder; BHI, BamHI; ERI, Eco RI; HIII, HindIII. Digests were loaded at 1 µg (left) and 3 µg (right) per lane. Molecular weights of the standard are indicated in kb. Fragments are marked putatively with letters,

alphabetically from largest to smallest as per convention, and putative submolar bands are indicted with a star.

For the *Bam*HI digest, *Hear*SNPV revealed the same number of fragments at the same relative migration distance indicating that this particular enzyme does not distinguish between our isolate and the major genotype (G4) of the Chinese isolate.

In the *Eco*RI digest fragments A and B, seem smaller in F1 than in G4, while one or two of the fragments T, U, V, or W, are significantly larger. The precise nature of the differences cannot be resolved with restriction enzyme digestion, but it is likely that there is an extra EcoR1 target sequence in fragments A and B, leading to the extra fragments, designated T and U between fragments S and T in the Chinese isolate. However, the overall pattern and sizes of F1 and G4 fragments are similar.

Again, the overall pattern of F1 and G4 isolates digested with *Hin*dIII compare very well. The difference lies in the J fragment of F1 that is substantially smaller, indicating extra restriction sites in this fragment, which is confirmed by the presence of fragments L and M, compared to G4.

4.4 Discussion

Results of electro-micrographs and gel-electrophoresis profiles of both HearNPV and PlxyGV isolates confirms that Kenya isolates are similar in morphology and in genomic make-up with other baculoviruses in the genera nucleopolyhedrovirus and granulovirus. The banding patterns of Plxy-F4 for Eco RI, HindIII and BamHI closely resemble the patterns in the publication by Subramanian *et al.*, (2008) on restriction

endonuclease profiles of four Kenyan and one Indian isolate with HindIII, PstI, BamHI, and EcoRI. In the case of EcoRI (lane 1 and 3 left and right panel, respectively) there is a typical pattern of two high molecular weight bands that are difficult to resolve, followed by three evenly-spaced bands and a band at a larger distance. This pattern most closely resembles those of isolates 1 and 2 of Kijabe, Kenya (Subramanian *et al.,* 2008; Fig 1, panel 4, lanes 4 and 5). The HindIII pattern seems to show a difference from the Kijabe in that the double band at approximately 9 kbp (Fig. 1, panel 1, lanes 4 and 5) is a single band in the report of Subramanian *et al.*, 2008. The rest of the pattern is very similar. Finally, the BamHI pattern is different from the Kijabe isolates in that the second fragment is closer to the third rather than closer to the top fragment as in the Kijabe isolates. Also, the F-4 isolate has an extra fragment at approximately 5.5 kb. It seems likely that there is an extra restriction site in fragment two.

The overall number of fragments and the spacing again indicates a high degree of similarity with the Kenyan isolates of Subramanian *et al*., (2008). In summary, the overall number of fragments generated with the three enzymes, the pattern as well as the total genome size, all confirm that isolate PlxyGV-F4 is a *Plutella xylostella* granulovirus isolate. The isolate somewhat resembles the Kijabe isolates, but it is a slightly different genotype. This is not surprising as reportedly 15 out of 16 Kenyan isolates turned out to be different in a single restriction enzyme digestion (Parnell *et al.,* 2002). The difference in genome among isolates could be extremely useful as a diverse genetic resource that could be exploited in the development of a GV for DBM control (Grzywacz *et al.,* 1998)

The REN profiles of the isolate analyzed in this work were very similar but not identical to those of the HearNPV isolates described by Figueiredo *et al.,* (1999), indicating that they constitute novel HearNPV strains. The overall pattern and size of the Kenya baculovirus isolate, HearNPV was also similar to that of the main genotype of Chinese isolate by Kadir *et al.,* 1999b, when digested with three restriction enzymes. It is evidence that HearSNPV-F1 is the *Helicoverpa armigera* single capsid nucleopolyhedrovirus, and that it is closely related to the Chinese isolate. The presence of two additional restriction cleavage sites in the F1 isolate explains the minor differences between the isolates; this is modest for inter strain variation. A mixture of different genotypic variants was detected, as indicated by the presence of submolar bands in their corresponding REN profiles. These results suggest that insects were not infected by single variants but rather a mixture of them in a single isolate. This is a common phenomenon in baculovirus wild-type isolates, which are usually formed by a mixture of genotypes in distinct proportions (Muñoz *et al.,* 1998).

Dose-mortality assays of PlxyGV isolates resulted in high mortality with LD_{50s} of 5.0×10^6 gran/ml and 5.0×10^6gran/ml. Grzywacz *et al.,* (1998) reported average LC_{50} values for 2nd instar DBM larvae varying from 2.36 x 10^6 OB/ml for Nya-01 *Plxy*GV to 3.95 x 10^7 OB/ml for Nya-40 *Plxy*GV. Compared to these Kenyan isolates was a Taiwan PlxyGV isolate, *Plxy*GV-Tw with LD^{50} of 1.55 x 10^7 OB/ml.

Dose-mortality assays of Kenyan HearNPV isolate resulted high mortality of up to 100% in 7-9 days post infection conforming to records of other NPVs against their noctuids host (Smits and Vlak, 1988). The LC_{50} values of four samples of the same virus differed by 2.0-fold to5.4-fold. This level of heterogeneity in HearNPV was also

reported by Zhang *et al.* (2005) between two Chinese isolates. The estimated LC50 values were similar to those reported by other authors for isolates from Africa (Ogembo *et al.*, 2005) and China (Sun *et al.*, 2004).

The bioassay results in this study suggest that Kenyan isolate of *Hear*NPV and PlxyGV are highly virulent virus against *H. armigera* larvae and *P. xylostella* respectively. The LD_{50} values of baculoviruses increase rapidly from the first to fifth larval instar (Bianchi *et al.*, 2000) thus HearNPV and PlxyGV may be useful in biological control programs of *H. armigera* and *P. xylostella* respectively especially the early instars, 1^{st}, 2^{nd} and 3^{rd} instars.

CHAPTER FIVE

5.0 DETERMINATION OF FIELD EFFICACY OF BACULOVIRUSES

5.1 Introduction

Baculoviruses are insect pathogens that when used as Biological controls may effectively suppress pests. They are considered to be very host-specific, mainly infecting members of the same host genus (Gröner, 1986). Their specificity is very narrow and is often limited to only one species. They have been used worldwide but their application as bio-insecticides has been limited. Expansion of baculoviruses as commercial insecticides was hampered by their slow killing action and technical difficulties for *in vitro* commercial production. Due to the slow killing action of baculoviruses, primary users (used to fast-killing chemical insecticides) regarded them as ineffective. Up to date the most successful project using baculoviruses was implemented in Brazil where over two million hectares of soybean are controlled by baculovirus AgMNPV (Moscardi, 1999; Moscardi and Santos, 2005).

Biological control can be potentially permanent because the natural enemies supplied from the outside will establish themselves in the pest population and are likely to exert long-term protection against the target pest species. The ways to implement biological regulation can be roughly divided into three major groups: importation, conservation or augmentation of natural enemies and application of microbial pesticides (Myers *et al*., 2000).

The most widely studied baculovirus is the *Autographa californica* nucleopolyhedrovirus (AcMNPV). Early work on AcMNPV was directed towards the

development of viral pesticides and construction of baculovirus-based expression vectors (reviewed by Wood and Granados, 1991)). Other baculoviruses that have been studied for virulence, efficacy and their general characteristics include *spodoptera litura* NPV (Nakai *et al.,* 2004), *Spodoptera exempta* NPV and *Mamestra brassica* NPV (Shigeyuki and Goto, 2010).

In this study, the efficacy of *Plutella xylostella* GV (PlxyGV) in the field was evaluated against its ability to control *P. xylostella*, DBM larvae in a broccoli field in Thika Kenya.

5.2 Materials and Methods

5.2.1 Baculovirus isolate

The baculovirus isolate, *Plutella xylostella* granulovirus, PlxyGV I0 obtained from DBM larvae collected from Ontulili farm Nanyuki Kenya, 0° 1' 0" N / 37° 4' 0" E, was evaluated for its efficacy as bio-control agent of the Diamondback moth in on-farm trials. The isolates were selected because of its high virulence.

5.2.2 Site description

The experiment was conducted on 50 m^2 experimental fields belonging to a commercial farm (AAA growers) in Thika district, latitude: 3′53″ and 1′45″ south of equator, longitude: 36′35″ and 37′25″ East. Altitude: 1450 m above sea level, ecological zone 4. The area is generally semi arid and receives low rainfall of 856mm with a bimodal distribution. The mean annual temperature is 20°C with the mean maximum temperature being 30°C.

5.2.3 Experimental design and layout

The baculovirus isolate, PlxyGV I0 was evaluated for their efficacy as bio-control agent of the DBM under Kenyan environmental conditions between July 2009 and September 2009. The experiments comprised of five treatments that included; three rates of PlxyGV I0t, a chemical control, Tracer 480 SC and untreated control. The treatments were laid out in a complete randomized block design where each treatment occurred in every block (Experimental layout: Appendix 4). There was a space, 1m wide between the blocks and 0.5m wide between plots. The blocks were surrounded by broccoli plants which acted as guard rows. The treatments were replicated four times.

5.2.4 Land preparation and planting

Land on the trial site was prepared by tilling and mixing with cow manure. Beds of length 10m and width of 1m were prepared. A space 1m wide was left between the beds. Transplanting holes, 10-15cm deep were dug 0.5 apart. Seedling raised on a 10m by 1m seedbed were hardened for a week by removing the shade and reducing watering to once in two days instead of once every day were transplanted. Watering was done daily by use of drip lines until the plants were well established. Gapping was done one week after transplanting to replace dead plants.

5.2.5 Field maintenance

Watering was done in the evenings once in two days by use of the drip irrigation method. Weeding was done manually twice during the entire broccoli growth period. Top dressing was done once before flowering of broccoli.

5.2.6 Field application of treatments

The GV stock suspension was diluted with 5 liters of water. Viral OB concentrations selected for the spray formulations were based on data of Okada (1977), 2.5×10^{13} OB/ha for the standard rate. A hydraulic knapsack sprayer (a manual sprayer, common for pesticides applications among small-scale farmers) was used to apply the virus to the plots; 5 L per 25 m^2 plot was a higher volume compared to the volume of chemical pesticide normally applied by farmers (approximately 500 L/ha) and was used to ensure complete foliar cover by the virus suspension. In the chemical control plot, treatments were applied two times (Tracer 480SC (Spinosad 480g/l) and Cimectacarb (pirimo) at week 4; the same day as the GV applications) and at week 9 after planting. In the control plot, 5 L of water containing teepol as a spreader was used. GV applications were made late in the afternoon to avoid OBs degradation by solar radiation (McGuire *et al*., 2001). GV applications were made using the same hydraulic knapsack sprayer where the control was applied first followed by low rate, 2.0×10^{14} OB/ha, the medium rate, 6.0×10^{14} OB/ha and finally the high rate, 1.0×10^{15} OB/ha. Chemical treatments, Tracer 480 SC (Spinosad 480g/l) and PIRIMOR WG, Pirimicarb 500g/kg, were applied using a different sprayer. PIRIMOR WG,

Pirimicarb 500g/kg; an aphid specific pesticide was applied during the 4th week after first treatment application to suppress aphids in the treatment plots.

5.2.3 Estimation of DBM larval population

Enumeration of DBM larvae in each plot began on the day before treatments were applied (week 0). Counting was done on seven randomly selected plants per plot each week after the first application and the number of larvae recorded for a period of eight weeks. Treatment applications were made each time the population was estimated to be higher than two larvae per plant (weeks 0, 1, 2 and 5 for the virus applications and weeks 0 and 5 for chemical applications).

5.2.5 Data Analyses

The numbers of DBM larvae per plot were analyzed by repeated measures ANOVA, to examine two factors (treatment and time of observation).

5.3 Results

5.3.1 Effects of GV application on Diamondback moth

The numbers of diamondback moth per plot for each treatment is show in Fig. 5.1 below. There were significant differences in the numbers of DBM larvae among the sampling times after GV application ($F=22.52$; $df= 4, 7$; $P<0.01$) and among the treatments ($F=59.14$; $df=4, 24$; $P<0.01$) by repeated measures ANOVA.

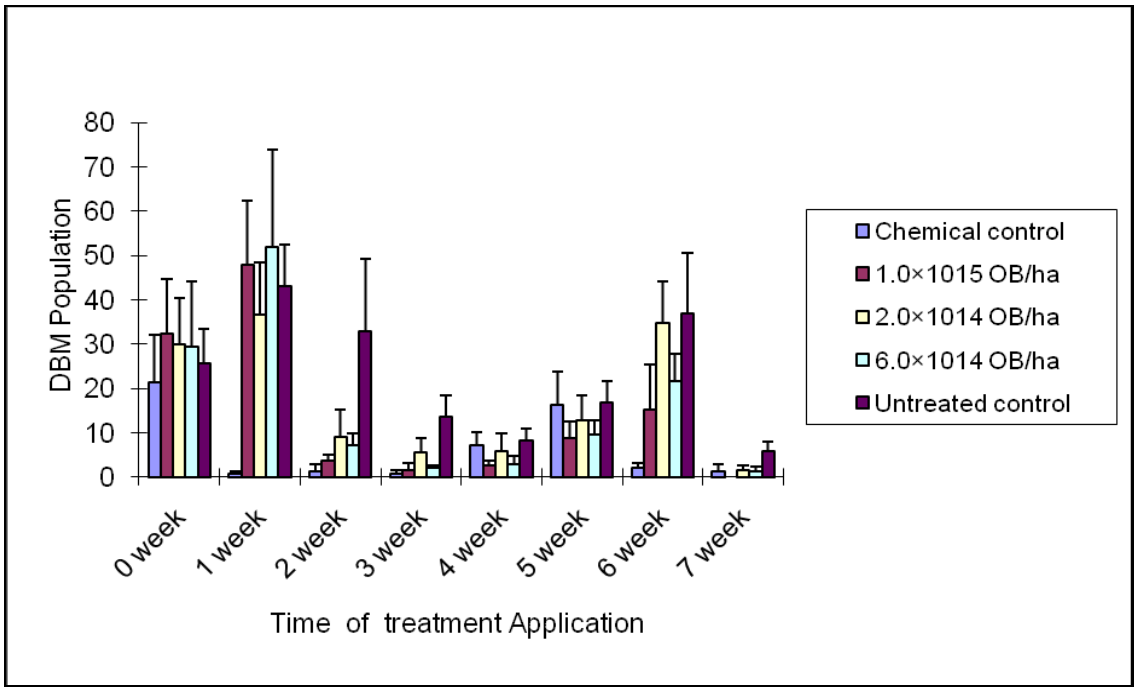

Figure 5.1 Mean Diamondback moth population over a period of seven weeks after application of three rates of Kenyan isolate of PlxyGV, 2.0×10^{14}, 6.0×10^{14} and 1.0×10^{15} OB/ha, a chemical control and untreated control. Bars are standard errors of five replicates obtained from seven plants per plot.

At the beginning of the trial, week 0, the mean population of DBM was not significantly different in all the five treatments ($F=0.71$; $df=4,24$; $P>0.05$) . Reduction in larval population was drastic in the chemical pesticide treatments. Population of DBM after first treatment application in this treatment was <2 larvae per plant.

Although the density of DBM larvae decreased drastically in the chemical pesticide treatment, the populations in untreated control, low rate, 2.0×10^{14} OB/ha, standard rate, 6.0×10^{14} OB/ha and high virus rate, 1.0×10^{15} OB/ha increased after first application. During the second and third weeks after the first spraying there was no

significant differences in the density on DBM larve on Chemical pesticide treatments and the three virus rates treatments (F=19.37; df=4,24; P>0.05). There was a signicant difference in the density of DBM larve in the untreated control and all the other treatments (P<0.05). The chemical standard had the lowest DBM population followed by the high rate virus treatment. During the fourth week there was no significant difference in the density on DBM larve between the chemical and the low rate virus treatment and between the high and the standard rate virus treatments but there was significant difference in the density on DBM larve in the untreated control and the other treatments. During the fifth week there was no significant differences in the numbers on DBM larve in all treatments (F=2.61; df=4,24; P>0.05).

In the sixth week there were no signicant differences in the density on DBM larve on chemical pesticide treatment and the high virus rate P>0.05 but there were signicant differences between the chemical pesticide treatment and the untreated control, low rate virus and the standard rate (F=12.34; df=4,24; P<0.05). In the seventh week after the first spay there were no significant differences in the dinsity of DBM larvae on chemical pesticide treatment, the low rate virus, the standard rate virus and the high rate virus(F=13.21; df=4,24; P<0.05). The density of DBM larve decreased from the first week for the chemical standard treatment and from the second week for all dthe other. Treatments application during the sixth week resulted to a reduction in the density of DBM/plant to an average of <2 insects per plant, a situation that prevailed till the harvest time at the 8^{th} and 9^{th} week after first treatments application.

5.4.2 Discussion

The average density of DBM larvae reduced in the chemical and virus treated plots and remained consistently higher in the untreated control plots throughout the experimental period. However, DBM larvae in the untreated control and chemical pesticide treatments showed considerable infection with PlxyGV two weeks after first application of treatments. The virus infection symptoms in untreated control and chemical pesticide treatments indicate effects of secondary infection as a result of rupturing and spread of viruses by primary infected insects (Grzywacz *et al.,* 1998). Baculoviruses as pathogens are known to be transmitted both vertically and horizontally (Boucias and Pendland, 1998) and this would have caused that mortality. DBM larvae density reduction in the untreated control would also be as a result of natural mortality caused by other pathogens.

Drastic decrease in the density of DBM larvae in the chemical pesticide treatment after the first application indicates their faster action against Diamondback moth larvae as compared to the granulovirus (*Plxy*GV) treatment. Chemical pesticides have knock out effect on pests hence the drastic decrease in DBM densities. Baculoviruses on the other hand are slow acting (Inceoglu *et al.,* 2001) hence the delay in the reduction of DBM population in the three virus rate treatments. There were no significant differences in the density of DBM larvae in the chemical and the virus treatments, a clear indication that *Plxy*GV is as effective as chemicals in the management of DBM in the field. A decrease in the mean population of the untreated control plots indicate that the population was not only affected by the chemical and PlxyGV treatments but also by the prevailing weather conditions e.g. temperature. In

addition to this, when DBM larvae infected with granulovirus die they rapture and release virus particles into the environment. This contributed to the mortality in the untreated plots. The effect of baculoviruses on insect population is long term as they are continuously released into the environment by other insects that die of virus infection. In this case, baculoviruses are better control agents compared to chemical controls.

The speed with which the first two sprays of *Plxy*GV initiated infection of DBM larvae reducing their density to <2 larvae/ plant could indicate that one or two applications of *Plxy*GV at the start of the season might be sufficient to start an epizootic infection in resident DBM populations. These results conquered with the results of trails done by Grzywacz *et al.* (1998) with Kenyan isolates of PlxyGV.

In conclusion, granulovirus (GV) isolated from Kenya-Nanyuki is highly efficacious against the population of *P. xylostella*. There was an application rate-mortality relationship where the higher PlxyGV rate, 1.0×10^{15} OB/ha had the highest mortality (Table 5). However, the density of *P. xylostella* larvae in all GV plots was not significantly different (P>0.05). The reduction of *P. xylostella* larvae is not only due to GV application but also to other mortality factors including natural enemies and weather conditions which reduce the density of *P. xylostella*.

CHAPTER SIX

6.0 GENERAL DISCUSSION CONCLUSION AND RECOMMENDATION

6.1 Discussion

Biological control of agricultural pests has gained importance in recent years due to increased pressure to reduce the use of synthetic chemicals and their residues in the environment and food. Baculoviruses have shown great potential to reduce pest population of various species without causing danger to human life or leaving residues on produce (Harper, 1986). Large scale production of baculoviruses in live hosts has necessitated development of cheap diets for artificial rearing of the hosts. The technique for rearing DBM on artificial diet was developed by Biever *et al*., (1971); Koshihara and Yamada (1976); Hsiao and Hou, (1978). *Plutella xylostella* was first reared on cabbage plants. This method was abandoned because it required large quantities of host plants and many rearing cages. Life and fecundity tables have been found to be important methods for analyzing and understanding the impact of an external factor, such as an artificial diet, upon the growth, survival, reproduction, and rate of increase of an insect population (Bellows *et al.*, 1992). These tools have been used to improve rearing techniques (Birch, 1948) and compare different food sources in diet (Hansen *et al.,* 1999).

The two diets; modified pieris diet and modified Multi-species Southland diet developed for rearing Diamondback moth and African bollworm respectively can be used to rear the two insects successfully. Average survival rates of 95% and 97% in African bollworm and diamondback moth respectively as compared to 90% survival

rates of both ABW and DBW on the imported multi-species southland diet reveal superiority of the modified diets over the standard diet. Dunhawoor and Abeeluck, (2003) recorded 75%, 44% and 52% survival rates on cabbage, Beiver's and soya diets respectively, all which are lower than on the modified diet in this study. The quality of insects reared on any particular diet depends on the quality of the initial colony, the environmental conditions and the diet on which they are reared on. Occasional contamination (microbial and mould) is almost inevitable and can cause high mortality in larvae and adults. This can be suppressed by maintaining a clean environment and sterilizing laboratory equipment, eggs and diets (Carpenter, 2002).

Baculoviruses isolated in Kenya have typical characteristics of those isolated and developed in other countries as bio-control agents against various crop pests. The *Plxy*GV isolate has single virions within the granulin while the HearNPV isolate has several virions within the polyhedra and only one particle per protein capsule hence is a singly embedded nucleopolyhedrovirus. The REN profiles of the isolates analyzed in this work were very similar but not identical to those of the HearNPV isolates described by Figueiredo *et al*. (1999), and PlxyGV isolates described by Parnel *et al*., 2002 indicating that they constitute novel isolates. The two isolates; HearNPV and PlxyGV are highly virulent with LD_{50}s of 4.17×10^{2} OB/larvae and 7.1×10^{3}granules/mm2 respectively and can be used either singly or in combination with other pesticides to manage DBM and ABW larvae.

In the field, the Kenyan baculovirus isolate, PlxyGV F0, is highly efficacious causing comparable DBM larvae control with the chemical pesticide. Iberian NPV isolates, just like Kenyan isolates, were reported to cause up to 95% mortality in insects

(Figuieredo *et al.*, 2009) hence indicating the efficiency of baculoviruses in pest control. The approximate speed to kill of the Kenyan isolates of baculoviruses of less than six days indicate that if applied on early instars larvae better control would be achieved. The Kenyan isolate of baculovirus, PlxyGV, can be used in the field to manage diamondback moth.

6.2 Conclusion

Rearing of insects on semi-synthetic diet provided a homogenous insect culture and was more reliable as compared to rearing on natural hosts. The diets developed for use in the rearing of ABW and DBM gave good quality insects.

Kenyan isolates of baculoviruses are similar both in morphology and in molecular structure with others isolated and developed in other countries as bio pesticides. They are also highly virulent causing up to 100% mortality in DBM and ABW depending on the concentration.

In the field, Kenyan baculovirus isolate, PlxyGV, is an effective control of DBM and is comparable to synthetic pesticide, Tracer 480S, in the management of the pest Therefore, Kenyan PlxyGv isolate has great potential as bio-rational agents against DBM.

6.3 Recommendations

Baculoviruses are species specific and therefore cause mortality in the insect species from which they are isolated. Apart from diamondback moth and African bollworm, other lepidoptera pests of importance need to be screened for their viruses. More

virulent isolates of PlxyGV and HearNPV against DBM and ABW respectively will also make their management more effective. This will make pest control wholesome and also reduce the dangers associated with over reliance of synthetic pesticides. Other options of making baculoviruses more efficacious need to be exploited especially in formulating the products.

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APPENDICES

Appendix 1: Diets ingredients

Pieris diet Ingredients

Ingredient	Quantity (g/l)
Casein	37.8
Wheat germ	32.4
kale powder	15
Sucrose	37.8
Cellulose	5.2
Wesson Salt	10.8
Vitamin mix	12
Ascorbic Acid	4.6
Choline chloride	1.06
Paraben	5
Agar	27
Sorbic acid	1
Linseed oil	5ml

Note: Modification of the diet was done by replacing the ingredient with locally obtained materials.

KBL standard diet ingredient

Ingredients	Quantity g/l
Wheat germ	34
Soy flour	39
Sucrose	34
Cellulose	5
Wesson salt	10
Vitamin mix	12
Ascorbic acid	4
Methyl paraben	5
Sorbic acid	1
Agar	17

Southland Multi-species diet

Ingredients	Quantity g/l
USDA mix	169
Linseed oil	6
Kale powder	5

Appendix 2: Diet preparation procedure

1. Prepare all ingredients before starting to make the diet, for instant precooking wheat and drying and grinding kale
2. Weigh out the required amounts of dry ingredients for the desired amount of diet, put them into a plastic bowl and mix thoroughly till all the cramps are broken.
3. Weigh out agar for the desired amount of diet and place the agar in a separate bowl.
4. Measure the desired amount of clean water into a 1 L graduated cylinder, add to the agar and stir to mix and boil in microwave for about 8-10 min.
5. Mix the dry components using 80ml warm water for 1L.
6. After boiling, let the agar cool down to about 60°C and add the mixture of dry ingredients. Mix thoroughly for 4 min and dispense into containers thoroughly.

Appendix 3: Restriction endonuclease analysis (REN) standard operating procedure

1. Restriction endonuclease profiling consists of three parts, DNA isolation and quantification, restriction enzyme digestion, and agarose gelelectrophorese. The procedures apply to both viruses unless otherwise specified.
2. Starting with purified viruses. Virus particles are liberated from the purified granula using the high pH of Na_2CO_3 to dissolve the granulin crystals.
3. Take approximately 2.5 x 10^{11} granula, or 5 x 10^{9} polyhedra in 1 ml water in a microfuge tube
4. Add 100 µl proteinase K (20 mg/ml) and 50 µl 10% SDS
5. Incubate at 37ºC for 1 hour (this is to destroy any remaining DNA in the sample)
6. Add an equal volume of 100 mM freshly prepared Na_2CO_3 to the suspension
7. Incubate until suspension is clear (if needed, incubate at 50ºC)
8. Pellet the envelopes in a microfuge at 5000 rpm for 2 min.
9. The remaining supernatant consists of liberated virus particles
10. Subject the supernatant to a series of phenol, phenol:chloroform:isoamylalcohol (50:49:1), and chloroform extractions as follows:

11. Add an equal volume of buffer-saturated phenol to the supernatant and mix the suspensions by rapidly inverting the tube for 3-5 min. Do not vortex.
12. Separate the phenol (bottom) and water phase by centrifugation at 10-12,000 rpm in the microfuge
13. Cut the tip off a 200 µl pipet and transfer the water phase to a clean tube, thereby avoiding to transfer any of the denatured proteins of the interphase
14. Add an equal volume of buffer-saturated phenol:chloroform:isoamylalcohol to the tube and mix by rapidly inverting
15. Spin as in 3.2
16. Transfer as in 3.3
17. Repeat steps 3.4, 3.5 and 3.6 until there is no or a barely visible interphase
18. Add 2 volumes 100% ice-cold ethanol mix and incubate for 1 hour to overnight in the freezer
19. Spin precipitate down at 12,000 rpm for 10 min and wash the pellet by adding 1 ml 70% ethanol and centrifugation as before
20. Pour the supernatant carefully off and spin for 10 seconds to collect remaining liquid in the bottom of the tube
21. Remove liquid with a P-20 pipettor without disturbing the pellet
22. Briefly dry the pellet by leaving the tube open (approx. 10 min) and check if there is no alcohol odour left
23. Add 50-100 µl dd water or TE buffer (10 mM Tris-EDTA, pH 7.6)
24. Let the DNA redissolve overnight at 4°C and check next morning if there is any precipitate remaining. If that is the case try to dissolve in a water bath at 60°C.
25. Take a 10 µl sample from the final supernatant and measure the DNA concentration in a spectrophotometer as per SOP or protocol of the particular piece of equipment.
26. High specific restriction endonucleases should be used depends on the particular virus under investigation, therefore see earlier internal reports or the scientific literature.
27. Reaction mixes for the restriction enzymes used should be prepared according to the recommendations of the manufacturers.
28. After the digestion is stopped by the addition of $1/6^{th}$ volume loading buffer, load 1-2 µl of the sample in a well of an agarose gel prepared as follows:

29. Heat a suitable quantity of 0.7% agarose in TBE buffer containing 10 µg/ml ethidium bromide in a microwave until all agarose is dissolved
30. Cool the mixture until it is hand warm
31. Pour the mixture in the tray of a gel box of at least 30 cm in length in which a comb is inserted. Make sure the tray is horizontal.
32. Let the agarose gel and once set place the tray in the electrophoresis box filled with the running buffer (TBE).
33. After equilibration for 30 min, remove the comb, and load the gel with the samples. The wells should be under the running buffer level. Note the order of the samples
34. On both sides of the samples, load the gel with a suitable molecular weight standard
35. Run gel for 16 h at 2.4 V/cm
36. Photograph the gel under UV light on a transilluminator

Appendix 4: Field Layout

T1R1	T4R2	T2R3	T5R4	T3R5
T2R1	T5R2	T3R3	T1R4	T4R5
T3R1	T1R2	T4R3	T2R4	T5R5
T4R1	T2R2	T5R3	T3R4	T1R5
T5R1	T3R2	T1R3	T4R4	T2R5

Treatments:

- Chemical standard - T1
- Untreated control - T2
- PlxyGV low rate - T3
- PlxyGV medium rate - T4
- PlxyGV high rate - T5

Appendix 5: DBM population over 7 weeks period for various treatments during field trial carried out at AAA farm in June/July 2009

Treatment	**Week 0**	**Week 1**	**Week 2**	**Week 3**	**Week 4**	**Week 5**	**Week 6**	**Week 7**
T1R1	9	0	4	2	11	15	0	0
T1R2	35	0	2	1	5	16	2	2
T1R3	16	1	0	0	9	24	3	4
T1R4	30	1	0	0	4	22	3	0
T1R5	17	1	0	0	7	5	2	0
Total/trtnt	**107**	**3**	**6**	**3**	**36**	**82**	**10**	**6**
Ave/trtnt	**21.4**	**0.6**	**1.2**	**0.6**	**7.2**	**16.4**	**2**	**1.2**
T2R1	22	37	26	19	6	12	30	6
T2R2	35	59	60	13	11	13	40	4
T2R3	15	37	23	14	10	17	24	4
T2R4	31	37	36	6	9	24	31	9
T2R5	25	45	20	16	5	18	59	6
Total/trtnt	**128**	**215**	**165**	**68**	**41**	**84**	**184**	**29**

Ave/trtnt	**34.3**	**38**	**23.3**	**10.9**	**12.5**	**16.8**	**36.8**	**5.8**
T3R1	14	35	10	4	9	7	40	2
T3R2	33	25	6	7	2	9	45	2
T3R3	43	44	14	1	11	15	20	2
T3R4	31	26	15	8	4	12	35	0
T3R5	29	53	0	8	3	21	34	2
Total/trtnt	**150**	**183**	**45**	**28**	**29**	**64**	**174**	**8**
Ave/trtnt	**30**	**36.6**	**9**	**5.6**	**5.8**	**12.8**	**34.8**	**1.6**
T4R1	21	78	6	1	1	7	20	2
T4R2	23	28	11	2	5	8	16	2
T4R3	50	50	9	2	5	15	16	0
T4R4	14	33	6	3	1	8	26	0
T4R5	39	70	4	2	2	10	30	2
Total/trtnt	**147**	**259**	**36**	**10**	**14**	**48**	**108**	**6**
Ave/trtnt	**29.4**	**51.8**	**7.2**	**2**	**2.8**	**9.6**	**21.6**	**1.2**
T5R1	27	66	3	0	3	7	3	0
T5R2	33	44	3	2	4	4	15	0
T5R3	31	43	5	0	3	12	9	0
T5R4	19	29	3	4	1	8	30	0
T5R5	52	58	5	2	2	13	19	0
Total/trtnt	**162**	**240**	**19**	**8**	**13**	**44**	**76**	**0**
Ave/trtnt	**32.4**	**48**	**3.8**	**1.6**	**2.6**	**8.8**	**15.2**	**0**

Appendix 6: *Hear*NPV purification procedure

1. Add approx. 5 ml of a 1% SDS solution for each gram infected larval cadavers
2. Homogenize cadavers in a blender, 15 sec
3. Let the mixture incubate at room temp for 15 minutes
4. Pour the mixture through a screen
5. Spin in a Centrifuge at 500 rpm for 5minutes, keep the supernatant discard the pellets
6. Spin int the centrifuge for 10 minutes at 5000 rpm, discard the supernatant and resuspend the pellets in 2 ml 100 mM Nacl for each gram of the original larvae
7. Centrifuge for 10 minutes at 5000 rpm
8. Repeat the last two steps twice
9. Finally resuspend the final pellets in a small volume of water or 10 mM Tris/HCl pH 7.0
10. Make counts and calculate the virus concentration

Appendix 7: *Plxy*GV purification procedure

1. Add approx. 5 ml of a 1% SDS solution for each gram infected larval cadavers
2. Homogenize cadavers in a blender, 15 sec
3. Let the mixture incubate at room temp for 15 minutes
4. Pour the mixture through a screen
5. Spin in a centrifuge at 3000 rpm for 15 minutes, discard the pellets and keep the supernatant
6. Spin the supernatant at 11000 rpm for 30 minutes, discard the pellets and keep the supernatant
7. Gently, resuspend the pellets in water
8. Repeat the last two steps twice
9. Finally, respend the pellets in a small amount of water or 10 mM Tris/HCl pH 7.0
10. Make counts and calculate the virus concentration